BUTTERFLY ASSEMBLAGES IN FRAGMENTED MEADOW HABITATS OF THE PRE-CARPATHIAN LOWLAND (BEREG PLAIN, SW UKRAINE)

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Abstract. The butterfly assemblages of grassland habitats in the Transcarpathian part of the Bereg plain were studied with standard transect walk methods in 2012 and 2013, in 6 different sites (14 transects), in all sites with 4 and 5 repetitions per year. To sum up, we observed 63 butterfly species in more than 4800 individuals. 25% of these species is under protection in Hungary. Considering the faunal types, the widely distributed and generalist Euro-Siberian species predominate, but with significant presence of Holo-Mediterranean and southern Continental elements. The less disturbed habitats surrounded by natural forests have shown the highest species diversity (Shannon-Wiener). The assemblages were compared with multivariate methods (PCA, cluster, Bray-Curtis) . Three main groups of habitats were separated and characterised by indicator species (IndVal). The positive effect of nature-like forest fringe and scrubby structures was demonstrated. According to our results the quality of habitats is more relevant for species diversity than size and/or isolation of habitats.

Keywords: *compositional diversity, dominants, biogeographical components, indicator species, protected species*

Introduction

Large parts of Europe suffer from loss of biodiversity (e.g. May et al., 1995; Hambler and Speight, 2004; Thomas et al., 2004; Thomas, 2005), despite of the efforts to stop or at least decelerate this process (see: Natura 2000). Especially in densely populated and industrialised West and Central European countries, the current extinction of many species is strongly connected to habitat fragmentation and loss (Andrén, 1996; Fahrig, 1997) resulting from the destruction of natural and semi-natural habitats (Wilcox and Murphy, 1985; Saunders et al., 1991; Harrison and Bruna, 1999). In other parts of Europe, the abandoning of traditional land use, connected with social changes represents the major threat for biodiversity (Warren, 1997; Wenzel et al., 2006; Schmitt and Rákosy, 2007).

Butterflies are considered as sensitive indicators of habitat changes since they are known to respond quickly to a number of environmental parameters, e.g. vegetation structure and composition (Erhardt and Thomas, 1991; Rákosy and Schmitt, 2011). They are also well suitable for monitoring of changes since they are well understood taxonomically, and easily recognized and marked in the field. Lepidoptera can also be used as umbrella species (sustaining habitat to conserve this species will also conserve many other taxa) for biodiversity conservation (New, 1997).

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The sites of our surveys are lying on the immediate continuation of the Pannonian lowland (Szatmár-Bereg plain) which is a traditional, low input agricultural region with several rests of the natural and semi-natural vegetation: lowland oak-hornbeam forests, hard- and softwood gallery forests, humid meadows and forest clearings (Simon, 1952), but recently also with abandoned pastures and fallow lands. A considerable part of the area is dissected by dreanage channels of the former huge peatland which existed here until the last decades of the XIX century. The climate of the Szatmár-Bereg lowland (Kormány, 1976, 2006) is slightly cooler (yearly average 8,9^{o} C) and more continental than the average of the Great Hungarian Plain (January —3,4^{o} C). The yearly sum of precipitation is also higher, about mm in average (609-640 mm). Biogeographically it belongs to the Pannonian region, however, with significant Carpathian influences in some groups of terrestrial invertebrates (land gastropods, ground beetles, see: Deli et al., 1997; Magura et al. 1997; Ködöböcz and Magura, 1999; Gálik et al, 2001). Our surveys were carried out near to the village Nagydobrony, partly in the „Nagydobrony Game Reserve" or in adjacent territories.

The aims of our surveys were:

- To record the species composition and species' relative frequencies of the butterfly assemblages.
- To compare the two years of survey considering whether has changed (i) the number of species, (ii) relative frequency of species, (iii) the Shannon-Wiener diversity index.
- To register the similarities and differences betwwen the composition of butterfly assemblages of different habitats.
- To search for indicator species of habitat types and for correlations between vegetation and butterfly assemblages.
- To evaluate the results for the nature conservation management of surveyed sites.

Material and Methods

The surveys were carried ot by standard transect walks in 2012 and 2013 in four and five repetitions, respectively (**2012**: 16-18. 06; 11-14. 07; 04-06. 08; 06-08. 09; **2013**: 05-06. 06; 05-08. 06; 05-08. 07; 21-22. 07.). We designated on 6 sites 14 transects (length 50 m) and recorded all butterflies 2.5 m to their right, 2.5 m to their left, 5 m ahead of them and 5 m above them. Visits were conducted when the temperature was above 20^{o} C in sunny weather, without strong wind and rain (see: Van Swaay et al., 2002). Most butterflies were readily identified by observation. The dubious specimens were captured by net and they were either immediately released after identification or preserved as voucher specimen for exact determination (e.g. some *Lycaenidae or Melitaea species*).

During each transect walks some standard values of vegetation were measured (species composition, relative cover in %, height of vegetation, etc.). From these data here we only use the relative cover of flowering forbs (dicotyledones) as a proxy of vegetation quality from respect the butterflies (*Table 1*).

The composition of butterfly assemblages was compared by Principal Component (PCoA) and cluster analysis in which the Bray-Curtis distance was used, in clustering the MISSQ (incremental sum of squares method of Ward and Orlóci) was chosen(Podani, 1997a). The assemblages were compared in each year separately and also with combined data of both years. The analyses were carried out with the programme package SynTax (Podani, 1997).

Table 1. *Cover of flowering herbaceous dicotyledones (%)*

Data 2012	Szapat	Körerdő	Felső-erdő	Reserve I.	Reserve II.	Kismakkos
16-19. 06.	21	24	53	37	48	41
11-14. 07.	25	36	61	43	57	47
02-06. 08.	24	38	66	44	63	53
08-10. 09.	22	28	45	39	41	44
Min-Max	21-25	24-38	45-66	37-44	41-63	41-53

Data 2013	Szapat	Körerdő	Felső-erdő	Reserve I.	Reserve II.	Kismakkos
05-06. 06.	29	27	55	41	47	45
06-08. 07.	36	34	62	48	54	52
21-23. 07.	38	41	68	52	57	54
13-15. 08.	33	38	61	49	51	51
30. 08-01.09	27	24	47	33	37	39
Min-Max	27-38	24-41	47-68	33-52	37-57	39-54

The quantitative character species, i.e. indicator species of the assemblages were classified by the IndVal method, using the programme package IndVal (Dufrêne and Legendre, 1997). We hierarchically classified the species according to their fidelity (constancy within group). The IV value of species is the highest (100) if the given species is present in all samples of the given group and is also exclusive for this group of samples. The program calculates the IV values of each species for each hierarchic level of clustering and the maximum value will be considered as indicator value of the given species. The significance of IV values was determined by randomisation (1000 iterations).

Results

Faunistic records

During our surveys we registered 63 butterfly species in more than 4800 individuals. From these, six species occurred in all habitats in both years: *Pyrgus malvae*, *Thymelicus silvestris*, *Pieris rapae, Polyommatus icarus*, *Coenonympha pamphilus*, *Maniola jurtina.* These are known as generalist species, three of them are connected to grasses (Poaceae) and three to herbaceous forbs. Three further species were observed in all habitats with the exception of the extremely dry ones: *L. sinapis/juvernica, Cupido alcetas, C. argiades*. They are connected to *Fabeceae species* (*Lotus, Lathyrus*, etc.). The migrant *C. argiades* was observed in both years constantly in relatively high individual numbers.

The species number was consequently the highest in the nearly undisturbed semi-humid meadow surrounded by natural hardwood forest with natur-like forest fringe (*transitional* in *Table 2*) and the lowest in artificially drained, disturbed abandoned pasture (*disturbed* in *Table 2*). It seems to be a general tendency that the nature-like forest fringes support a higher diversity of species, faunal elements and ecological types. These values seem to be independent from the extension of the sampling sites (*Table 2*). Of course, the number of observed individuals was the smallest on the site with the smallest extension. Furthermore, the species number and the number of

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observed individuals (with one single exception) were slightly higher in 2013 than in 2012, possibly due to higher number of repetitions (5 vs. 4).

***Table 2**. Basic data of the sampling sites and observed species and individuals*

	disturbed,	transitional	humid	sum
Mean area	5.2	3.5	1.9	3.8
Number of sites (samples)	2 (4)	3 (6)	1 (2)	6 (12)
Species richnes (Stotal)	33	59	34	63
Mean species/site (α) (±SD)	20.8 (3.3)	34.0 (3.6)	32.0 (1.4)	29.3 (7.0)
Whittaker's S/α	1.6	1.7	1.1	2.2
S/αmax	1.3	1.6	1.0	1.7
Number of specimens	1018	3371	420	4809
Specimen/site (±SD)	254.5 (72.6)	561.8 (182.7)	210.0 (38.2)	400.8 (212.8)

The whole species composition of assemblages is added as *Electronic Appendix* to the publication. More than 25% of the observed species are protected in Hungary, it means that at least some of our sampling sites have a nature conservation importance.

Biogeographical composition of butterfly assemblages

The sites of our surveys are enbedded into an agricultural landscape and are mostly surrounded by anthropogenic habitats. Thus, the bulk of the fauna is formed by widely distributed Euro-Siberian species with broad ecological tolerance. They do not have any food plant specialisation and occur, as a rule, also in disturbed or anthropogenically transformed habitats. Special biogeographic elements, as Holo-Mediterranean (-West Asiatic), Ponto-Mediterranean, Southern Continental or Boreo-Continental species are much less represented (*Fig. 1*). The number of migrant („extra-Palaearctic") species is relatively high.

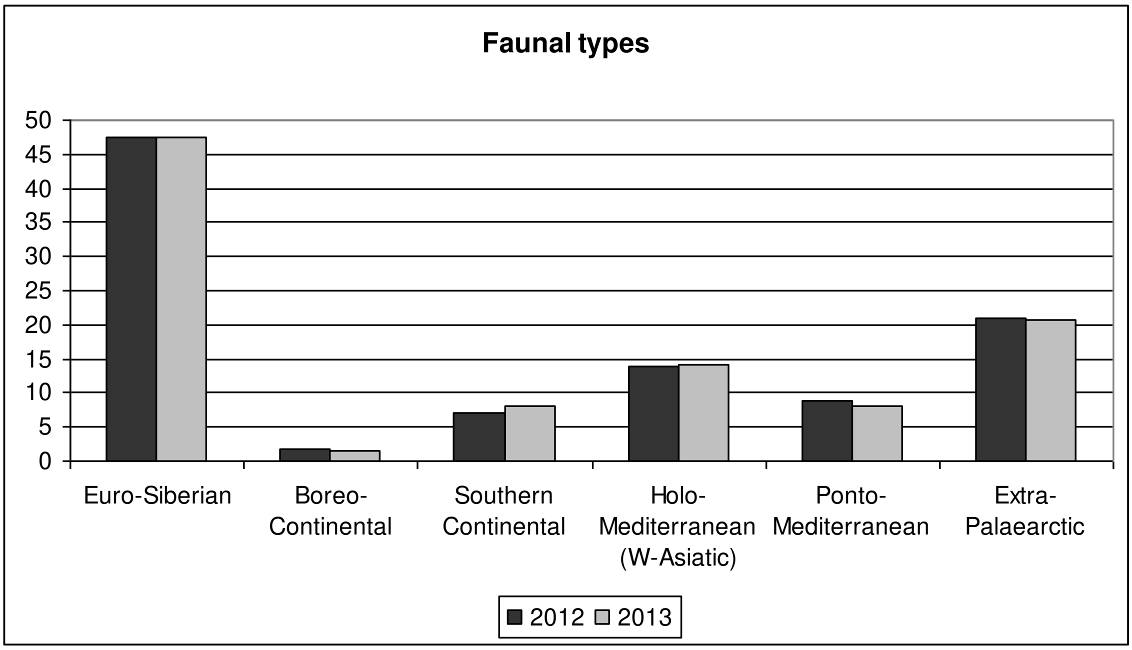

***Figure 1**. The relative frequency of species numbers belonging to different faunal types.*

The classification of the faunal components was based on the concept of Varga (1977), recognising that the bionomy of the lepidopterans is primarily influenced by the life history of the caterpillars, the larval hostplants, etc. and therefore it can be characterized by a certain type of habitat (like humid vs. dry sward, humid tall forb formations, softwood vs. hardwood forest, etc.). The composition of faunal components (*Fig. 2*) also proved to be essentially similar to the faunal types since the highly tolerant, euryoecious species predominate. The specialists are mostly connected either to humid habitats, e.g. *Lycaena dispar rutila, Boloria selene* or scrubby forest skirt formations, as *Satyrium species*, *Neptis sappho, Brenthis daphne*, etc. In these respects we could not find any differences between the two consecutive years.

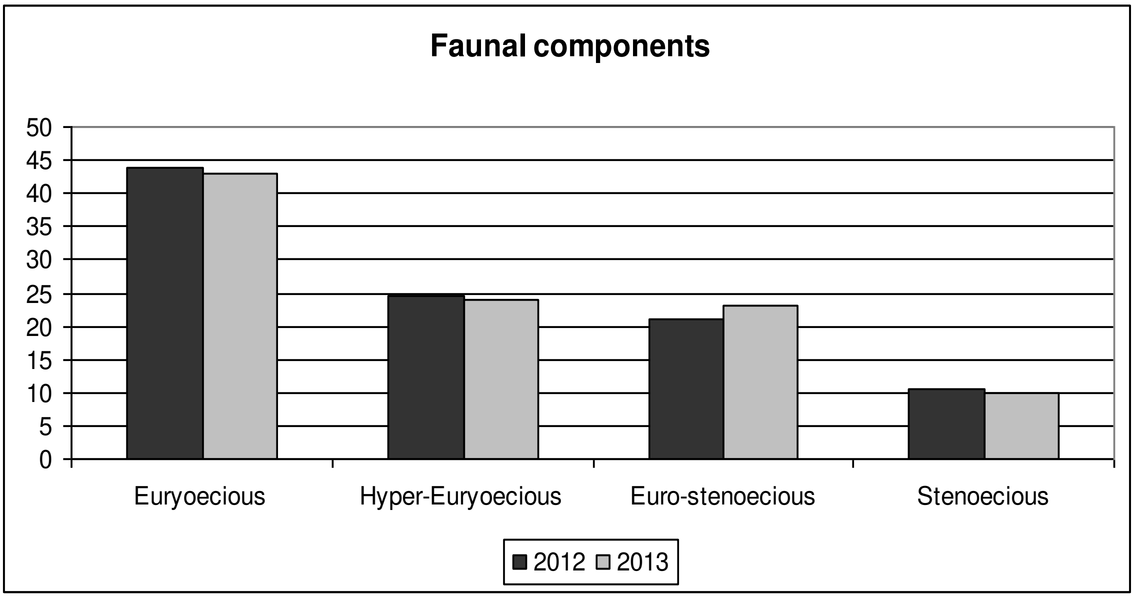

***Figure 2**. The relative frequency of generalist vs specialist species*

Compositional diversity and similarity of butterfly assemblages

The values of Shannon-Wiener diversity show an increasing gradient from the heavily disturbed (drained and formerly overgrazed) pasture („Szapat”) to the mosaic-like habitats with scrubby patches and/or nature-like forest fringes (Reserve/Rezervátum I and „Kis-Makkos”). The species diversity of the sites surrounded by forests or nature-like forest fringe structures is nearly at the same level (*Fig. 3*, see sites 3-6). No significant difference was observed between 2012 and 2013.

The compositional similarity of the assemblages was compared by Principal Component Analysis (PCoA) and they were clustered based on Bray-Curtis distances (programme package SynTax 2000). The ordination was carried out separately for each year but also using the pooled (2012-2013) data of sampling localities.

Three groups of sites were clearly differentiated (*Figs 4-5*). The drained and disturbed habitats with lower diversity are clearly separated, but also the mostly closed, scrubby habitat, surrounded by forest, seems to be distant from the other ones. The configuration of the sites is essentially the same in both years, the importance of the axes was changed, however. The ordination based on both years also confirms the separation of the three main groups.

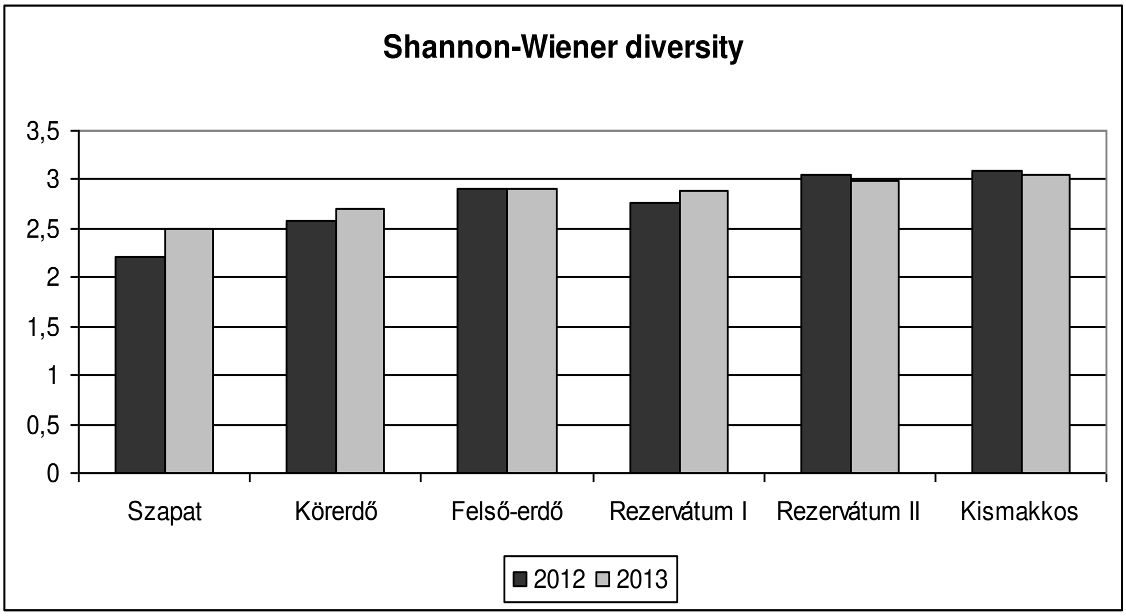

Figure 3. *The Shannon-Wiener diversity of butterfly assemblages*

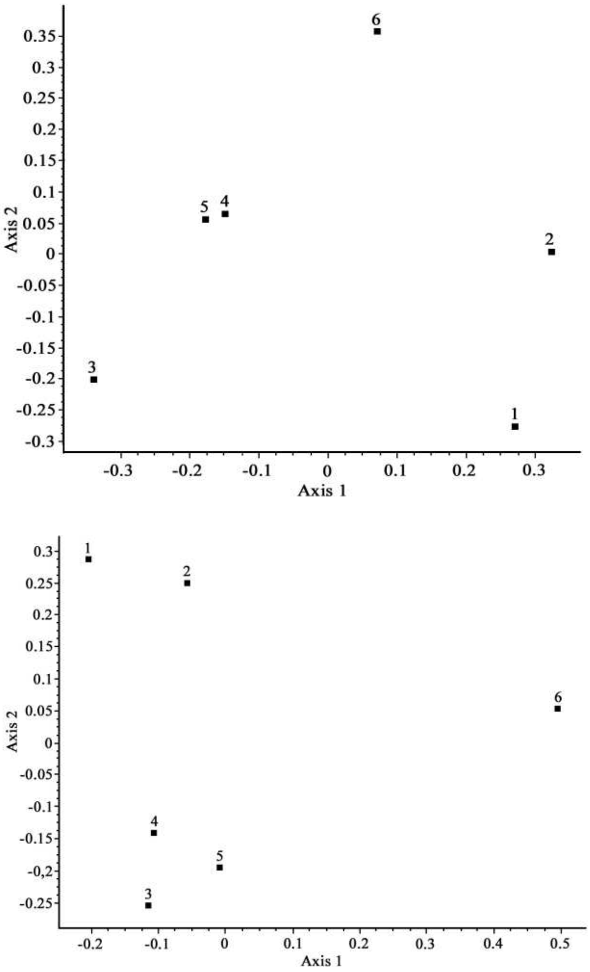

Figure 4. *Ordination of the sampled assemblages in 2012 and 2013 (Bray-Curtis)*

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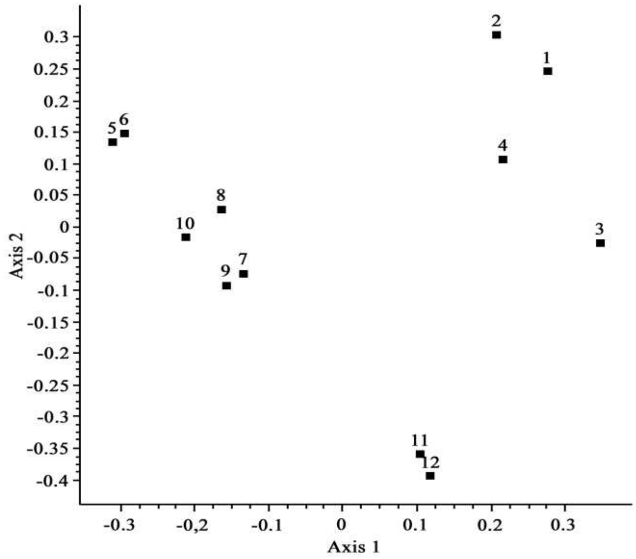

***Figure 5**. Ordination of the sampled assemblages in both years (Bray-Curtis)*

The clustering of sampling sites clearly shows the same pattern (*Fig. 6*). The basic split is between the disturbed, drained vs. more nature-like or semi-natural habitats. The data of the sampling sites from the both years are usually clustered together.

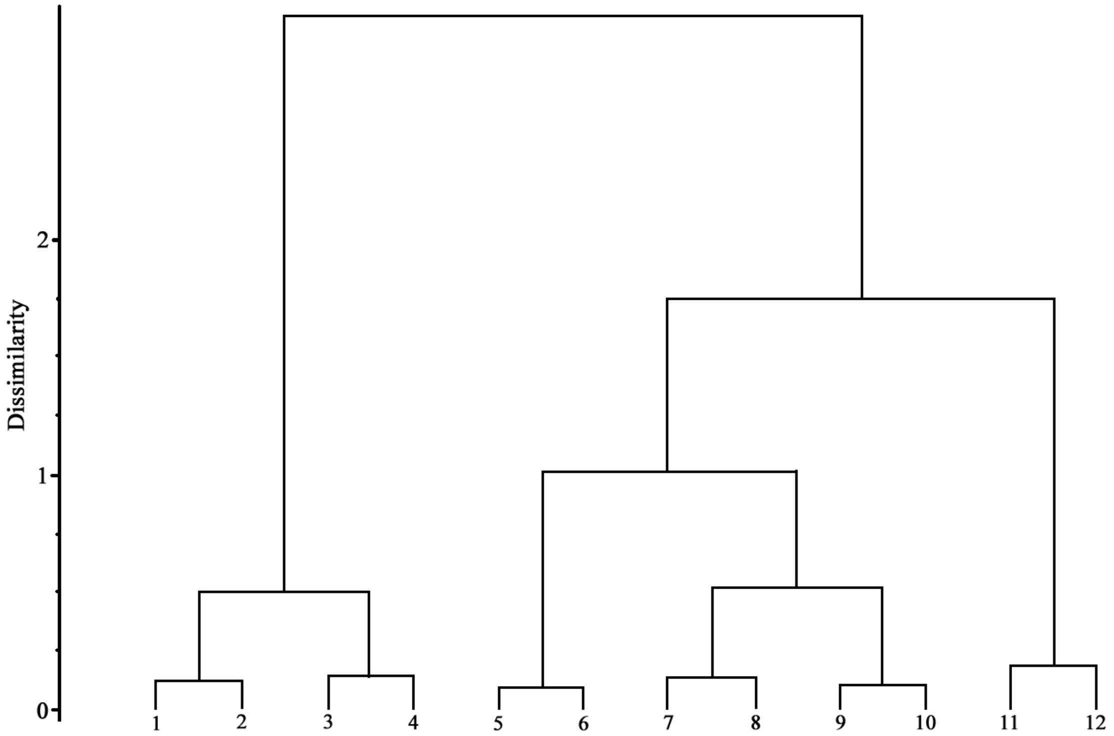

***Figure 6.** Clustering of sampling sites (Bray-Curtis)*

The dominance-identity of sites shows a completely similar pattern as the Bray-Curtis dendrogram. The most disturbed sites (Szapat, Körerdő) show the highest similarity to each other and they are also very dissimilar to all other sites. The second group is formed by sites (5-10) where the meadow is surrounded by nature-like hardwood forests. The third habitat type is mosaic-like, with semi-dry grassy and scrubby patches and nature-like forest fringe structures. This habitat shows a striking differentiation mostly in 2013 from the other ones.

The ordination of the sites was completed with an Indicator Species Analysis (IndVal) (*Fig. 7*). Seven generalist species are typical for all sampling sites, while 5 vs 5 species characterise the disturbed pasture-like and the more meadow/hayfield-like sites, respectively. We could only differentiate a third hierarchic level of splitting: between mesic-humid habitats surrounded by hardwood stands (6 species) and the patchy-scrubby semi-dry habitats (4 species). The conservation biologically significant species are to be found in the species groups typical for mesic and/or scrubby, semi-dry sites. We could not differentiate further sub-groups since the indicator species of the lowest hierarchic level would be very weakly supported.

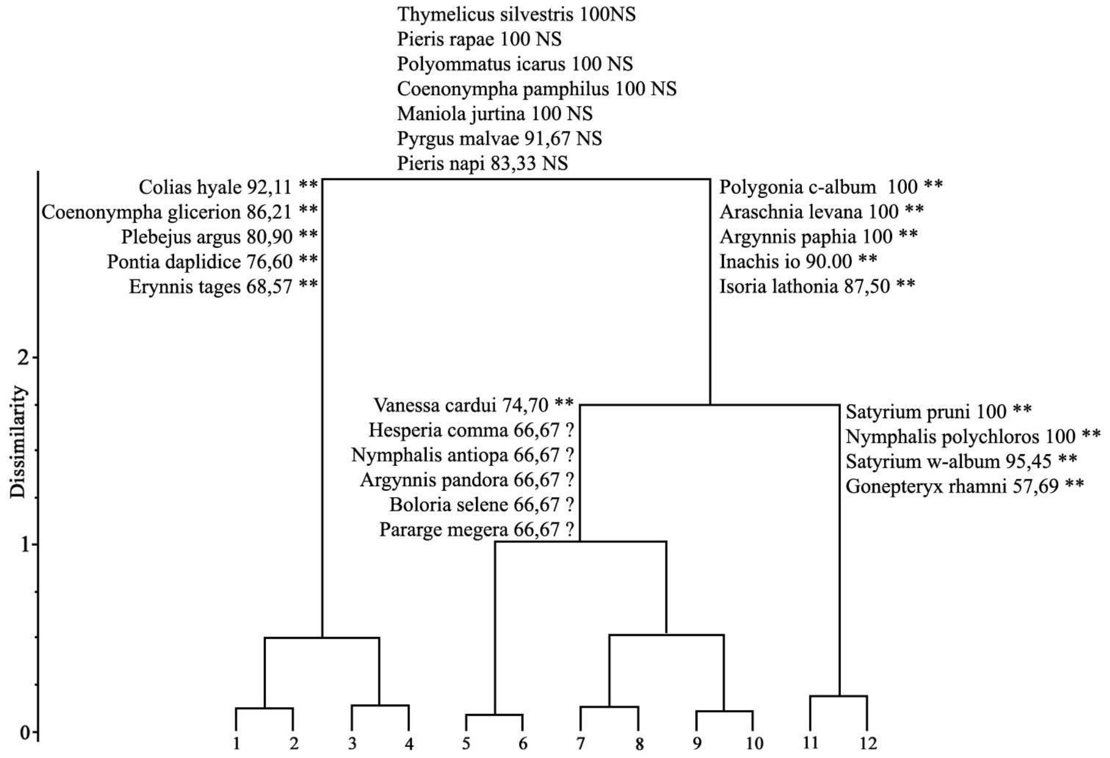

***Figure 7**. Indicator species of the clustered sites*

Discussion

Habitat fragmentation and loss are known to reduce species richness and to change the community structure (Wilcox and Murphy, 1985; Steffan-Dewenter and Tscharntke, 1999, 2000; Tscharntke et al., 2002a, b). These negative processes are mostly connected with the alteration in land use practices causing habitat loss and fragmentation and hence they are also the main factors modifying species communities (Foster and Boose,

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1992; Austrheim et al., 1999; Cousins and Eriksson, 2001; Maes and Van Dyck, 2001). These changes are particularly dramatic for butterfly species (Bourn and Thomas, 2002; Van Swaay, 2002; Wenzel et al., 2006). The decline of many butterfly species across Europe (León-Cortés et al., 2000; Maes and Van Dyck, 2001; Van Swaay et al., 2011) highlights the need to identify the factors influencing species diversity and characteristic habitats for contemporary conservation action.

The agriculturally dominated mosaic landscapes are mostly suitable to identify the situations in which species diversity is high and can be sustained and in which the species diversity is in decline. Riverine lowlands like the Bereg plain, may represent especially valuable fields for such surveys. In such landscapes the traditional land use often preserved more or less natur-like gallery forests and floodplain habitats but the regulation of waterways, the drainage of plains with fluctuating humidity and also the abandoning the traditional land use practices strongly transformed or destroyed large areas. Thus we have contrasting habitats in different extension. Therefore we have to put and discuss numarous questions.

It is one of the most frequently declared working hypotheses that the strong disturbance leads to dominance of generalist species and causes the decline of the more specialist ones, and these processes are usually connected with some homogenisation in the composition of species assemblages. Here we can observe a situation in which the two components can be separated, The dominance of generalist species seems to be evident in both respect: in geographical range and in the lack of connection to special habitats. We cannot observe however, any homogenisation of assemblages, as we can see it from the ordinations. But, the other side of the coin is the unexpected high frequency of migrant species of extra-Palearctic origin. Of course, we need further, at least mid-term surveys to clarify: is there a trend connected with the climate change or we only have seen occasional events.

The other frequent question is whether the size vs quality of habitat patches is more relevant for sustaining species diversity. Here we have seen essentially three different habitat types in very different extensions. Our results clearly demonstrate that the extended but heavily disturbed, drained site(s) cannot support any species rich assemblages. Oppositely, the site with smaller extension but with nature-like forest-fringe structures can sustain a nearly so rich assebly than the much larger meadows of the „Reserve". To measure the „quality" we used the simplest proxy, the relative cover of flowering herbaceous forbs in the times of the transect walks (*Table 1*). We can see that these data are essentially similar in all habitats with the exception of the first to sites (Szapat and Körerdő) which were also ahown as habitats of species-poor assemblages. Although we only have some „anecdotic" information on the earlier land use practices, we can believe that these more disturbed sites with low level of diversity were grazed (or even overgrazed and trampled) while the other meadows were suitable for mowing and were used as hayfields. The more detailed study of soil and vegetation will be necessary in the next step of surveys.

In this connection we also have seen that the forests, and especially the fringe structures can positively influence the species diversity, since numerous butterflies feeding on nectar sources of meadows are connected by their larval foodplants to forest fringes or light-penetrated parts of forests (e.g. Theclini: *Satyrium spp*., *Argynnis paphia, Brenthis daphne*). Some other species possibly prefer meadow patches surrounded by forests from micro-climatic reasons, e.g. sheltering from wind, humidity, less extreme fluctuation of temperature, etc. Unfortunately, these questions cannot be answered yet. References show that in contrast to broad-scale climatic impacts on biodiversity (e.g. Parmesan, 2006), small-scale impacts influencing the composition of insect assemblages are much less understood. The survey of the small-scale effects of micro-climate, vegetation structure, etc. would essentially improve the understanding of composition in natural communities, especially which harbour specialist species. Moreover, it would enhance the effectivity of conservation measures maintaining species diversity under climate change.

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APPENDIX

Electronic Appendix: Transect 2012-2013

		Disturbed					Transitional							Patchy			
		Szapat		Körerdő			Felső-erdő		Rezervátum I.		Rezervátum II			Kismakkos			
		2012	2013	2012	2013		2012	2013	2012	2013	2012	2013		2012	2013		
Pyrgus malvae	*pyrmal*	9	29	11	15	64	23	5	5	0	8	5	46	8	11	19	129
Pyrgus armoricanus	*pyrarm*	0	0	0	0	0	2	0	0	0	0	0	2	0	0	0	2
Thymelicus lineola	*thylin*	0	0	0	4	4	3	17	0	7	0	0	27	0	0	0	31
Thymelicus silvestris	*thysil*	9	5	13	14	41	4	3	5	5	7	4	28	6	4	10	79
Hesperia comma	*hescom*	0	0	0	0	0	9	3	0	0	6	4	22	0	0	0	22
Erynnis tages	*erytag*	0	8	3	5	16	0	0	0	3	0	0	3	0	0	0	19
Ochlodes sylvanus faunus	*ochsyl*	0	0	4	7	11	0	6	0	0	14	9	29	7	5	12	52
Papilio machaon	*papmac*	1	2	0	0	3	0	0	0	0	1	2	3	0	2	2	8
Iphiclides podalirius	*iphpod*	0	0	0	2	2	0	0	0	0	5	9	14	1	3	4	20
Leptidea sinapis /juvernica	*lepsin*	0	0	6	5	11	35	17	11	17	23	21	124	11	7	18	153
Aporia crataegi	*apocra*	0	1	0	3	4	0	0	0	0	0	0	0	0	3	3	7
Pieris brassicae	*piebra*	0	2	5	6	13	0	3	0	0	4	10	17	2	16	18	48
Pieris napi	*pienap*	13	14	16	23	66	22	10	12	14	23	36	117	0	0	0	183
Pieris rapae	*pierap*	15	27	15	21	78	51	48	19	21	13	5	157	9	5	14	249
Colias crocea	*colcro*	6	6	1	2	15	7	9	0	0	0	0	16	0	0	0	31
Colias hyale	*colhya*	9	16	3	7	35	0	0	0	0	6	0	6	0	0	0	41
Gonepteryx rhamni	*gonrha*	0	0	0	0	0	0	0	10	1	0	0	11	2	3	5	16
Pontia daplidice	*pondap*	5	3	6	4	18	3	2	0	0	0	0	5	2	4	6	29
Lycaena phlaeas	*lycphl*	2	4	0	0	6	8	4	0	0	0	0	12	0	0	0	18
Lycaena thersamon	*lycthe*	0	0	0	0	0	2	2	0	2	0	0	6	0	0	0	6
Lycaena dispar rutilus *	*lycdis*	0	0	0	0	0	1	3	0	0	0	0	4	0	0	0	4
Satyrium pruni	*satpru*	0	0	0	0	0	0	0	0	0	0	0	0	2	3	5	5
Satyrium spini	*satspi*	0	0	0	2	2	0	0	2	1	0	0	3	0	0	0	5
Satyrium ilicis	*satili*	0	0	0	0	0	0	2	0	0	0	0	2	0	0	0	2
Satyrium w-album	*satwal*	0	0	0	0	0	0	0	0	1	0	0	1	2	5	7	8
Cupido alcetas	*cupalc*	0	0	1	1	2	6	5	0	0	5	11	27	3	1	4	33
Cupido argiades	*cuparg*	0	0	4	5	9	7	2	8	11	10	16	54	4	3	7	70
Cupido minimus	*cupmin*	4	11	0	0	15	0	0	0	0	0	0	0	0	0	0	15
Celastrina argiolus	*celarg*	0	0	2	0	2	8	15	7	3	0	1	34	3	14	17	53
Plebejus argus	*plearg*	6	14	7	9	36	3	7	0	0	0	0	10	2	5	7	53
Polyommatus icarus	*polica*	37	74	22	46	179	48	92	26	53	29	52	300	12	9	21	500
Polyommatus semiargus	*polsem*	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	2
Polyommatus bellargus	*polbell*	0	5	0	0	5	0	0	5	8	0	0	13	0	0	0	18
Hamearis lucina	*hemluc*	0	0	0	0	0	0	0	7	18	0	0	25	0	0	0	25
Apatura ilia	*apaili*	0	0	0	0	0	0	0	13	36	0	0	49	4	3	7	56
Neptis sappho	*nepsap*	0	0	0	3	3	0	0	0	3	7	16	26	3	1	4	33
Polygonia c-album	*polcal*	0	0	0	0	0	5	4	6	8	4	7	34	3	4	7	41
Nymphalis antiopa	*nymant*	0	0	0	0	0	0	0	1	1	1	2	5	0	0	0	5
Nymphalis xanthomelas	*nymxan*	0	0	0	0	0	0	0	0	2	0	3	5	0	0	0	5
Nymphalis polychloros	*nympol*	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4	4
Inachis io	*inacio*	1	3	0	0	4	14	6	11	6	16	11	64	5	3	8	76
Inachis urticae	*inaurt*	0	0	0	0	0	0	0	2	6	0	0	8	0	0	0	8
Vanessa atalanta	*vanata*	0	0	0	0	0	5	3	12	4	0	0	24	4	5	9	33
Vanessa cardui	*vancar*	2	5	0	0	7	1	3	1	1	12	13	31	0	0	0	38
Araschnia levana	*aralev*	0	0	0	0	0	19	15	29	30	27	16	136	7	2	9	145
Issoria lathonia	*isslat*	0	0	0	0	0	9	5	6	13	8	6	47	0	33	33	80
Argynnis aglaja	*argagl*	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	2
Argynnis paphia	*argpap*	0	0	0	0	0	94	73	96	65	64	74	466	19	9	28	494
Argynnis pandora	*argpan*	0	0	0	0	0	3	2	0	0	3	6	14	0	0	0	14
Brenthis daphne	*bredap*	0	0	0	0	0	4	12	7	6	0	3	32	3	7	10	42
Boloria selene	*bolsel*	0	0	0	0	0	38	31	0	0	2	4	75	0	0	0	75
Boloria dia	*boldia*	0	0	0	0	0	20	32	0	0	0	3	55	0	0	0	55
Melitaea phoebe	*melpho*	0	0	0	0	0	4	0	0	3	26	18	51	10	14	24	75
Melitaea athalia	*melath*	0	0	1	0	1	28	22	0	0	13	7	70	1	10	11	82
Coenonympha pamphilus	*coepam*	79	67	32	52	230	79	81	38	54	31	58	341	14	16	30	601
Coenonympha glicerion	*coenon*	17	23	1	9	50	0	0	2	2	7	5	16	0	0	0	66
Maniola jurtina	*manjur*	12	18	16	31	77	30	45	20	47	30	45	217	25	13	38	332
Aphantopus hyperantus	*aphhyp*	0	0	0	6	6	84	53	0	0	35	29	201	4	11	15	222
Minois dryas	*mindry*	0	0	0	0	0	127	123	0	0	0	0	250	0	0	0	250
Lasiommata maera	*lasmae*	0	0	0	0	0	0	0	0	0	1	2	3	0	0	0	3
Parage aegeria	*paraeg*	0	0	0	3	3	3	0	2	5	0	0	10	4	0	4	17
Pararge megera	*parmeg*	0	0	0	0	0	0	1	6	7	2	0	16	0	0	0	16
M. galathea	*melgal*	0	0	0	0	0	0	3	0	0	0	0	3	0	0	0	3
	N	227	337	169	285	1018	809	769	369	464	443	517	3371	183	237	420	4809
	S	17	21	20	25	33	36	38	28	34	32	36	59	31	33	34	63
	Átlag N					254,5							561,833			210	400,75
	Átlag S					20,75							34			32	29,25
	Max S					25							38			33	38
	Össz S					33							59			34	63
	SDN					72,5787							182,7			38,1838	212,811
	SDS					3,30404							3,57771			1,41421	6,99513

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VEGETATION OF ULUBEY CANYON (USAK, TURKEY)

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Abstract. The phytosociological and phytoecological features of the vegetation of Ulubey Canyon in Uşak (Turkey) were investigated. The vegetation of the area was analysed by Braun-Blanquet method. Two new associations from forest and shrub vegetation were determined. The nomenclatural, floristical, and ecological features of the identified associations were evaluated. The associations and their higher syntaxa are as below:

Quercetea-*Pubescentis* Doing-Kraft ex Scamoni and Passarage 1959
Querco-Cedretalia libani Barbéro, Loisel and Quézel 1974.
Abieto-*Cedrion* Akman, Barbéro and Quézel 1977
Forest vegetation
1. Thymo lycaonico-Pinetum brutiae ass.nova.
Shrub vegetation
2. Euphorbio dendroidi-Quercetum cocciferae ass.nova

Keywords: *Ulubey Canyon,Vegetation, Phytosociology, Uşak, Turkey.*

Introduction

The bioclimatic, geomorphological and pedological diversity of Turkey, support a different vegetation types (Parolly, 1998), and also makes the concerned area significant for vegetation investigations. The data related to vegetation studies is more recent according to the floristic studies in Turkey. In the beginning of the 20th century, Handel-Mazetti in the North Anatolia and Czeczott in the Northwest Anatolia were carried out the first phytosociological studies in Turkey (Çetik, 1985).

Many syntaxonomical studies were conducted in the localities close to the research area by Ocakverdi and Çetik (1982), Vural et al. (1985), Gemici (1988), Ekim and Akman (1991), Şanda and Küçüködük (2000), Kurt (2002), Şık and Gemici (2009) and Sağlam (2013). However, syntaxonomical investigation in this study area has not been reported in literature so far.

The aim of this study is to complete the phytosociological classification of the plant formations of Ulubey Canyon and to determine their potential based on environmental factors such as climate and soil structure, and also to provide recommendations for the protection of vegetation.

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Description of the study area

Research area was located in Usak province of the Aegean Region and the Southern West Anatolia *(Figure 1).* Ulubey Canyon was bordered Usak province, Ulubey and Karahallı district. Canyon to be opened to nature tourism and is a place to be protected.

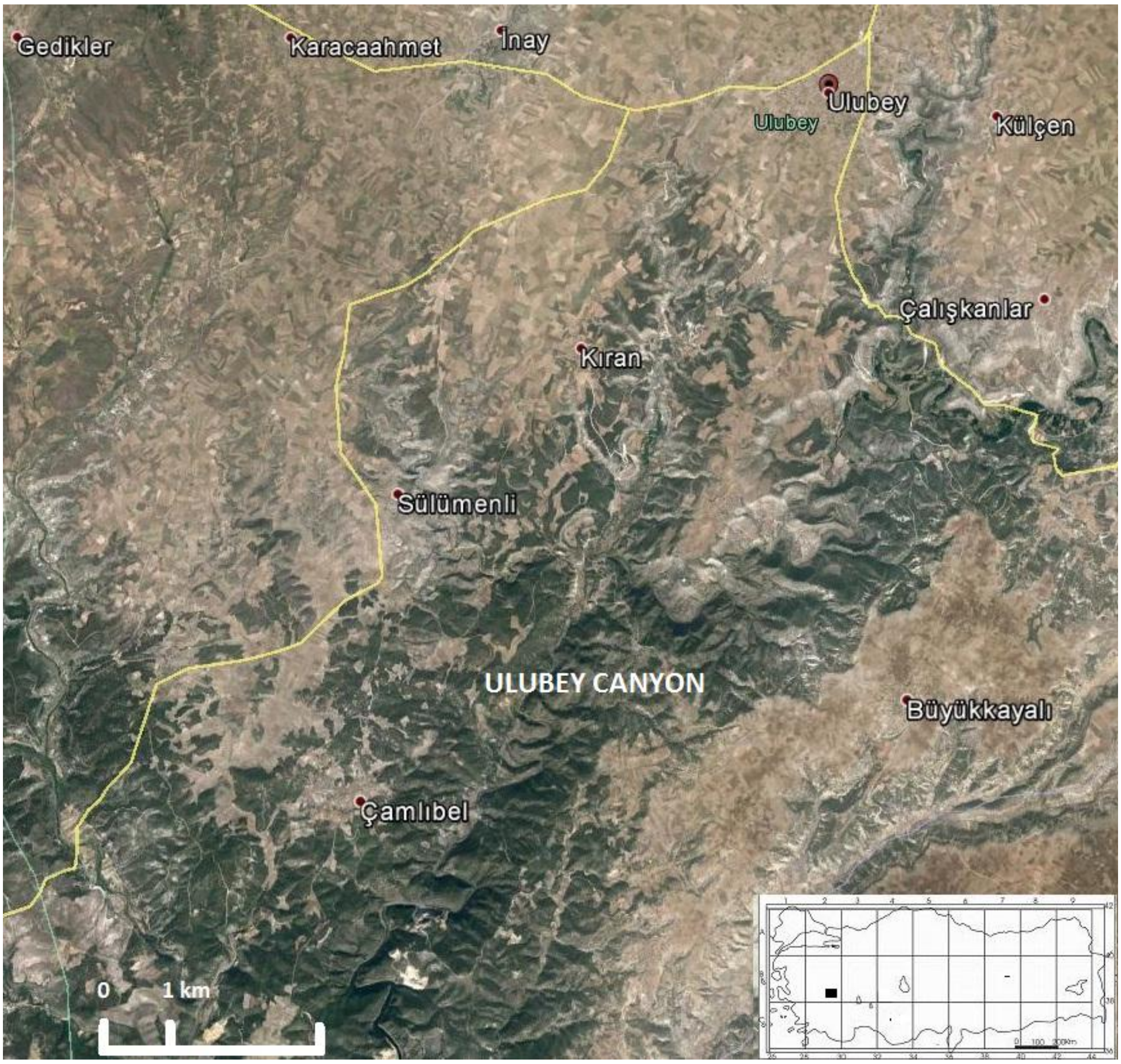


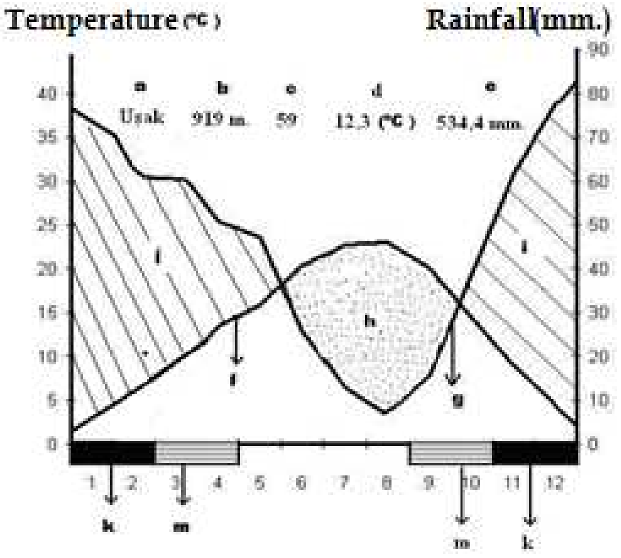
Figure 1.*Topographic map of the study area (revised from Google Earth data)*

Dokuz Sele Stream (Gain Stream) and Ulubey River canyon formed by the outer height of 750-900 meters altitude. Canyons are 100-500 meters wide, 75 km long and 135-170 meters deep. The shape of the limestone canyon system had been occured before 4.5 million by chemical and mechanical effects (Atalay, 2005).

The study area is located in B2 square according to the grid system adopted in the Flora of Turkey by Davis (Davis, 1965-1985; Davis and et al, 1988). This area is situated in the Euro-Siberian region both geographically and phytogeographically (Akman, 1993). Ulubey Canyon formation, with the collapse of the Büyük Menderes, karstic formations are associated with the process. The host rock of the area is limestone and travertine (Çetik, 1985). There are brown forest soil in area. The geographical location of the study area is under the influence of a typical Mediterranean climate in terms of natural flora and vegetation. Mediterranean climate is extratropical climate, of which has daily and seasonal photoperiodism, and collected its precipitation in the cold season (Akman,1990). Area has the type of rainy and upper cool of Mediterranean climate, and the precipitation regime of Winter-Autumn-Spring-Summer (W. A. Sp. Sm.) according to the climate data of the study area (*Table 1*). The research area has "Winter cold, semi-arid Mediterranean climate" as climatic data. Climatic data and biclimatic diagram of research area are represented in *Table 2* and *Figure 2.*

Table 1. *Meteorological data of Usak Province*

DATA	MONTHS												
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Annual
Mean temperature	2	2.9	5.7	10.7	15.6	19.9	23.2	23.3	18.7	13.1	8	4	12.3
High temperature	6.5	7.8	11.4	16.7	21.7	26.1	29.9	30.3	26.1	20.1	13.9	8.5	18.3
Low temperature	-1.5	-1	0.8	4.7	8.7	12.1	14.5	14.7	11.2	7.2	3.6	0.7	6.3
Rainfall	76.6	65.2	57.8	42.9	49.2	24.8	15.2	.9.1	16	36.4	57.7	83.5	534.4



Abbrevations
a-Location of weather station
b-The station's altitude
c-Observation period
d-The average annual temperature (ºC)
e-The average annual rainfall (mm)
f-Temperature curve (based on monthly average)
g-Precipitation curve (based on monthly average)
h-Arid period
i-Rainy period
k-Frost days
m-Frost, which may be months

Figure 2. *Bioclimatic ombrotermic diagram of Usak province*

Material and Methods

The vegetation was carried out during vegetation periods from 2010 to 2012 using Zurich-Montpellier approach (Braun-Blanquet, 1932). 80 homogenous quadrat were sampled to determine the interaction between plant communities and environmental factors. The size of the releves was determined according to the minimal area method, which was chosen 1000 m^2 for *Pinus brutia* community, 600 m^2 for *Quercus coccifera* community. Taxonomic nomenclature followed Davis (1965-1985), Davis et al. (1988), Tutin et al. (1964-1980), Güner et al. (2000, 2012), and Özhatay et al. (2009, 2011). The plant specimens collected from the quadrat were stored in the Uşak University. The new syntaxa were named by the International Code of Phytosociological Nomenclature (Weber et al., 2000). Syntaxonomical interpretations of taxa for the forest vegetation were made according to Quézel (1973), Akman et al. (1978a, 1979), Barbéro et al. (1979), and Quézel et al. (1978, 1980). The associations that contains same dominant taxon in previous studied publications on near the research area were compared by Sorensen's similarity index (1948).

The climate of the area was examined using the datas comprising the average of many years from the meteorology stations in Uşak (Anonymous, 1984). The climatic datas were applied to Emberger's formula of rain and temperature factors.

The soil samples were collected from the top 0-20 cm and 20-40 cm of the profile of the vegetation type. Soil samples were analysed in Laboratory of Soil Department, Agriculture Faculty, Ege University.

Results

Vegetation Types of Research Area

Two new associations belonging to forest and shrub vegetation have been described. The results of the analysis of soil samples from different releves are given in *Table 2* in order to give comparative details about the soils where the plant associations have developed.

The forest vegetation is the most common vegetation type in the area, and is represented by community of *Pinus brutia* between 600 and 700 meters altitude.

The shrub vegetation is represented by community of *Quercus coccifera* between 700 and 900 meters altitude.

Table 2. *Physical and chemical properties of soil samples*

		Thymo lyacaonico-Pinetum brutiae		*Euphorbio dendroidi-Quercetum cocciferae*	
Physical values	Depth	0-20 cm	20-40 cm	0-20 cm	20-40 cm
	Clay (%)	13.04	15.04	19.04	33.24
	Silt (%)	22.72	18.72	28.72	12.72
	Sand (%)	64.24	66.24	52.24	54.24
	Texture (Structure)	Sandy-Tin	Sandy-Tin	Sandy-Tin	Sandy-Clay-Loam
Chemical values	Electrical conductivity (ECx103 25 C)	367	320	269	232
	Total salt (Mikro s/ cm.)	0.023	0.020	0.017	0.015
	Lime (CaCO) %	75.50	69.79	77.66	60.22
	Phosphorus (kg/dek)	0.44	0.31	0.40	0.26
	Organic matter %	5.83	2.73	0.98	0.77
	pH	7.87	7.77	7.76	7.95

Thymo lyacaonico-Pinetum brutiae Şahin and Şanda ass. nova.

This association (holotypus: *Table 3*, quadrat number: 47) spreads locally on the Dutluca Village, Ulubey-Karahallı highway, Kazancı Stream Bridge, Hasköy hire and

Clandras hire from the canyon between 600 and 700 m altitude. This association is thought to occur by the effect of the micro-climate depends on the topography. The soil of this association has basic character (7.77-7.87), organic matter (2.73-5.33%), and clayey (13.04-15.04%) in texture. While there is no lime in the soil at a depth of 0-20 cm, the soil in depth of 20-40 cm has 69.79-75.50% of lime (*Table 2*). This association exhibits tree, shrub and herb layers. Total coverage of tree, shrub and herb layers are 80-85%, 10-15%, 30-40%, and 29-50 m, 1.2-2 m, 50–90 cm in the maximum lengths in quadrats, respectively.

The characteristic and differential species of the association are *Pinus brutia*, *Thymus zygioides var.lycaonicus* and *Teucrium chamaedrys* subsp.*tauricolum*.

Table 3. *Thymo lyacaonico-Pinetum brutiae Şahin and Şanda ass.nova.*

(Holotypus: Quadrat number: 47)

Quadrat Number	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	**Presence**
Altitude (m)	600	600	600	500	500	500	500	600	600	600	700	700	600	500	600	
Quadrat size (m²) x10	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
Exposure	E	E	E	E	NE	NE	NE	NW	NW	NW	S	S	S	SW	SW	
Inclination (º)	30	30	30	30	20	25	20	30	35	30	10	10	15	30	35	
General cover (%)	100	90	90	90	85	90	95	100	90	95	90	100	90	95	90	
Tree cover (%)	85	85	80	80	85	85	85	80	80	85	85	85	80	80	85	
Tree Length (m)	25	25	20	25	25	25	20	20	25	25	20	20	25	25	25	
Shrub cover (%)	10	10	15	10	15	10	10	15	10	10	10	10	10	15	15	
Shrub Length (m)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
Herb cover (%)	40	30	30	40	40	30	30	40	30	40	40	30	30	40	30	
Herb Length (cm)	80	90	80	80	70	50	60	80	70	80	80	70	80	50	60	
Rock									**LIMESTONE**							
Characteristic and differantial taxa of association																
Pinus brutia	44	44	44	44	44	44	44	44	44	44	44	44	44	44	44	V
Thymus zygioides var.lycaonicus	13	13	13	13	.	13	13	13	13	13	13	13	13	.	13	V
Teucrium chamaedrys subsp.*tauricolum*	1	1	1	1	1	.	1	1	1	1	.	1	1	1	1	V
Characteristic taxa of *Abieto-Cedrion*																
Cyclamen cilicium var. *cilicium*	11		11	1	1	11	.	1		11		1		1	1	IV
Thlaspi perfoliatum	1	1	1			1	.	1	1		1	1	1		1	IV
Vicia cracca subsp. *stenophylla*	1	1	1	1	1	.	1	.	.	1	1	.	1	1	.	IV
Dorynium pentaphyllum subsp. *anatolicum*	.	1	1	1	1	.	1	.	1	1	1	.	.	1	1	III
Bunium microcarpum subsp. *microcarpum*	.	11	.	11	.	11	11	11	.	.	.	11	11	11	.	III
Juniperus excelsa	33	.	34	.	.	.	33	.	33	.	.	33	33	.	.	III
Characteristic taxa of *Quercetea pubescentis*																
Quercus pubescens	13	14	13	13	13	14	13	13	13	13	13	14	13	13	14	V
Crataegus monogyna	13	13	23	23	13	23	.	13	23	.	13	13	23	23	13	V
Trifolium physodes var. *physodes*	1	.	1	.	1	1	.	1	1	1	.	1	1	.	1	IV
Coronilla varia subsp. *varia*	1	.	1	1	.	1	1	1	.	.	1	1	1	.	1	IV
Briza humilis	.	1	1	.	1	1	1	1	.	1	1	.	1	1	1	III
Thesium bergeri	.	1	.	1	.	1	1	1	.	1	1	.	.	1	1	III
Populus tremula	44	44	34	.	.	44	44	.	44	.	.	44	.	.	44	III
Paronchia argyroloba	.	1	1	.	.	1	1	1	.	.	1	1	.	.	1	III
Alyssum sitrigosum subsp. *cedrorum*	1	1	.	.	1	.	.	1	1	1	1	1	.	.	1	III
Galium peplidifolium	.	1	.	1	.	.	1	1	1	.	.	1	1	.	.	III
Buxus sempervirens	44		44		34			34		34			44	44		III
Scilla bifolia	1	1	.	.	1	.	.	1	1	.	.	1	.	1	.	III
Prunus divariaca subsp. *divariaca*	44	.	.	44	.	44	.	.	44	.	.	44	.	.	.	III
Teucrium chamaedrys subsp. *chamaedrys*	1	1	1	1	1	.	.	1	.	1	.	1	1	1	.	III
Pyrus elaegnifolia	14	14	.	.	14	.	.	14	.	.	.	14	.	14	.	II
Characteristic taxa of *Quercetea ilicis*																
Quercus coccifera	33	33	33	33	33	23	13	33	33	23	13	33	13	13	13	V
Pistacia terebinthus	11	11	12	12	2	11	11	12	.	.	11	11	.	12	12	IV
Jasmium fruticans	11	21	11	11	11	.	.	21	21	11	2	11	11	.	11	IV
Rhus coriaria	11	11	.	11	.	12	.	.	2	11	.	11	.	11	12	IV
Crataegus aronia var. *aronia*	13	13	13	.	.	13	.	.	13	13	.	.	.	.	.	III
Characteristic taxa of *Onobrychido armenea - Thymetalia leucostomi*																
Acantholimon acerosum	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	V
Ziziphora tenuior	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Scabiosa argentea	1	.	1	1	.	.	1	1	.	1	1	1	.	.	.	III
Anthemis tinctoria var. *tinctoria*	.	1	1	.	1	1	.	.	1	.	.	.	1	1	.	III
Characteristic taxa of *Astragalo-Brommetea*																
Dianthus zonatus var. *zonatus*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Globularia trichosantha	12	12	12	.	.	12	.	12	12	.	12	12	.	12	12	IV
Asyeneuma limonifolium subsp. *limonifolium*	.	.	1	1	.	1	1	1	1	.	1	1	.	1	1	IV
Astragalus ongustifolius subsp. *angustifolius*	12	12	12	.	12	.	12	.	.	.	.	12	.	.	12	III

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Thymus spyleus subsp. *spyleus*	12	.	12	12	12	.	12	.	.	12	12	.	.	12	.	III
Globularia orientalis	1	1	1	.	.	1	1	1	.	.	1	.	1	1	.	III
Euphorbia macroclada	11	11	.	11	.	11	11	.	11	.	11	.	.	11	.	III
Sideris montana	1	1	.	.	1	.	.	1	.	.	.	1	.	.	.	III
Polygala anatolica	1	1	1			1		1		1	1			1		III
Koelaria cristata	.	1	1	.	.	1	1	.	.	.	1	1	.	.	.	III
Companions																
Wiedamanniana orientalis	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Capsella bursa pastoris	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Aegilops triuncialis	1	1	1	1	1	1	1	1	1	1	1	1	1	.	1	V
Papaver rhoedas	1	1	1	1	1	1	1	1	1	1	.	.	1	1	1	IV
Scorzonera eriophora	1	1	1	1	.	1	1	1	1	1	.	1	.	1	.	IV
Bromus joponicus	1	1	1	1	1	1	1	1	1	1	1	.	.	1	.	IV
Erygium campestre	12	.	12	12	.	12	12	.	12	12	.	12	12	.	12	IV
Anchusa officinalis	.	1	1	1	.	1	1	1	.	1	1	.	1	.	1	IV
Carex flacca subsp. *serulata*	1	.	1	1	1	.	1	1	.	1	.	1	.	.	1	IV
Poa bulbosa var. *vivipara*	.	1	.	1	1	.	1	1	1	1	.	1	.	1	1	IV
Carex flacca subsp. *serulata*	1	.	1	1	1	.	1	1	.	1	.	1	.	.	1	IV
Poa pratensis	1	.	1	.	1	1	1	.	1	1	.	1	1	.	1	IV
Adonis flammea	1	.	1	1	.	1	1	1	1	1	.	1	1	.	1	IV
Centaurea solstitialis subsp. *solstitialis*	1	.	1	1	1	1	1	1	.	.	1	1	.	.	1	IV

Note: Because of the table's size, taxa of I-II presence in companions are not presented here. See the full version of Table 3 in the Electronic Appendix.

Other trees and shrubs of the associarions are as follows; *Pyrus elaegnifolia, Quercus pubescens, Juniperus excelsa, Populus tremula, Buxus sempervirens, Prunus divaricata* subsp.*divaricata, Quercus coccifera, Crataegus aronia* var.*aronia, Jasmium fruticans, Pistacia terebinthus, Rhus coriaria, Ulmus glabra (Table 3).*

Euphorbio dendroidi-Quercetum coccifera Şahin and Şanda ass. nova.

This association (holotypus: *Table 4*, quadrat number: 1) spreads locally on the Kazancı Stream Bridge, Hasköy and Calıskanlar village from the canyon between 700 and 900 m altitude. The soil of this association has basic character (7.76-7.95), organic matter (0.77-0.98%), and clayey (19.04-33.24%) in texture. While there is no lime in the soil at a depth of 0-20 cm, the soil in depth of 20-40 cm has 69.22-77.66% of lime (*Table 2*). This association exhibits shrub and herb layers. Total coverage of shrub and herb layers are 40-60%, 10-35% and 2-2.5 m, 40–80 cm in the maximum lengths in quadrats, respectively.

The characteristic and differential taxa of the association are *Quercus coccifera, Euphorbia dendroides, Dranculus vulgaris, Phlomis viscosa, Capparis ovata* var. *herbacea.*

Q.coccifera of the association after the dominant trees and shrubs; *Pyrus elaegnifolia, Quercus pubescens*. Association travertine bedrock is located on brown soil. Herb least because of the amount of overlap, the association is suitable for erosion .

Discussion

Pinus brutia community

The dominant species of the association is *Pinus brutia* and widely occured in the Mediterranean and Aegean regions of Turkey. Phytogeographically, plant associations belong to the forest vegetation that spreads along West and South Anatolia were included within the classes of *Quercetea pubescentis* and *Quercetea ilicis.* According to Akman et al., 1978a,b (*P. brutia* communities that occur in the Supra-Mediterranean zone of North-west Anatolia have been included in *Querco-Carpinetalia orientalis* and

Quercetea pubescentis. According to Akman (1995), the *Quercetea ilicis* class is represented by many xerophilic and deciduous forest species in the Mediterranean region of Turkey. The forest formations belong to 0-1000 meters Mediterranean Vegetation Zone are included in *Quercetea ilicis* class. This association should be included in *Quercetea pubescentis* Doing-Kraft 1955 class, *Querco-Cedretalia libani* Barbéro, Loisel, Quézel, 1974 order, Abieto-Cedrion alliance due to environmental conditions of the stands occupied by this association, because of floristic properties like high cover and presence values of diagnostic species of these upper syntaxa units. Bioclimatic terms, pine, semi-arid mediterranean climate on the floor (which is quite rare here), rainy and very wet floors in the Mediterranean climate of hot, cool and cold types spread. This kind in Turkey, may develop on different parent material. Generally, marl and marly limestone rocks are dominant over, but Amanos and ophiolitic rocks in the Toros Mountains (serpentine, gabbro, peridotite) also develop on. Hard limestones, sandstones and shales, do not develop (Akman, 1995).

Also, *Pinus brutia* communities in Turkey are connected to different plant sociology.

1. Red pines are located in a warm and genuine Mediterranean are connected to **Quercetalia ilicis,**
2. Located in the upper Mediterranean red pines are connected to **Querco-Cedretalia libani** and **Querco-Carpinetalia orientalis**.

Table 4. *Euphorbio dendroidi-Quercetum cocciferae Şahin and Şanda ass. nova*

(Holotypus: Quadrat number: 1)

Quadrat Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	**Presence**
Altitude (m)	750	750	750	800	800	850	750	800	850	900	700	750	750	900	850	
Quadrat size (m²) x10	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
Exposure	E	E	E	NE	NE	NE	W	W	W	W	NE	NE	NE	S	S	
Inclination (°)	30	30	40	60	60	60	45	45	45	45	30	30	30	15	20	
General cover (%)	60	60	60	60	65	65	60	60	65	70	60	60	70	80	85	
Shrub cover (%)	50	50	40	50	50	50	60	50	55	50	40	40	50	60	50	
Shrub Length (m)	2	2,5	2	2	2,5	2	2	2	2,5	2	2	2,5	2,5	2	2	
Herb cover (%)	10	10	20	10	15	10	10	10	10	20	20	20	20	20	35	
Herb Length (cm)	40	45	50	80	80	75	80	80	70	75	80	85	70	60	70	
Rock	**TRAVERTINE**															
Charactaristic and differential taxa of association																
Quercus coccifera	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	V
Euphorbia dendroides	12	22	13	22	12	13	12	12	12	12	22	13	13	22	22	V
Dranculus vulgaris	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	V
Phlomis viscosa	11	11	1	1	11	11	12	12	12	1	1	11	12	12	1	V
Capparis ovata var. *herbacea*	1	1	1	1	.	1	1	1	1	.	1	1	1	1	1	V
Characteristic taxa of *Abieto-Cedrion*																
Asyneuma amplexicaule	1		1		1	1	1		1		1	1		1		III
Thlaspi perfoliatum	1	1		1	1	1	1	1		1		1		1		III
Euphorbia macrostegia	13	13			12			12	12		12		12			III
Characteristic taxa of *Querco - Cedralia libani*																
Bunium microcarpum subsp. microcarpum	11	11	11	11	11	11	11	11	11	11	11	11	.	.	.	IV
Dorynium pentaphyllum subsp. *anatolicum*	1	1	1	1	1	1	1	.	.	.	.	1	1	.	.	III
Vicia cracca subsp. *stenophylla*	1	.	1	.	1	.	.	1	.	.	1	.	1	.	1	III
Viola modesta	1	1		1		1			1			1				III
Characteristic taxa of *Querctea pupescentis*																
Quercus pubescens	44	34	33	33	34	44	.	34	34	34	44	33	44	.	34	V
Pyrus elaeagnifolia	14	14	.	13	14	13	14	13	14	.	14	13	13	13	.	V
Coronilla varia subsp. *varia*	1	.	1	.	1	1	1	.	1	1	1	.	1	1	1	V
Paliurus spina-christi	33	34	34	44	34	44	34	34	34	33	34	44	34	44	.	V
Alyssum sitrigosum subsp. *cedrorum*	1	1	1	1	1	1	1	1	1	1	.	.	1	.	.	IV
Briza humilis	.	1	.	1	1	1	1	1	.	.	1	1	1	.	.	IV
Trifolium physodes var. *physodes*	1	1	1	.	.	1	.	1	.	1	.	1	1	1	1	III
Thesium bergeri	1	1	1	.	.	1	1	.	.	1	1	.	1	1	.	III
Scilla bifolia	1	1	.	.	.	1	1	1	.	.	1	1	.	.	.	III
Paronchia argyroloba	.	.	1	.	1	.	.	.	1	.	.	.	1	1	.	II

Galium peplidifolium	1	.	.	1	.	.	1	.	.	1	1	.	1	.	.	II
Teucrium chamaedrys subsp. *chamaedrys*	1	.	1	.	1	1	.	.	1	1	.	.	.	.	.	II
Cotinus coggygria	34	.	.	34	.	.	.	34	.	.	.	.	.	34	.	I
Characteristic taxa of Onobrychido armenea - *Thymetalia leucostomi*																
Acantholimon acerosum	12	12	12	12	12	12	12	12	12	12	.	.	.	12	.	IV
Hedisarum varium	.	1	1	1	.	1	1	.	1	1	1	.	1	1	1	IV
Anthemis tinctoria var. *tinctoria*	1	1	1	1	.	.	1	1	.	1	1	1	.	1	1	IV
Onosma aucheranum	1	1		1	1			1	1		1	1	1		1	IV
Scabiosa argentea	1	1	.	.	1	.	1	.	.	1	.	1	.	.	1	III
Phlomis armeniaca	.	1	.	1	.	.	.	1	.	.	.	.	1	.	.	III
Characteristic taxa of Astragalo- Brometea																
Dianthus zonatus var. *zonatus*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Ziziphora tenuior	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Lappula barbata	1		1	1	1		1		1		1	1		1	1	IV
Ziziphora capitata	1	1		1		1	1		1	1		1	1	1		IV
Globularia orientolis	1	.	1	.	.	.	1	1	.	.	1	.	.	1	.	III
Anthemis cretica subsp. *albida*	1	.	.	1	.	1	.	1	.	.	1	.	.	.	1	II
Companions																
Rosa canina	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	V
Verbascum cheiranthifolium	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	V
Globularia trichosantha	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	V
Valerianella alliarifolia	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Wiedamanniana orientalis	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Moenchia mantica subsp. *mantica*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Capsella bursa pastoris	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Knautia integrifolia var. *bidens*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Poa bulbosa var. *vivipara*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Carduus nutans	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Euphorbia macroclada	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	V
Lotononis genistoides	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	V
Dactylus glomerata	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	V
Crataegus monogyna	13	13	13	13	13	.	.	13	13	13	13	13	13	13	13	IV
Centaurea solstitialis subsp. *solstitialis*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	.	IV
Adonis flammea	1	.	1	1	1	1	1	.	.	.	1	1	1	1	1	IV
Erygium campestre	12	.	12	12	.	12	.	12	12	.	12	12	12	.	.	IV
Anchusa officinalis	1	1	1	.	.	1	1	.	.	.	1	.	.	1	1	IV
Geranium robertianum	1	.	1	.	1	1	1	1	.	.	1	1	1	.	.	IV
Hypericum cerastoidea subsp. *confertum*	1	.	.	1	.	.	1	.	1	1	1	1	1	.	.	IV
Hordeum bulbosum	1	.	.	1	1	1	.	.	1	1	1	.	1	1	1	IV

Note: Because of the table 's size, taxa of I-II presence in companions are not presented here. See the full version of Table 4.in the Electronic Appendix.

Accordingly, the Mediterranean and Aegean regions red pine communities, **Quercetalia ilicis**, Aydin-Eskişehir-Adapazari passing over the line in the northwest red pine communities, **Querco-Carpinetalia orientalis** and that line the southeast with the high areas red pine communities, the **Querco-Cedretalia libani** is connected to.

Real Mediterranean cypress floor, the top floor of the Mediterranean to the red pine trees, crossing six major changes in the flora forest manifests itself. Chamaephytes nanofonerofit and the characteristics of the Mediterranean gariga (except *Juniperus oxycedrus* subsp. *oxycedrus* and *Quercus coccifera*) in place to gradually leaves quite abundant herbaceous species.

Thymo lyacaonico-Pinetum brutiae should be included in *Quercetea pubescentis* Doing-Kraft 1955 class, *Querco-Cedretalia libani* Barbéro, Loisel, Quézel, 1974 order, Abieto-Cedrion alliance due to environmental conditions of the stands occupied by this association, because of floristic properties like high cover and presence values of diagnostic species of these upper syntaxa units. Floristic similarity between the ***Thymo lyacaonico-Pinetum brutiae*** association and previously described *P. brutia* associations ranges from 5% to 18%. The highest similarity percentage (18%) was found in the study by Gemici (1986) due to its proximity to the research area. (*Table 5*). *P. brutia* community in the Ulubey Canyon should be distinguished as a new association, namely ***Thymo lyacaonico-Pinetum brutiae*** ass. nova (*Table 3*).

Table 5. *The comparison of the associations with the similar studies according to Sorensen's similarity (Is) formula [Is = 2W100 / (A + B)]. W, number of species in both sites; A, number of species in first site; B, number of species in second site.*

Previous investigations	**Associations** *Thymo lyacaonico-Pinetum brutiae*	 *Euphorbio dendroidi-Quercetum cocciferae*
Seçmen(1977) Nif Mountain(İzmir)	5	
Gemici (1986) Akdağ (Çivril-Sandıklı-Dinar)	18	
Şık (1992) Yunt Mountain (Manisa)	8	
Aksoy (1992) Mahmut Mountain (Kemalpaşa)	7	
Serin ve Eyce(1994) Aladağ (Hadim-Konya)	6	5
Oluk (1999) Babadağ (Denizli)	13.5	9
Serin (1996) Dedegöl (Anamas)		8.3

Quercus coccifera community

Quercus coccifera (kermes, pirnal oak) wide spread on Mediterranean maquis with a wide distribution across the Mediterranean Basin. The structure and the floristic composition of *Q. coccifera* have been identified in Mediterranean shrublands of Greece by climate and the anthropogenic factors (Tsiourlis et al., 2009). *Quercus coocifera* community of Ulubey Canyon is wide spread, particularly in travertine areas of the research area. Numerous steppic and xerophilous species penetrated into this association. *Quercus coocifera* belonging to *Quercetalia ilicis* from Eumediterranean and *Quercetalia pubescentis* from Submediterranean zone. *Q. coccifera* associations described in Aladağ (Serin and Eyce, 1994), in the east of Dedegöl (Anamas) Mountain (Serin, 1996), in Babadağ (Oluk, 1999) were categorised under the *Querco-Cedretalia libani* order. The floristic composition of ***Euphorbio dendroidi-Quercetum coccifera*** is well presented by the characteristic species of the order *Querco-Cedretalia libani* and the class *Quercetea pubescentis*. For these reasons, the association should be belonged to order *Querco-Cedretalia libani* and the class *Quercetea pubescentis*. The similarity percentages between the associations described in the present study and those of the other, previously defined areas (*Table 5*) range from 5% to 9%. The highest similarity percentage (9%) was found in the study by Oluk (1999) due to its proximity to the research area.

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APPENDIX

Electronic Appendix: Full versions of Table 3 and Table 4

EFFECT OF LAND USE CHANGE ON LAKE WATER QUALITY IN DIFFERENT BUFFER ZONES

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Abstract. Most previous studies are based on one year data, it is insignificant and not enough to demonstrate the correlation between land use change and water quality. Such studies have also focused on a single buffer zone, whose influence on water quality is minor compared with that of different buffer zones. According to the above-mentioned problems, we chose Meiliang Bay of Taihu Basin in China as the study area. Meiliang Bay was one of the Taihu lake divisions that faced with serious eutrophication problem, and algae blooms happened from 1987. Five buffer zones were established within 10km catchment along the lakeshore. The relationship between land use and water quality was identified with an exponential model based on 10 year data from 1995 to 2006(data of 1996, 1997 were missing). At last, we analyzed the difference among buffer zones. Finding that the effects of land use types on water quality mainly reflected in 2km and 4km buffer zones, and the effects of spatial patterns mainly reflected in 8km buffer zone. And we also found that the critical factors had a big difference in each buffer, constructed land and farm land were always the critical factors in buffer 2 and buffer 3, but it did not always happen in other buffers. Moreover, this study could also provide practical basis for the critical lake conservation region identification and recognition.

Keywords: *land use change, lake water quality, Meiliang bay, effective buffer zones, critical factors*

Introduction

The rapid economic development and the accelerated urbanization in recent decades have resulted in the serious degradation of water quality in China, where the deterioration of lake water quality is a typical environmental problem. In particular, the deterioration of lake water quality in Lake Taihu Basin, as a region with a relatively developed economy and high land use intensity, is rather serious.

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A significant correlation exists between land use and water quality (Solbe, 1986; Mander et al., 1998; Su et al., 2012; Wan et al., 2014), and this correlation is influenced by land use types and their spatial patterns. Extensive research has been conducted on the relationship between land use and water quality in the watershed scale (Jun, 2011; Seeboonruang, 2012; Jun, 2013). Seeboonruang (2012) found that different land use types have different effects on water quality. Basnyat (1999) and Wang (2001) found that industrial land and agricultural land decrease water environment quality and forest land and grassland have a negative influence on water pollutant concentration. Silva and Williams (2001) found that urban area and residential land have larger influence on water chemistry than other land classes. Conversely, Tong and Chen (2002) found that forest land contains lower inorganic ions and plays an important role in relieving the degradation of water quality. Silva and Williams (2001) found that farm land greatly influences the concentrations of NO^{3-} and $SO4^{2-}$.

In the watershed scale, considerable research has confirmed the correlation between land use and water quality (Hanratty and Stefan, 1998; Brezonik and Stadelmann, 2002), but a controversy exists on the effect of land use on water quality in different buffers. Jun and Xia (2008) considered that a significant difference exists on the relationship between land use and water quality among different buffers. They found that Farm Land significantly, negatively influences the specific conductance (SC) concentration in the watershed scale but significantly, positively affects the SC concentration in 200m buffer. Sawyer (2004) found that the land use in 30m buffer has larger influence on water quality than the whole watershed for it has better correlation between the buffer and water biological community structure change. However, Hunsaker and Levine (1995) found that the correlation between land use and water quality in the watershed scale is stronger than in 200m or 400m buffer. Similarly, Silva and Williams (2001) showed that the effect of land use change on water quality in the watershed scale is stronger than in 100m buffer. By contrast, Xiang (1995) found that land use has a significant influence on water quality only in 8m to 175m buffer.

These inconsistent conclusions are attributed to various reasons. First, land use pattern is of great difference in various research regions. Second, the use of different water quality indices may contribute to diverse results. Nevertheless, studies have agreed that the effect of land use on water quality varies in different buffers (Hanratty and Stefan, 1998; Amiri et al., 2009; Guo et al., 2010; Jun, 2011). Numerous previous studies, however, have used a single buffer as the research region, making no in-depth comparative analysis of the effect of land use on water quality in different buffers. Many studies are also based on one year data, but the land use change in a year is insignificant and is not enough to illustrate the relationship between land use change and water quality (Hanratty and Stefan, 1998; Sawyer et al., 2004; Guo, 2010). To solve these problems, the current article is based on multiple buffers and multiple time series data of land use. Furthermore, we use a stepwise regression model to show the correlation between land use and water quality in different buffer zones.

Our objectives are (1) to identify the critical factors in different buffer zones and (2) to determine the differences among buffer zones on land use change and water quality.

Materials and Methods

Study Area

Taihu Basin is a region with the most concentrated population, the most developed economy, the most intensive industry, and a high urbanization level in China. Algal bloom has occurred frequently in this area, particularly in Lake Meiliang (Ma, 2008), in recent years. The degradation of Lake Taihu water quality is rather serious. The algal bloom outbreak in Lake Meiliang in 2007 led to water crisis that affected more than 200 people in Wuxi, caused great economic losses, and generated extensive concern from society.

Given that 10km area of Meiliang lakeshore is located in Lake Taihu Basin, we chose this lakeshore as the study area to compare the land use effects on water quality among different distances (*Figure 1.*). The study area was divided into five buffers, namely, 2km (buffer1), 4km (buffer 2), 6km (buffer 3), 8km (buffer 4), and 10km (buffer 5).

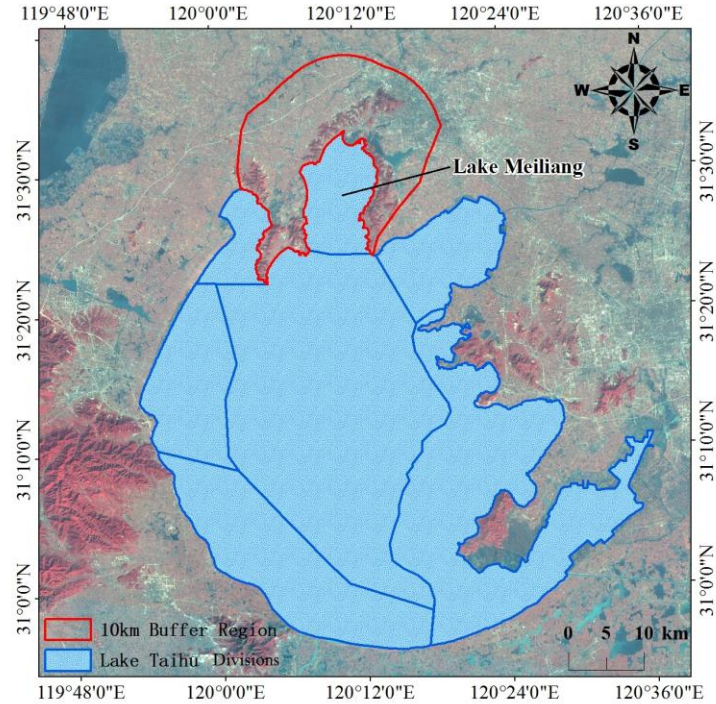

Figure 1. *Location of study area. Lake Taihu can be divided into 7 parts according to Taihu Basin authority. Lake Meiliang is a part of Lake Taihu and is located on the north of Lake Taihu*

Land Use Classification

Land use data were extracted through a 30m TM image by object-oriented method interpretation. The data were from 1995 to 2006 (1996 and 1997 data are missing). Owing to the low resolution of the TM image, and considering that automatic interpretation results will have a large error, we modified the interpreted results for many times after mining points in the field. The modified classification system employed the following six general categories: original forest (OF), artificial forest (AF), constructed land (CL), Farm Land (FL), wetland (WL), and other land (OL). OF includes evergreen broad-leaved forest, deciduous broad-leaved forest, and evergreen coniferous forest; AF includes tree garden, shrubs, garden land and planted economic forests, green belts, etc.; FL includes paddy field and dry land; CL includes residence, quarry, industrial land, traffic land, and commercial land; WL includes reservoir/pits, lakes, rivers, canals, and channels; OL includes grassland and bare land.

Land use change includes the change of land use types and spatial patterns. Area percentage was used to characterize the change of land use type, and landscape indices were used to characterize the change of spatial pattern. Fragsats 3.0 software was used to extract the representative indicators, Shannon diversity index (SHDI), Shannon evenness index (SHEI), and fractal dimension (FRACT). SHDI reflects the landscape heterogeneity, is especially sensitive to the unbalanced distribution of various patches, and reflects the complexity and variability of all kinds of patches. SHEI reflects the homogeneous degree of landscape component distribution. FRACT is a measure of complex shape irregularity.

Determination of Water Quality Indicators

The main land use types are CL, FL, and WL, and the main components of the land runoff pollutants included total nitrogen (TN), total phosphorus (TP), NH_3-N, and dissolved oxygen (DO) (Basnyat et al., 2000; Bhaduri et al., 2000). The monthly average monitoring data used were from the Taihu Basin authority. Five monitoring stations locate in Lake Meiliang (*Figure 2.*). The data on water quality were scattered points, and the average value was acquired. We selected five water quality indicators, namely, TN, TP, NH_3-N, DO, and chemical oxygen demand (COD).

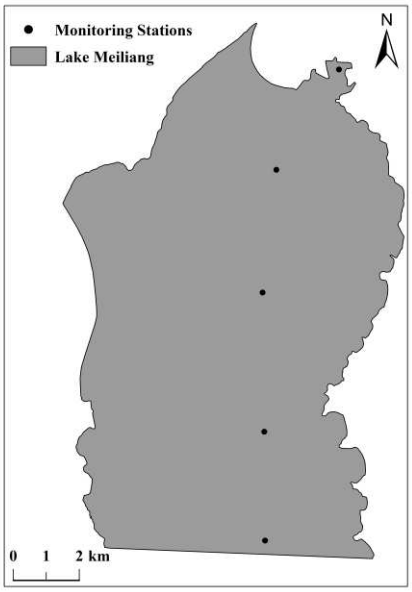

Figure 2. *Distribution of monitoring stations in Lake Meiliang*

Building a Correlated Model of Land Use and Water Quality

The land use indicators included six land use types, i.e., the area proportions of Original Forest, Artificial Forest, Constructed Land, Wet Land, Farm Land, and Other Land, and three landscape parameters, namely, SHDI, SHEI, and FRACT. Considering that the exponential model can accurately represent the effect of land use pattern on water quality (Basnyat et al., 2000), we also use this model to build the relationship between land use and water quality. The expression is as follows:

$$WQI_i = a \times \exp(b_1 * OF_i + b_2 * AF_i + b_3 * FL_i + \cdots + b_{9*} SHEI_i) \qquad \text{(Eq.1)}$$

Where a is constant, and b1, …, b9 are the coefficients that depict the direction and strength of the relationship between land use indicators and WQI_i. When the value of b is negative, this land use type or landscape index has a negative effect on water quality. When the value of b is positive, this land use type or landscape index has a positive effect on water quality. We modeled the relationship between land use and water quality in each buffer using the stepwise regression method. The correlation coefficient (R^2) was used to compare the correlation between land use and water quality in different buffer zones.

Results

Land Use Characteristic

Six land use types were classified in this study. The classification results of land use in five buffer zones from 1995 to 2006 along the lakeshore were generated. However, only the results of land use classification in 1995, 2000, and 2006 are listed (*Figure 3.*).

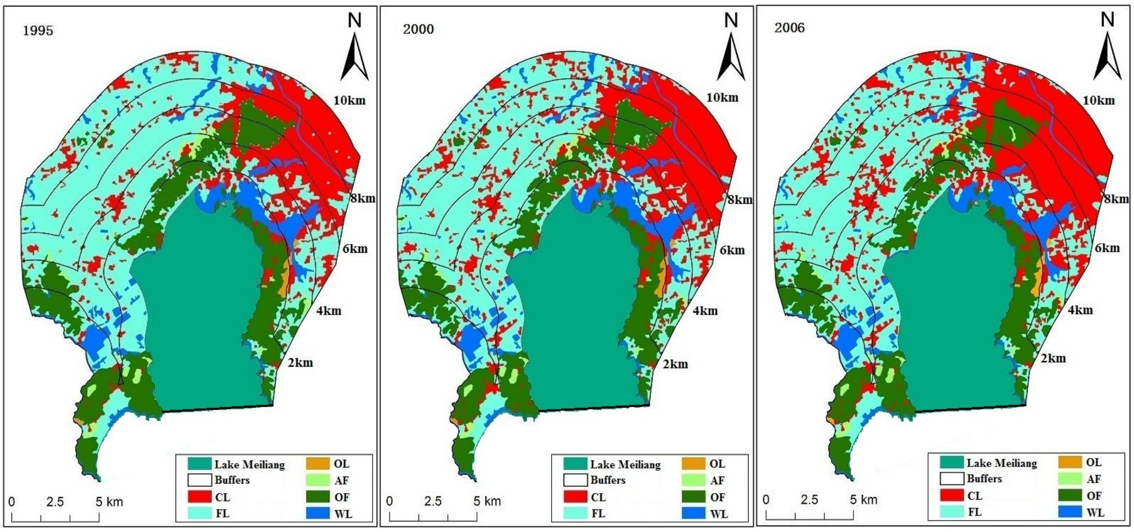

Figure 3. *Results of land use classification in 1995, 2000, and 2006*

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The land use change from 1995 to 2006 mainly embodied in the following three land use types: (1) the area of CL had increased rapidly, especially in the north of Lake Meiliang from 1995 to 2000; (2) the area of FL decreased fast in the northwest of the study area and was fragile; (3) OF was increasingly fragile, especially in the west of Lake Meiliang. The land use change was analyzed in details on two sides, namely, the change of proportions of land use types and the landscape index in different buffer zones.

With the increase of buffer distance, the change of the proportion of land use type was manifested in two main classes, namely, Constructed Land and Farm Land. However, Farm Land changed mainly because of Constructed Land; thus, we selected only Constructed Land for analysis. From *Figure 4* we can see that, Constructed Land expanded in each buffer, but a significant difference was found in various buffers, as well as in different years. For example, a proportion of CL increased by 13.27% in buffers 1 to 4 but decreased by 9.16% in buffers 4 and 5 (*Figure 4*). The proportion of Constructed Land had an irregular fluctuation in 1995 and 2000 but had a significant upward trend in 2006.

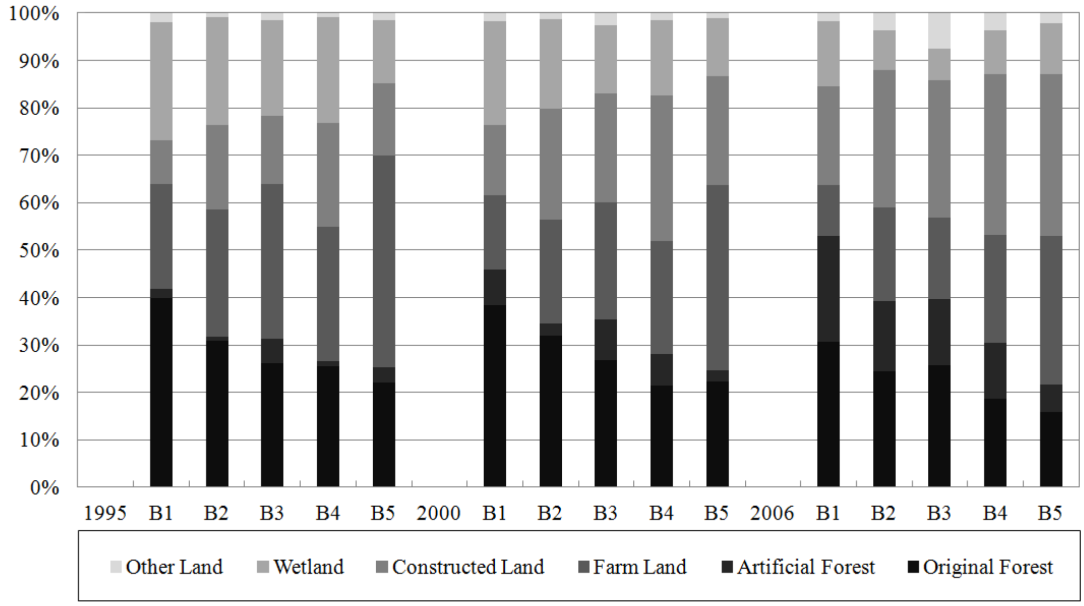
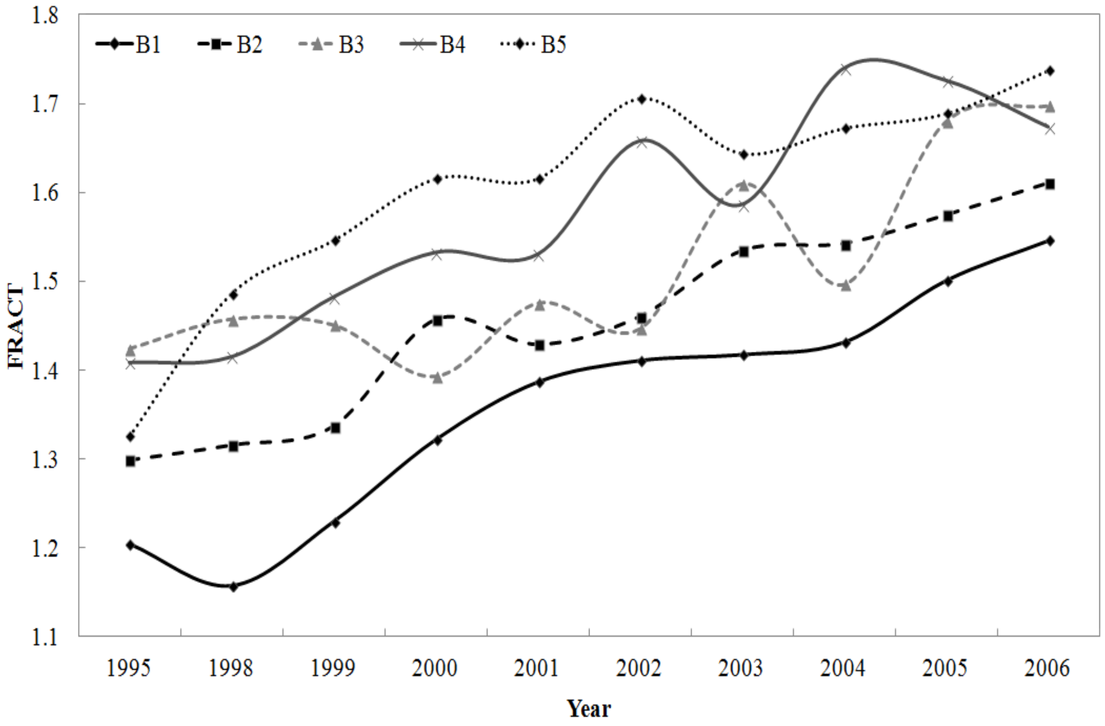
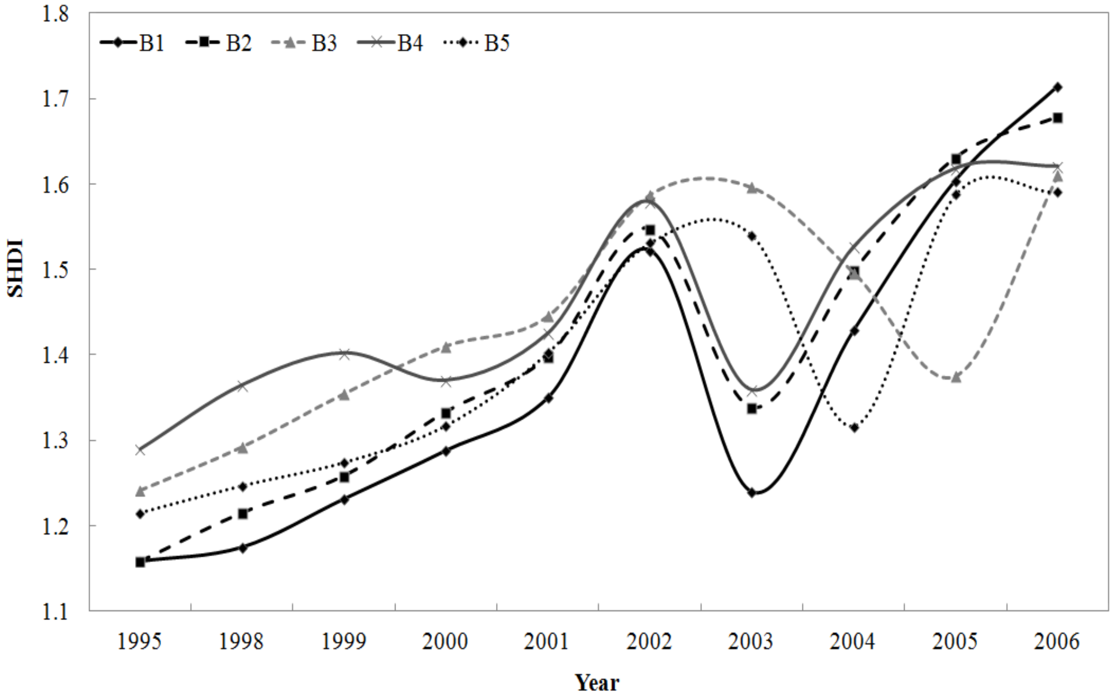


Figure 4. *Land use proportions in five buffer zones in 1995, 2000 and 2006. Land use proportions varied with increasing size of the buffer zone*

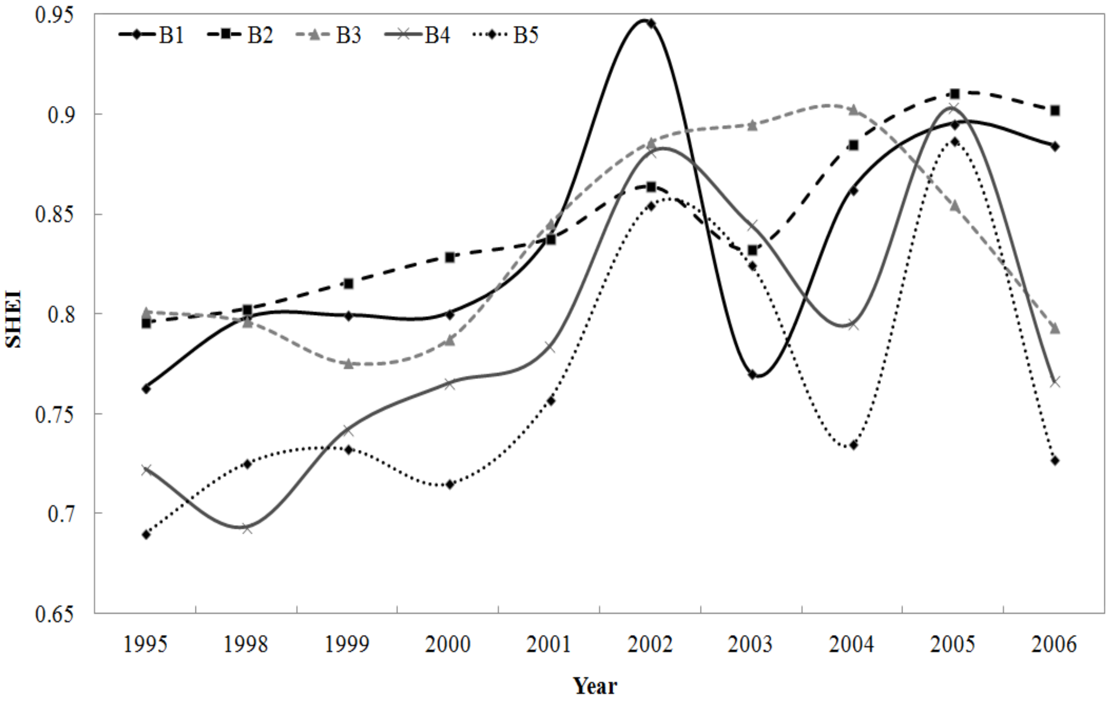
As the change of time, FRACT value indicated a gradually upward trend in buffers 1 and 2 and an irregular fluctuation in buffers 3 to 5 (*Figure 5(a)*). FRACT values in buffers 4 and 5 were higher than other buffers. The minimum value distribution differed with time. For example, the minimum value in buffer 1 was about 1.15, but it was 1.42 in buffer 3 and 1.3 in buffer 2. The symbols of SHDI in all buffers were two “V”-shaped curves (*Figure 5(b)*). For example, the minimum value appeared in buffers 1, 2, and 4 in 2003, but it appeared in buffer 3 in 2005 and buffer 5 in 2004. Compared with the other two landscape indices, the change of SHEI was more complex (*Figure 5(c)).* Except for buffers 2 and 3, we found a significant fluctuation in the curves of SHEI in other buffers.



(a)



(b)

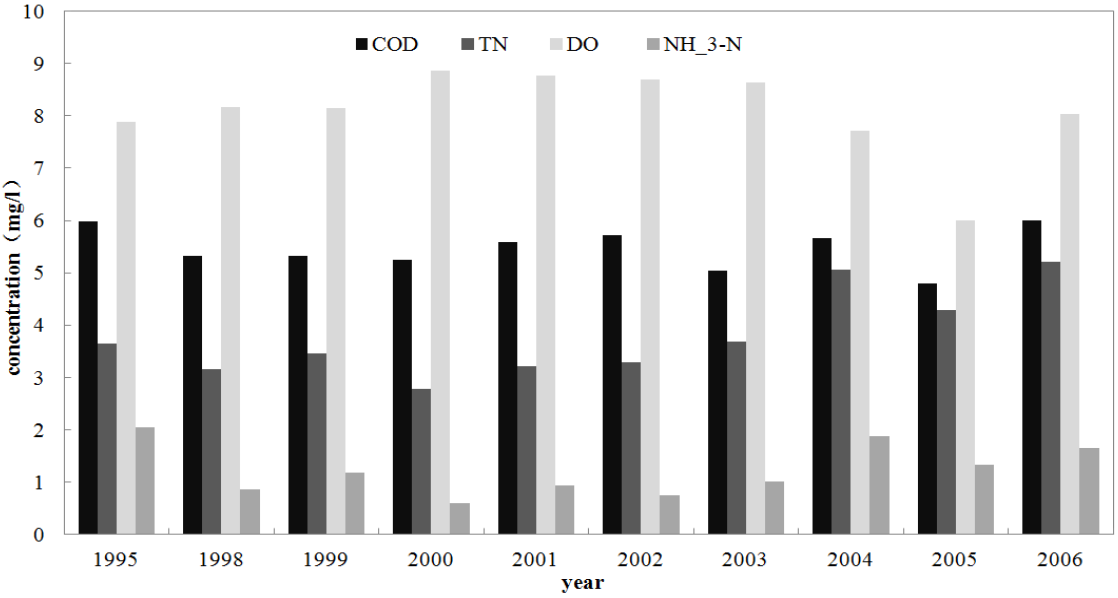


(c)

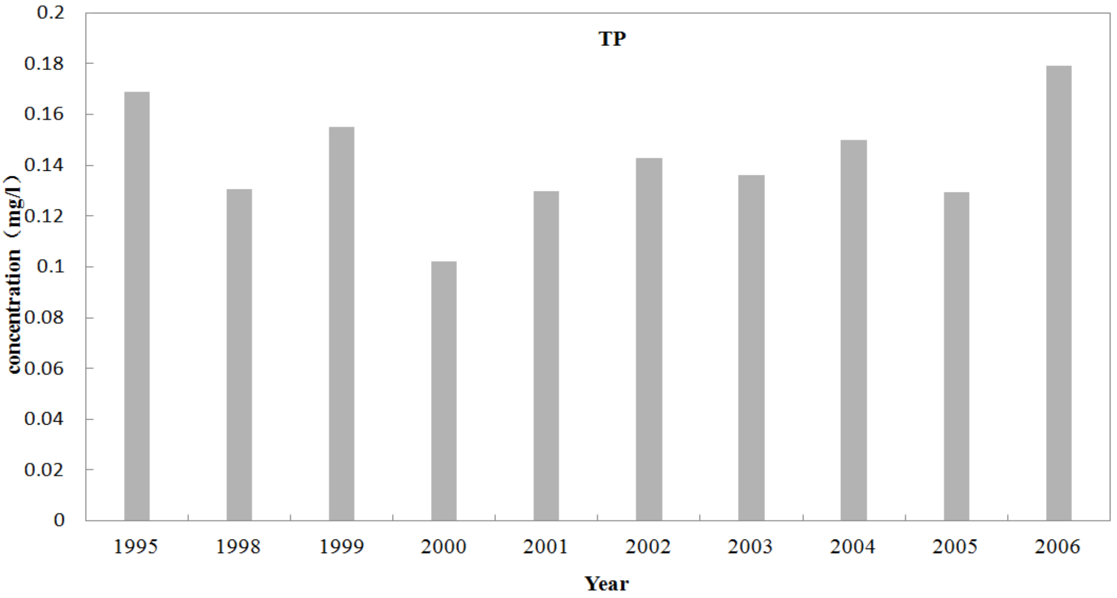
Figure 5. *Typical trends of landscape indices with the change of time in five buffer zones. Figure 5(a) is the curve of FRACT, Figure 5(b) is the curve of SHDI and Figure 5(c) is the curve of SHEI*

Change of Water Quality

In this article, we chose five indicators (*Figure 6*) to analyze the change of water quality in Lake Meiliang. The levels of TN, TP, COD, NH_3-N, and DO were over the national grade V (according to GB3838-2002 by EPA of China, 2002), and the degradation of water quality was serious (*Figure 6(a)*). The variability of these parameters in different years was remarkable. For example, the change of COD concentration values was very small from 1998 to 2002 but had a regular fluctuation from 2002 to 2006. The NH_3-N concentration values sharply dropped from 1995 to 1998 and returned to their peak in 2004. The changing trends of DO and COD were similar, and their minimum values appeared only in 2006. The NH_3-N concentration values were maximized in 2004. The TP concentration values obviously fluctuated from 1995 to 2006, the maximum value appeared in 1999 and 2004, and the minimum value appeared in 2000 (*Figure 6(b)*).



(a)



(b)

Figure 6. *Changes in the surface water quality of COD, NH_3-N, DO, TN, and TP in Lake Meiliang. Figure 6(a) is the results of COD, NH_3-N, DO and Figure 6(b) is the results of TP and TN*

Relationship between Land Use and Water Quality

In this article, we used R^2 to indicate the relationship between land use and water quality in various buffer zones (*Figure 7*) (Sliva, 2001). R^2 of COD decreased from buffers 1 to 5, that of NH_3-N decreased in buffers 1 and 2 but increased in buffers 3 to 5,

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that of DO was similar in buffers 1 and 2 and in buffers 4 and 5, that of TN appeared a minimum value in buffer 3, and that of TP appeared a maximum value in buffer 3.

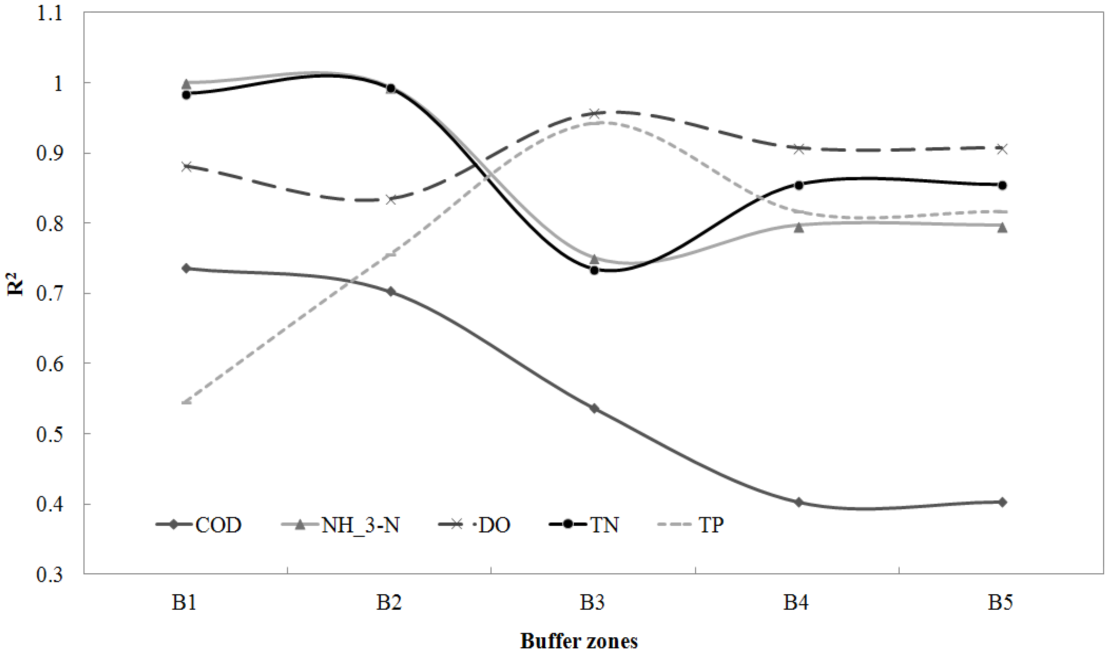

Figure 7. *R^2 curves appearing in the multiple regression model from buffers 1 to 5*

Regarding the correlation between the specific factors of land use and water quality, this study considered TN as an example. The land use factors that had a significant effect on water quality differed in various buffer zones (*Table 1*). Original Forest, Artificial Forest, Constructed Land, and Wet Land had a significant correlation with TN in buffer 1, but only Constructed Land and Wet Land had a significant correlation with TN in buffer 2. Farm Land and the SHDI of spatial pattern had a significant effect on TN. The correlation between land use types and TN decreased in buffer 4, where only Wet Land, Original Land, and FRACT had a significant correlation with TN. Buffer 5 was almost similar to buffer 4, where Farm Land had a significant effect on TN.

Table 1. *Results of multiple stepwise regression of land use change and lake water quality from buffer 1 to buffer 5 (Results are significant at $p<0.05$). The change of land use type and landscape index has a positive (expressed as a "+") and negative (expressed as a "−") effect on lake water quality.*

Buffers	**Indicators**	**Land Use Indicators**						**Landscape Indicators**					
		OF	AF	FL	CL	WL	OL	FRACT	SHDI	SHEI	R	R^2	P
B1	COD	+		-			+				0.858	0.736	0.002
	PH		-	-	-						0.976	0.952	<0.001
	NH_3-N		+		-	+					1.000	1.000	<0.001
	DO	-	-	+							0.939	0.882	<0.001

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	TN		+			+		+			0.993	0.985	<0.001
	TP			+	+	+					0.738	0.545	0.006
B2	COD			+		-	-				0.838	0.703	0.001
	PH		+			+	+				0.984	0.963	<0.001
	NH_3-N	+			-	-					0.997	0.994	<0.001
	DO			+			+			+	0.914	0.835	<0.001
	TN	-				+				+	0.681	0.463	0.015
	TP	-	+					-			0.869	0.755	0.010

Table 1. (continued)

Buffers	Indicators	Land Use Indicators						Landscape Indicators			R	R^2	P
		OF	AF	FL	CL	WL	OL	FRACT	SHDI	SHEI			
B3	COD	-			-			+			0.733	0.537	0.03
	PH					+		+	+		0.732	0.536	0.031
	NH_3-N	-				-			-		0.867	0.751	0.002
	DO						-	-	-		0.978	0.957	<0.001
	TN	-				-			-		0.857	0.735	0.001
	TP						-	-	-		0.971	0.943	<0.001
B4	COD						-		+	-	0.685	0.403	0.018
	PH				+	+	-				0.802	0.643	0.002
	NH_3-N				+	+	-				0.893	0.797	0.012
	DO				-	-	+				0.953	0.908	0.002
	TN					-	+	-			0.925	0.855	0.001
	TP						-	+		+	0.904	0.817	0.002
B5	COD						-		+	-	0.635	0.403	0.018
	PH				+	+	-				0.802	0.643	0.002
	NH_3-N				-	-	+				0.893	0.797	0.012
	DO				-	-	+				0.953	0.908	<0.001
	TN					-	+	-			0.925	0.855	0.001
	TP						-	+		+	0.904	0.817	<0.001

Discussions

Critical Factors in Each Buffer

The critical factors in each buffer are the land use indicators that significantly affect water quality. Land use has a positive (expressed as a “+”) and negative (expressed as a “–”) effect on water quality. With the increase of buffer zones, the critical factors vary.

Critical factors have two kinds, namely, land use types and landscape indices. Land use type had a significant effect on water quality in buffer 1, where Wet Land, Farm Land, and Original Forest were the critical factors (*Table 1*). *Figure 3* indicates that Original Forest was the main land use type in buffer 1 and could have cushion for pollutants. The main class of Wet Land in buffer 1 was water. This land use type is located in the east of Wuxi and directly connected to Lake Meiliang. The pollutants could easily flow into the lake through water, and Original Forest hardly controlled them. Except for TN and TP, other water quality indicators were affected by the land use types on both positive and negative sides.

Wet Land, Farm Land, and Constructed Land were the critical factors in buffer 2. Wet Land had a significant, negative effect on the water quality indicators in buffer 2, such as COD, NH_3-N, and TN. Farm Land and Constructed Land had a significant, positive effect on water quality and released pollutants, whereas Wet Land diluted and adsorbed such pollutants.

Compared with buffer 2, the effects of land use types on water quality decreased in buffer 3 and mainly acted as negative effects. Original Forest, Farm Land, and Constructed Land were the critical factors in buffer 3. Original Forest had the most significant effect on water quality and had a negative effect on COD, NH_3-N, and TN. The area of Original Forest is the largest among all the land use types and distributed along the lakeshore and could have a cushion for the pollutants from Farm Land and Constructed Land. The critical factors in buffer 4 are Constructed Land, Wet Land, and Original Land. Among them, Other Land had the most significant effect on water quality. Other Land had a negative effect on TP, COD, and NH_3-N and a positive effect on TN and DO. Buffer 5 was similar to buffer 4, where Other Land had a positive effect on NH_3-N, DO, and TN.

The critical factors of spatial patterns varied in different buffer zones. The number of critical factors increased with increasing buffer zones. For example, only one critical factor was found in buffer 1, but approximately two critical factors were found in buffers 2 to 5 (*Table 1*). FRACT was the only critical factor that had a significant effect on water quality in buffer 1. SHEI and FRACT had a critical effect on water quality in buffer 2, and the critical factors in buffers 3 to 5 were FRACT, SHDI, and SHEI. The reason for this result was that more land use types exist in buffers 3 to 5 than in buffers 1 and 2 (*Figure 3*). Farm Land and Constructed Land were the main land use types in buffers 3 to 5, and the land use change mainly reflected on Farm Land and Constructed Land. The change of land use would result in the change of spatial patterns, which would cause water quality degradation indirectly.

Comparison among Different Buffers on the Effect of Land Use on Water Quality

This study found that Farm Land had a significant, positive effect on COD and TN and that Constructed Land had a significant, positive effect on NH_3-N and DO at the watershed scale (*Table 1*). Sonzogni (1980) showed that the concentrations of COD, TP, and TN from intensive agriculture and urban areas were normally 10 times to 100 times greater than those from forested and idle lands; the pollutants from these land use types would constantly flow into the lake, which would result in the degradation of water quality. Bannerman et al. (1993) and Tong and Chen (2002) found that Constructed Land areas, including commercial, industrial, street, and high-density residential land, were the main pollutant sources. Similarly, Guo (2010) showed that the proportions of rural habitation and commercial land use had a positive correlation with TN, TP, NH_3-N, COD, and Se. Our results also indicated that Wet Land had a negative effect on NH_3-N, TN, and Dissolved Oxygen. Original Forest had a negative effect on NH_3-N and TP, which could be due to the function of the classes in Wet Land and Original Forest that absorbed or retained the pollutants.

With the change of buffer zones, the correlation between land use and water quality varies. Original Forest had a significant, positive effect on COD in buffer 1, but this effect was not found in buffers 2 to 5 (*Table 1*). Original Forest had both positive and negative effects on the water quality indicators in buffers 1 and 2, but it only had a negative effect in buffers 3 to 5. Original Forest had a significant, negative effect on COD, NH_3-N, and TN in buffer 3. Farm Land had a positive correlation with Dissolved Oxygen in buffers 1 and 2, but this correlation did not exist in buffers 3 to 5. Constructed Land had a positive influence on NH_3-N, TN, and TP in buffer 1, but it had a significant effect on no more than two water quality indicators in the other buffer zones.

We used only one water quality indicator to analyze the difference of the effects of land use types on water quality among buffer zones. As TN is an important indicator that can significantly affect the degradation of water quality, we used TN to analyze the change on the relationship between land use and water quality. As shown in *Table 1*, TN was only significantly affected by Constructed Land in buffers 1 and 2, by Farm Land in buffer 3, and by Wet Land in all buffers.

The land use classification results (*Figure 3*) indicate that Original Forest, Wet Land, and Farm Land are the main land use types in buffer 1. Given Original Forest is distributed along the lakeshore and had a negative effect on water quality, this land use type could act as a buffer zone of pollutants and help protect the water quality of Lake Taihu. Farm Land, Constructed Land, Wet Land, and Original Forest exist in buffer 2, of which Farm Land and Original Forest are the main classes. As Farm Land had a significant, positive effect on water quality, the pollutants from Farm Land could flow into lake when farming activity is frequent and the use of fertilizer is unreasonable. The main land use types in buffers 3, 4, and 5 are Constructed Land, Farm Land, and Original Forest, of which the first two classes mainly had a positive effect on water quality. Although Farm Land had larger area than other classes, Constructed Land could produce a large amount of pollutants and had such a great influence on water quality as Farm Land.

Spatial patterns also affect water quality. They had an insignificant effect on water quality in buffers 1 and 2. Only FRACT had a significant, negative effect on TN. This finding might be related to the restriction of land use by the local government along the lakeshore. However, the effect of spatial pattern on water quality reached peak in buffer 3, and SHDI was the landscape index that had the most significant effect on water quality. With the diversity of land use in buffer 3, and given that this buffer zone is located in the water source protection zone set by the local government, the indicator of spatial pattern mainly had a negative effect on water quality. SHDI, FRACT, and SHEI were the critical factors in buffers 4 and 5. SHDI had a significant, positive effect on COD, and SHEI and FRACT significantly affected TP. The reason for such effects was that the proportion of Farm Land and Constructed Land increased, as well as the amount of pollutants. The land use intensity was also high and could play an important role in enhancing the effect of spatial pattern.

Overall, the effect of land use type on water quality mainly reflected in buffers 1 and 2, and the effect of spatial pattern mainly reflected in buffers 3 to 5.

Conclusions

In this study, we model the relationship between multi-temporal land use data and the corresponding water quality within 10km land area to explore the effect of land use change on the water quality of Lake Meiliang. The 10km land scope was divided into five buffer zones. We studied the differences of the effect of land use change on water quality among different buffer zones and identified the critical factors in each buffer zone.

This study indicated that land use types and spatial patterns greatly affect water quality and that a significant difference exists in different buffer zones. The effects of land use types on water quality mainly reflected in buffers 1 and 2, and the effects of spatial patterns mainly reflected in buffers 4 and 5. We found that (1) the proportions of Farm Land and Original Forest were the critical factors in buffers 1 to 3, (2) the proportion of Wet Land was the critical factor in buffers 1, 2, 4, and 5, (3) the proportion of CL was the critical factor in buffers 2 to 5, (4) the effect of spatial pattern reached peak in buffer 3, and SHDI, SHEI, and FRACT were the critical factors in buffers 3 to 5. Our study indicated that the identification of the effective buffer zones could provide new information and ideas for planning and management.

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APPENDIX

Electronic Appendix: Result Data

SOIL ORGANIC MATTER QUALITY, STRUCTURE AND ACTIVITY OF THE DENITRIFIERS COMMUNITY AS INFLUENCED BY DECAYING MULCHED CROP RESIDUES

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Abstract. Our experiment evaluated the effect of maize and peanut mulched residues upon soil organic matter and denitrifying community over a five-week incubation in soil microcosms. Soil organic matter under peanut and maize debris contrasted in biochemical quality throughout incubation, despite identical soil C contents that were significantly increased by both organic amendments. Moreover, only soluble extracts originating from the peanut treated soils could significantly fuel semi-potential denitrification. Accordingly, the composition of the *nirK* and *nosZ* communities was also specifically affected by mulch type, and the strongest impact was registered within the *nosZ* gene-bearing denitrifiers under peanut residues. Residue enriched soils displayed temporal patterns of potential denitrification activity that were also dependant on residue type. The highest overall emission rates were recorded with soils under maize mulch but they were not mirrored by direct N_2O contents of the microcosm's headspace. Overall, both soil organic matter and denitrifiers community changes were conditioned throughout the decomposition kinetics by the original quality of mulched crop residues. However, the denitrification activity was decoupled both from the composition of the denitrifying community and from the soil organic matter quality.

Keywords: *soil denitrification, mulched crop residues, dissolved organic matter, nitrous oxide*

Introduction

Nitrous oxide constitutes around 6% of the greenhouse effect (Bouwman, 1998) and contributes to the depletion of the stratospheric ozone (Tabazadeh et al., 2000). In 2005, agriculture was estimated to account for about 60% (2.8 Gt CO_2-eq yr^{-1}) of global N_2O anthropogenic emissions (IPCC, 2007). Organic fertilization with animal wastes and crop residues has been identified as a potent factor responsible for agricultural N_2O emissions. In soil, N_2O is released as a by-product mainly during the biological

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processes of nitrification and/or denitrification according to the water soil content. There is a consensual body of evidence in literature to identify bacterial denitrification as the predominant process of N_2O production when water-filled pore space values become higher than 60% (Bouwman, 1998; Bateman and Baggs, 2005).

The stimulating effects of organic fertilization on denitrification rates have been identified for long (Mosier et al., 1991; Philippot et al., 2007). The extent and temporal pattern of gaseous N losses often appear to be governed by the biochemical quality of the organic inputs, their placement and loading rates. In particular, crop residues with low C:N ratios, such as that of legume, are often considered to trigger higher N_2O emissions (Baggs et al., 2001; Huang et al., 2004; Millar et al., 2004) by delivering more readily available N compounds to soil microorganisms. Conversely, plant materials with high lignin or polyphenol contents were shown to lower these nitrogen losses by decreasing N availability for the involved microbial processes (Palm et al., 1991; Millar and Baggs 2004; Garcia-Ruiz and Baggs 2007). As soil dissolved organic matter (DOM) mainly originates from litter and humus, it can be influenced by crop residues retention and further impacts on microbial processes (Kalbitz et al., 2000; Chantigny, 2003). DOM is known to stimulate microbial growth as exemplified with the soluble fraction of labelled ryegrass residues whose carbon was shown to be incorporated into the soil microbial biomass (McMahon et al., 2005). A stimulating role of DOM in the denitrification process was already observed with mineral soils amended with DOM extracted from organic soils (Katz et al., 1985). Moreover, the *in vitro* nitrate reductase activity level of an *Azospirillum brasilense* strain proved to be dependant on the type of soluble carbon sources (Sadasivan and Neyra, 1988). More recently, a range of single C substrates of increasing recalcitrance also proved to differentially impact on soil potential denitrification (Murray et al., 2004). Several studies reporting on detritusphere, defined as the soil layer influenced by the adjacent litter, have evidenced that diverse microbial activities (e.g. respiration, xylanase, phosphatase, protease) were strongly enhanced along a sharp gradient from the soil surface in response to soluble organic substances released from the residues into the soil poral network (Gaillard et al., 1999; Kandeler et al., 1999; Poll et al., 2006). Such a stimulation of microbial activities in the detritusphere is likely to lower oxygen concentration and generate simple organic molecules that would be both beneficial to denitrification.

While numerous studies aimed to estimate the impact of organic inputs on N losses by denitrification, little research has been performed to explore the functional link between denitrification activity and denitrifying community composition and/or size. A reference *in vitro* study however clearly stated that the N_2O/N_2 ratio of N losses could be altered according to the diversity of the denitrifiers isolates (Cavigelli and Robertson, 2000, 2001). Since then a few *in situ* investigations have characterized how organic fertilization can impact the denitrifying community structure and activity (Enwall et al., 2005; Dambreville et al., 2006; Patra et al., 2006) but have failed to evidence any robust link between these community traits. Therefore, there is still a need to clarify to which extent the biochemical characteristics of organic inputs are likely to influence the denitrification rates and N_2O losses through the control of the denitrifying community composition. In particular, the ability of the organic matter pool within the detritusphere, as influenced by translocation of organic substrates from surface decaying plant debris, to influence the composition and the activity of denitrifiers has barely been investigated in mulch-based systems.

In this study, we aimed to investigate the impact of surface application of crop residues on soil organic matter quality, denitrifiers community composition and activity. We hypothesized that under non-limiting nitrate supply and low oxygen availability : (i) the biochemical quality of distinct decaying mulched plant materials would specifically drive soil organic matter and denitrifiers community features, (ii) the denitrification activity would be correlated to soil organic matter quality and denitrifiers community structure. Shifts in soil organic matter pool were monitored by means of near infrared reflectance spectroscopy (NIRS), evaluation of C content and ability of soil DOM to fuel denitrification. The *nirK* and *nosZ* genes encoding the copper nitrite reductase and the nitrous oxide reductase were used as molecular markers of the denitrifiers community. Sequence polymorphism of amplified *nirK* and *nosZ* genes fragments was revealed by means of denaturing gradient gel electrophoresis (DGGE) and restriction fragment length polymorphism (RFLP), respectively. Activity of the denitrifiers community was investigated by monitoring potential and direct N_2O emissions.

Materials and methods

Biological materials

The agricultural soil was collected during dry season in the Senegal's Peanut Basin (Thyssé Kaymor) from the 0-20 cm layer in a field previously cropped with sorghum. The soil was sieved at 2 mm, air-dried and hermetically stored at room temperature until use. It was classified as a Lixisol (FAO, 1998). Its main characteristics were : sand 55.3%, loam 34.3%, clay 10.4%, pH (H_2O) 5.8, cationic exchange capacity 5.4 meq g^{-1}, total C 0.75% and total N 0.07%. The total above-ground parts of maize (*Zea mays* L., C/N=117) and peanut (*Arachis hypogaea* L., C/N=17), both plants commonly cropped in this Senegalese region, were sampled at maturity, dried at 55°C for 24 h, grinded at 2 mm and hermetically stored at room temperature. These plant materials displayed two distinct NIRS signatures before incubation (data not shown).

Experimental design

The microcosms consisted in 120 ml glass flasks (diameter 5 cm) containing 25 g equivalent dry soil that could be sealed with a rubber septum allowing headspace sampling with a needle. The height of the soil column was around 1 cm which encompasses the size of the detritusphere whose spatial limits have previously been estimated between 3 and 5 mm away from the decaying residues (Gaillard et al., 1999, Kandeler et al., 1999; Poll et al., 2006). This parameter was chosen so as to minor the volume of soil not affected by residues. Biological reactivation of the soil, collected during the dry season, and stabilisation of its respiration rate were obtained by bringing soil gravimetric water holding capacity (WHC) to 40% (+ 3 ml H_2O per flask) for 4 weeks at 28°C before adding crop residues. CO_2 emissions were daily measured on 10 flasks initially chosen at random. Soil respiration was stabilized around 0.4 µg CO_2-C g^{-1} h^{-1} after 14 days of incubation. Soil WHC was then raised to 70% by adding 2.25 ml of a 0.2 M KNO_3 solution to all microcosms to ensure soil conditions conducive for denitrification (sub-oxic conditions, non-limiting nitrate supply). Three treatments (n=4 replicates) were then applied on these soils supplemented with nitrate: control soil without mulch, soil covered by a maize mulch and soil covered by a peanut mulch. Maize and peanut residues were equally humidified with sterile distilled water and applied onto the soil surface at a rate of 900 mg C per microcosm (ca. 1 t residue ha^{-1}). All microcosms were then assigned a reference weight and further incubated opened in a randomized design in the dark at 28°C for five weeks. Microcosm moisture was checked three times a week on a weight basis and adjusted with sterile distilled water.

Soil and gases sampling procedures

Microcosms were destructively sampled every week (n=4 per treatment, *Fig. 1*). Flasks atmosphere was renewed with several ambient air flushes and 2 x 4 ml of the microcosm headspace were then sampled in sterile 3 ml Labco® vials (Labco, Buckinghamshire, UK) for determination of initial CO_2 and N_2O levels. Flasks were then sealed air-tight prior to a 3 h incubation at 28°C in the dark. Afterwards, flasks atmosphere was mixed by withdrawing and injecting headspace gas three times. Final CO_2 content was then directly analysed and 4 ml of the headspace were sampled in a Labco® vial for determination of final N_2O level. All decaying residues were then retrieved from the flasks with tongs and spatula. Soil was homogenised with a spatula and aliquoted for the subsequent analyses.

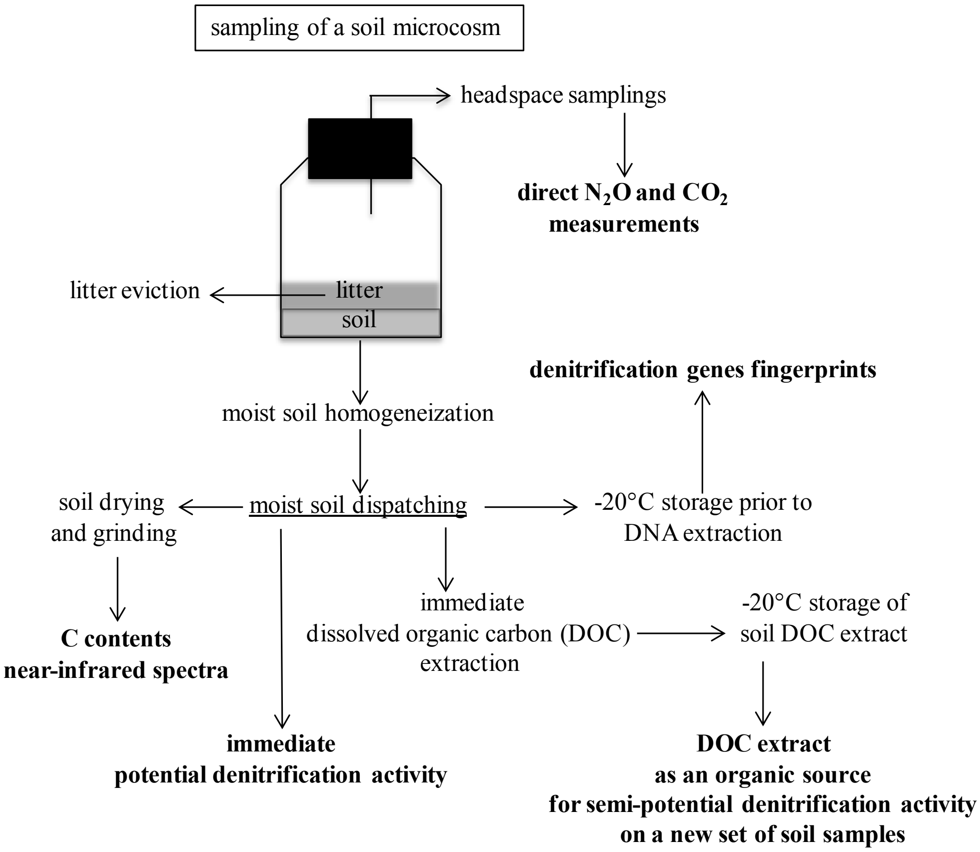

***Figure 1**. Analytical flowchart of a soil microcosm.*

Aliquots dedicated to molecular analysis were stored at –20°C before soil DNA extraction. Aliquots dedicated to C content and NIRS analyses were dried at 45°C and grinded in a mortar with a pestle. The remaining fresh soil samples were immediately processed for measurement of potential N_2O emission rates and extraction of their

DOM as follow. Moist soil (10 g equivalent dry soil) was suspended in 25 ml distilled water and shaken for 1 h at room temperature on a ping-pong shaker at 100 rpm. Soil slurry was centrifuged (8,000 × *g*, 10 min, 4°C) and supernatant was sterilized by filtration through 0.2 µm cellulose acetate Nalgene® syringe filters and stored at -20°C before use. All these DOM extracts collected each week were further used simultaneously in a modified denitrification activity protocol. Hence, microbes and particulate organic matter were excluded from these solutions by 0.2 µm filtration and neither additional substances (e.g. salts) nor heating were employed during the extraction process. Moreover, it is recognized that a rapid extraction at room temperature with distilled water enable an efficient recovery of soil DOM (Jones and Willett, 2006).

Soil physical and chemical analyses

Total C contents of grinded soil samples were determined by dry combustion with a CHN analyser (ThermoFinnigan Flash EA 1112 series) by US Imago (IRD, Senegal, Dakar). NIRS spectra, that characterize the diversity of covalent bonds in organic molecules, were used to fingerprint soil organic matter (Hedde et al., 2005). NIRS signatures of grinded dry soil samples packed into a quartz-glass cell were obtained between 1100 and 2500 nm at 2 nm intervals using a Foss NIRSystems 5000 spectrophotometer (Silver Spring, MD, USA). The data sets were averaged every five consecutive spectral points yielding 140 data points per spectrum. Spectra were processed using second derivatives and a standard normal variate transform with detrending (Brunet et al., 2007).

Direct N2O and CO2 levels in the microcosm atmosphere

Gas samples stored in Labco® vials were analysed for N_2O with a gas chromatograph fitted with an electron capture detector (CP-3800 Varian, Les Ulis, France). Column and detector temperatures were 100 and 300°C, respectively. Chromatograms were integrated by using Station Star 5.52 software (Varian). Net direct N_2O emissions over the 3 h incubation period were calculated by subtracting N_2O values obtained for initial ambient air to that of microcosms at the end of incubation. CO_2 values were obtained with a gas chromatograph fitted with a thermal conductivity detector (P200 MTI Analytical Instruments) at the start (from gas samples in Labco® vials) and at the end of the 3 h incubation (directly from the microcosm headspace). A similar calculation as for N_2O was applied to evaluate net direct CO_2 emissions.

DNA extraction and molecular fingerprints

Three out of four microcosm replicates per treatment were randomly chosen for molecular analyses. Total soil DNA was extracted using the method described by Ndour et al., (2008). Triplicate DNA extracts (from three 500 mg equivalent dry soil) were pooled for each microcosm, purified using the Wizard® DNA Clean-Up kit (Promega, Charbonnières, France) and quantified by agarose (2%) electrophoresis with a calibration curve based on serial dilutions of a calf thymus DNA standard. For all denitrification genes, PCR reactions were performed with a GeneAmp PCR System 9700 (Applied Biosystems, Courtaboeuf, France) in 25 µl mixtures elaborated with the HotStarTaq® Master Mix Kit (Qiagen, Courtaboeuf, France) and containing 10 ng

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of template DNA. To ensure the DGGE segregation of *nirK* and *nirS* amplicons, a 33 bp GC-clamp was added to the 5' end of the reverse primer. Primer pairs F1aCU/R3Cu-clamp for *nirK* gene, cd3aF/R3cd-clamp for *nirS* gene and nosZ-F/nosZ-R for *nosZ* gene were used at 1 μM (Throbäck et al., 2004). Bovine serum albumin (Promega) was added to the reaction mixture at a rate of 600 and 1000 ng μl^{-1} PCR for *nirK*/*nosZ* and *nirS* genes, respectively. Thermal profiles were similar to that of Throbäck et al., (2004) with slight modifications. The initial denaturation step was set at 95°C for 15 min to activate the DNA polymerase and annealing temperatures were 53 and 58°C for *nosZ* and *nirK*/*nirS* primers, respectively. Specificity of PCR products was checked by agarose (2%) gel electrophoresis and ethidium bromide staining. All PCR amplifications were done in duplicate, pooled and further purified with the MinElute® PCR purification Kit (Qiagen) only in the case of the DGGE analysis. The *nirS* gene could not be detected in any sample in contrast to the *nirK* and *nosZ* genes which displayed enhanced amplification rates in amended treatments. Amplicons obtained for the *nirK* gene were resolved by DGGE using 8% acrylamide gels and a gradient of 45-70% denaturant (Djigal et al., 2010). Agarose slices containing pooled *nosZ* amplicons were processed with the Wizard® SV Gel Clean-Up System (Promega). Retrieved *nosZ* amplicons were quantified by agarose (2%) gel electrophoresis and 100 ng of purified PCR products were then digested with *Alu*I at 37°C for 2 h (Mounier et al., 2004). Restriction fragments were then resolved by electrophoresis on 6% acrylamide gels (acrylamide-bisacrylamide 40%, 29:1, Sigma-Aldrich, Saint-Quentin Fallavier, France) at room temperature in 1 × TBE buffer for 7 h at 90 V - 17 mA to generate RFLP patterns. Staining of the DGGE and RFLP gels was performed with 0.5 × SYBR® Green I (Invitrogen, Cergy Pontoise, France). Scanning was described elsewhere (Ndour et al., 2008).

Denitrification activity measurements

Potential N_2O accumulation in presence of acetylene was determined on moist soil (5 g equivalent dry soil) immediately after microcosm sampling in sterilized 60 ml flasks. Flasks were sealed with a rubber septum, vacuumed for 1 min and flushed with He for 1 min. Soil samples were then enriched with 5 ml of a 0.2 μm-filtered and degassed substrate solution containing KNO_3 (0.25 mg N g^{-1} dry soil), glucose (1 mg C g^{-1} dry soil) and succinate (1 mg C g^{-1} dry soil). Ten per cent (vol/vol) of the headspace volume was replaced with an equivalent volume of acetylene to inhibit N_2O reductase activity. Soil slurries were incubated at 100 rpm and 28°C on an horizontal shaker. N_2O concentrations were monitored with the P200 gas chromatograph along a 24 h incubation period.

The ability of the soil DOM, as qualitatively and quantitatively influenced by decaying residues, to fuel denitrification (semi-potential denitrification assay) was tested on 5 g equivalent dry soil contained in flask. The soil used for this purpose originated from the initial stock but in no case from the microcosms used for testing crop residues. Hence, its chemical and microbial properties were identical between all DOM treatments. Prior to DOM spiking, it was incubated at 40% WHC and 28°C in 60 ml flasks for four weeks to lower the potential interference of autochtonous soil organic matter with the DOM assay. In this background, any DOM extract originating from a microcosm replicate of the mineralisation kinetics was individually associated to a single DOM flask replicate (n=4). All frozen DOM extracts, collected from control, maize and peanut microcosms, were thawed and applied together on soil flasks. They were subjected to the same procedure as for the above-mentioned potential denitrification activity protocol but the glucose-succinate mixture was alternatively replaced by 5 ml of the soil DOM extract. Each DOM extract was mixed with 250 µl of a sterile KNO_3 solution so as to bring 0.25 mg N g^{-1} dry soil. Lastly, four additional soil flasks were processed according to the regular denitrification activity protocol in order to compare N_2O accumulation under non-limiting C supply (i.e. glucose-succinate) to that obtained with DOM solutions.

Statistical analyses

Comparisons of C contents, CO_2 and N_2O emission rates were performed using the Tukey test ($\alpha=0.05$) in XLSTAT (v2012.6.02, Addinsoft^{TM}, Paris, France). Data were tested for normality and log-transformed before statistical analysis when necessary. Soil initial state (Day 0) refers to microcosms sampled right after the four-week pre-incubation step at WHC 40%. DGGE and RFLP fingerprints were encoded in matrices related to the presence-absence of bands and their proportional intensities using TotalLab TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Similarity of encoded fingerprints and NIRS signatures was assessed by hierarchical cluster analysis (dissimilarity matrix, Euclidian distances, Ward aggregation method) in XLSTAT. Relationships between the denitrification activity levels (N_2O accumulation in presence of acetylene) and the *nirK* community composition, or the soil organic matter quality, were evaluated by linear regressions between N_2O emission rates and principal component analysis ordinations (PCA) of DGGE fingerprints or NIRS signatures in XLSTAT (Rich et al., 2003; Rich and Myrold, 2004). Lastly, co-inertia analyses were performed to test the correlations between the molecular fingerprint and the NIRS data sets by using the ADE-4 software (Thioulouse et al., 1997).

Results

Soil chemical properties

Soil C content was lowered from 0.75 to 0.55% at the end of the pre-incubation period and was significantly increased in presence of mulched residues as soon as the first week and during the entire incubation by comparison with the control soil (data not shown). Values for control, maize and peanut treatments ranged from 0.46 to 0.53%, from 0.52 to 0.72% and from 0.72 to 0.75% during the mineralisation kinetics, respectively. No significant differences were noticed between organic treatments at any time. C contents in amended soils were constant throughout incubation (except at the last sampling with a lower C content under maize mulch (0.52%) similar to that of the control).

Soil NIRS signatures as influenced by peanut residues were completely segregated from soil spectra measured for the control and maize treatments (*Fig. 2*). Surprisingly, NIRS signatures associated with the soil amended with maize residues displayed strong similarities with that of the unamended soil. Nevertheless, the dendrogram branches representative of maize treatment spectra formed two sub-clusters that were distinct from that of control spectra. For all treatments, the topology of the branches associated with the 5 consecutive sampling dates was inconsistent with a chronological distribution pattern. Moreover, discrimination of spectral signatures associated with two contiguous decay stages was not systematically observed.

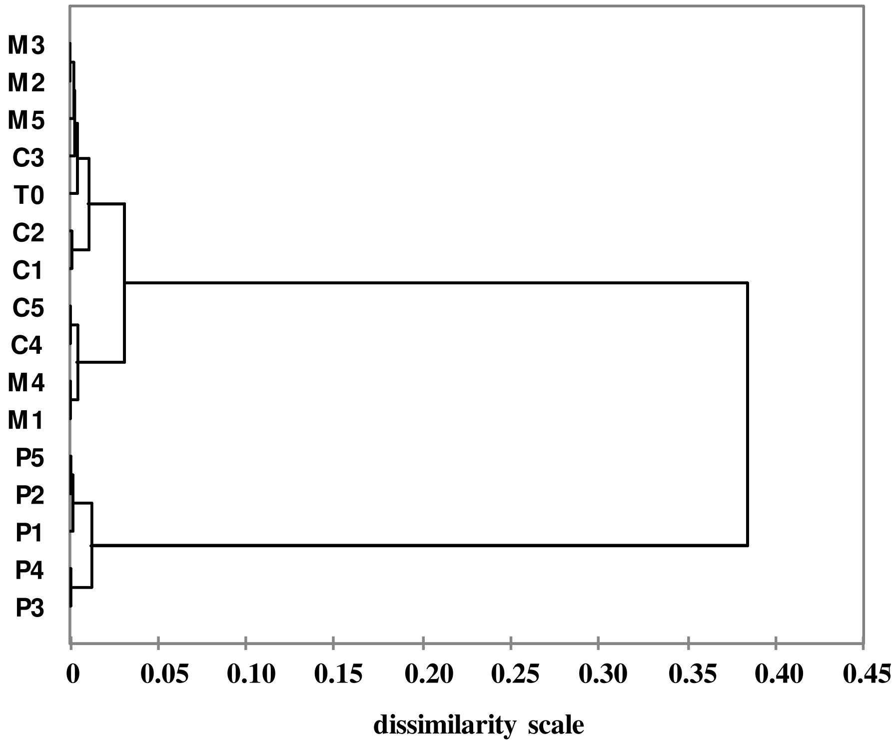

Figure 2. *Impact of mulch type and decay stage on soil NIRS signatures compared by hierarchical cluster analysis. Owing to the size of the dendrogram obtained with the complete set of data (64 branches), replicate spectra were averaged (n=4) before analysis. C : control ; M : soil under maize mulch ; P : soil peanut mulch ; 1-5 : week 1-week 5 ; T0 : initial state.*

Direct CO2 and N2O emissions

Respiration rates of unamended soils varied little throughout incubation (from 0.11 to 0.28 mg CO_2-C kg^{-1} dry soil h^{-1}) and were significantly lower than those of residue enriched soils (*Table 1*). CO_2 emission rates from organic treatments were residue specific at all times, except at week 2. The respiration profile of the peanut treatment followed a declining trend (from 6.35 to 1.66 mg CO_2-C kg^{-1} dry soil h^{-1}), while CO_2 emissions under maize peaked at the third sampling (from 2.40 to 4.53 mg CO_2-C kg^{-1} dry soil h^{-1}) and declined thereafter to 3.36 mg CO_2-C kg^{-1} dry soil h^{-1}. The highest levels were recorded in peanut microcosms during the first two weeks but from the third week, they were associated with maize microcosms.

The highest direct N_2O emission rates were systematically associated with the maize treatment, especially at the first week with an average peak value of 41 µg N_2O-N kg^{-1} dry soil h^{-1} (*Table 1*), but variation among these particular maize microcosms was high. From the second week, direct N_2O emissions in maize microcosms abruptly decreased and ranged from 5.23 to 2.00 µg N_2O-N kg^{-1} dry soil h^{-1} while the rates estimated for the control and peanut treatments fluctuated from 1.15 to 1.53 µg N_2O-N kg^{-1} dry soil h^{-1}. Emission rates were similar between control and peanut microcosms and close to that of bare soil just before mulches were applied (soil initial state). Owing to the size and shape of microcosms, the 5.23 µg N_2O-N kg^{-1} dry soil h^{-1} rate for instance would correspond to ca. 0.67 g N_2O-N ha^{-1} h^{-1}.

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Table 1. *Direct N2O and CO2 emission rates as influenced by mulch type and decay stage (mean value ± SE , n=4).Different capital index letters within a column indicate significant differences (P < 0.05) between mulch treatments for a given gas, at a given sampling date. Different lowercase index letters within a column indicate significant differences (P < 0.05) between sampling dates for a given gas and a given mulch treatment.)*

Sampling date	Treatment	Direct N_2O emissions (N_2O-N kg^{-1} dry soil h^{-1})	Direct CO_2 emissions (mg CO_2-C kg^{-1} dry soil h^{-1})
Day 0	**Initial state**	1.24 ± 0.08	0.09 ± 0.01
Week 1	**Control**	$1.23 \pm 0.06^{A, a}$	$0.15 \pm 0.01^{C, a}$
	Peanut mulch	$1.05 \pm 0.02^{A, a}$	$6.35 \pm 0.81^{A, a}$
	Maize mulch	$41.10 \pm 20.08^{A, a}$	$2.40 \pm 0.29^{B, b}$
Week 2	**Control**	$1.35 \pm 0.03^{B, a}$	$0.28 \pm 0.05^{B, a}$
	Peanut mulch	$1.15 \pm 0.02^{B, a}$	$3.91 \pm 0.61^{A, b}$
	Maize mulch	$5.23 \pm 1.38^{A, b}$	$2.56 \pm 0.07^{A, b}$
Week 3	**Control**	$1.53 \pm 0.22^{AB, a}$	$0.16 \pm 0.01^{C, a}$
	Peanut mulch	$1.15 \pm 0.02^{B, a}$	$2.74 \pm 0.28^{B, bc}$
	Maize mulch	$2.04 \pm 0.20^{A, b}$	$4.53 \pm 0.58^{A, a}$
Week 4	**Control**	$1.23 \pm 0.01^{A, a}$	$0.26 \pm 0.07^{B, a}$
	Peanut mulch	$1.18 \pm 0.01^{A, a}$	$1.52 \pm 0.14^{B, c}$
	Maize mulch	$2.00 \pm 0.38^{A, b}$	$3.64 \pm 0.56^{A, ab}$
Week 5	**Control**	$1.32 \pm 0.04^{B, a}$	$0.11 \pm 0.01^{C, a}$
	Peanut mulch	$1.46 \pm 0.28^{B, a}$	$1.66 \pm 0.29^{B, c}$
	Maize mulch	$3.10 \pm 0.57^{A, b}$	$3.36 \pm 0.14^{A, ab}$

Denitrification activity

N_2O emissions were significantly favoured in soils covered by mulched residues as compared to control soil for which no emission could be detected, except with bare soil before plant debris were applied (*Fig. 3*). Emission levels were residue specific at each sampling date and were the lowest under peanut residues during the first three samplings. The highest overall rate was associated with the maize treatment that induced an emission peak around 10 µg N_2O-N g^{-1} h^{-1} at the first stage, in contrast to 3 µg N_2O-N g^{-1} h^{-1} in the case of the peanut treatment. As N_2O emissions from control microcosms were below our detection threshold, PCA of soil NIRS signatures was only performed with spectra obtained from organic treatments. In this case, N_2O emission rates were not correlated with the PCA scores of the corresponding soil NIRS signatures (R^2=0.16 and 0.11 for axis 1 and 2, data not shown), suggesting an absence of relationship between N_2O emissions and quality of soil organic matter as influenced by decaying mulches.

The soil DOM extracted from peanut microcosms triggered the highest denitrification activities whatever the sampling date, as compared to those obtained with DOM extracted from control and maize microcosms (*Fig. 4*). These highest scores ranged from 0.29 to 0.47 µg N_2O-N g^{-1} h^{-1}. For all treatments however, N_2O emission rates were significantly inferior to that of the reference soil enriched with the glucose-succinate solution (ca. 1.2 µg N_2O-N g^{-1} h^{-1}). C contents of DOM extracts could not be measured due to methodological limitations. We speculated they could be proportional to C contents of the corresponding soil samples and in this case no correlation was found between those C contents and N_2O levels sustained by the corresponding DOM extracts (data not shown).

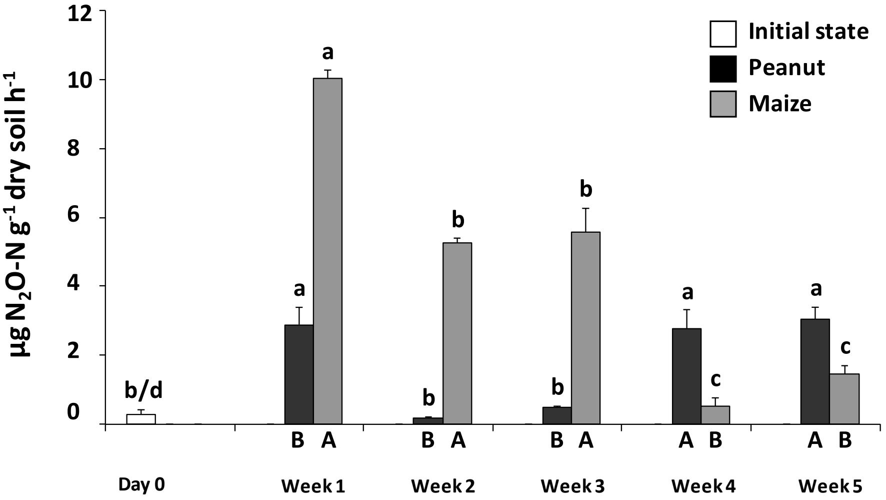

***Figure 3**. Soil denitrification activity as influenced by mulch type and decay stage (mean and standard error, n=4). No column was drawn for the control treatment as no N2O could be detected. Different capital letters at the bottom of columns indicate significant differences (P < 0.05) between treatments at a given sampling date. Different lowercase letters at the top of columns indicate significant differences (P< 0.05) between sampling dates within a given treatment. At the top of the first column (day 0), the two lowercase letters refer to the statistical comparisons between soil initial state and peanut / maize treatments, respectively.*

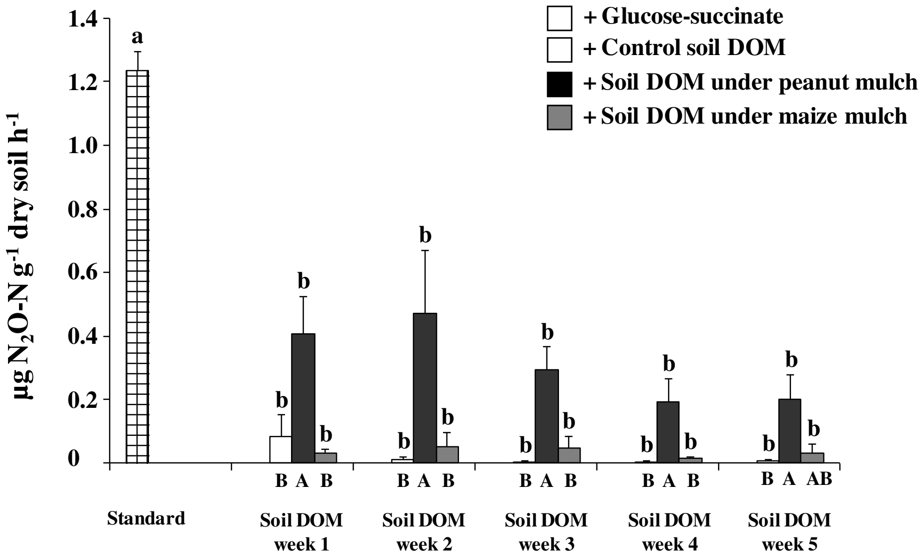

***Figure 4.** Denitrification activity of the reference soil as influenced by spiking with soil DOM extracted from control and mulch microcosms during the mineralization kinetics (mean and standard error, n=4). The hashed column stands for soil spiking with the glucose-succinate solution (non-limiting C supply). Capital and lowercase letters hold the same signification as in Fig. 3. At the top of the first column (standard), the lowercase letter refers to the statistical comparison with all treatments.*

Denitrifiers community structure

Amplification of *nirS* gene fragments from soil DNA failed with all soil samples despite attempts to improve amplification parameters, especially combinations of increased amounts of template DNA, bovine serum albumine and primers, as well as lower annealing temperatures. A similar situation occurred with the *nirK* gene but only in the case of control soil. By contrast, *nosZ* gene fragments could be successfully amplified from all soil samples.

The *nirK* gene fingerprints were not identical among replicates as observable in *Fig. 5A*. Against that background, banding patterns were segregated in two monospecific clusters corresponding to the maize and peanut treatments (*Fig. 5B*).

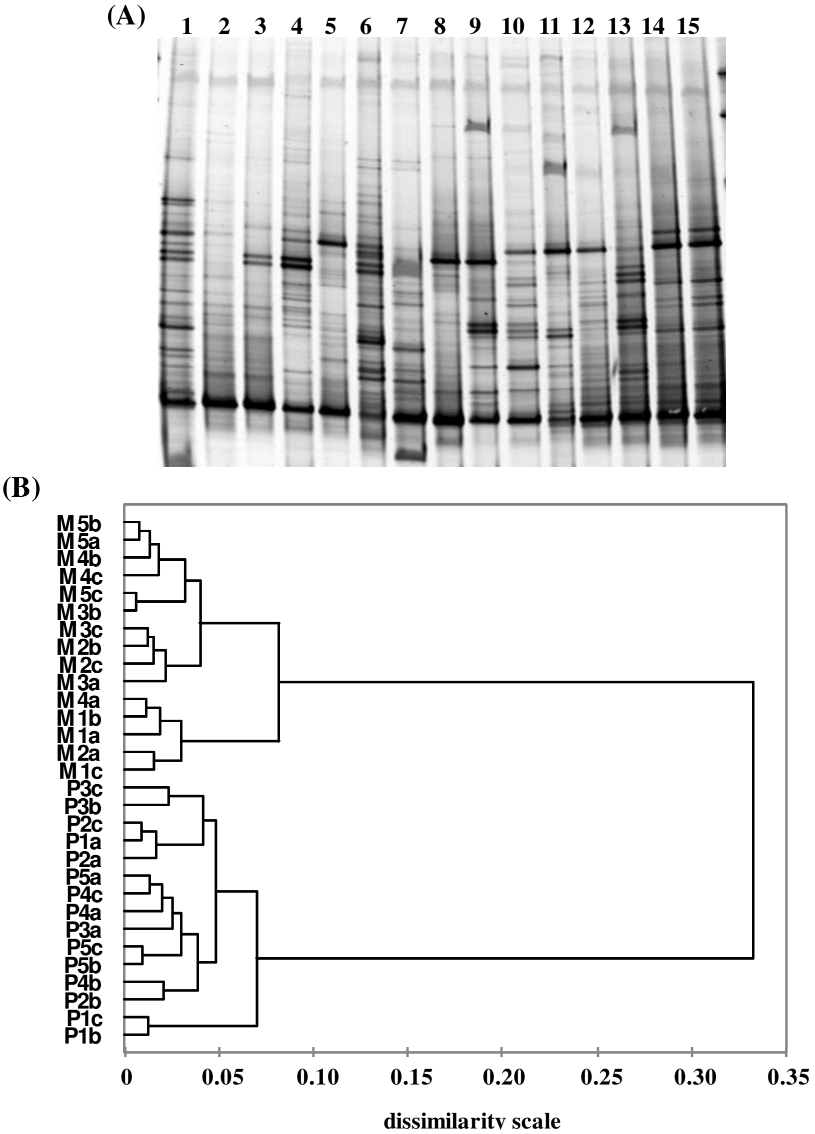

***Figure 5**. (A) DGGE migration profiles resolving the diversity of nirK gene fragments amplified from soil DNA of peanut microcosms (n=3). Lanes 1-3: week 1, lanes 4-6: week 2, lanes 7-9: week 3, lanes 10-12: week 4, lanes 13-15: week 5. (B) Impact of mulch type and decay stage on nirK-DGGE fingerprints compared by hierarchical cluster analysis. M : soil under maize mulch ; P : soil peanut mulch ; 1-5 : week 1-week 5 ; a-c : triplicate number.*

In both cases, no gradual shift of fingerprints related to the 5 consecutive sampling stages could be evidenced as the topology of each cluster did not fit a chronological distribution. N_2O emission levels from maize and peanut treatments (see *Fig. 3*) were not correlated with PCA ordination of the corresponding *nirK* fingerprints (R^2=0.24 and 0.07 for axis 1 and 2, data not shown), possibly indicating a poor link between N_2O production and the genetic structure of the copper nitrite reductase community. Co-inertia analysis revealed that the mulch type effect on soil *nirK* fingerprints was highly correlated to that on soil spectral signatures ($P<0.0001$).

The *nosZ* gene fingerprints were rather similar among triplicates as exemplified in *Fig. 6A*.

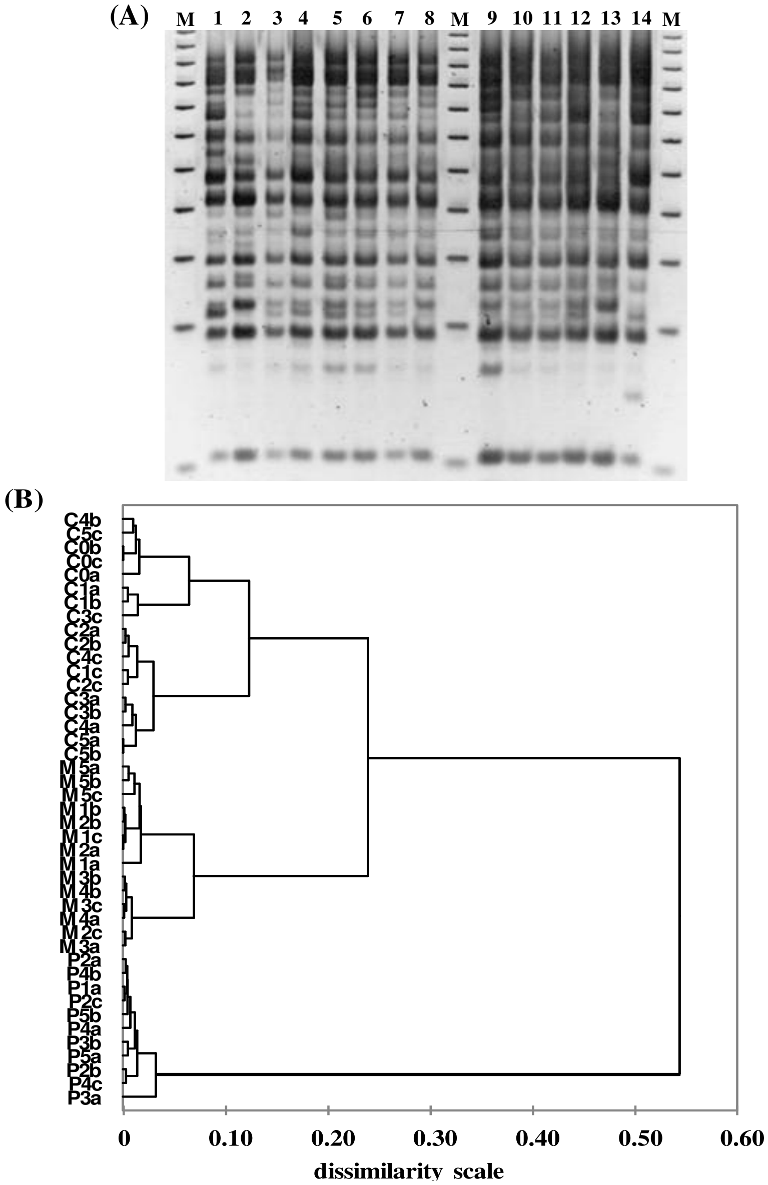

***Figure 6**. (A) RFLP migration profiles resolving the diversity of nosZ gene fragments amplified from soil DNA of maize microcosms (n=3). Lanes 1-3: week 5, lanes 4-5: week 4, lanes 6-8: week 3, lanes 9-11: week 2, lanes 12-14: week 1. M: 50 bp molecular weight marker. (B) Impact of mulch type and decay stage on nosZ-RFLP fingerprints compared by hierarchical cluster analysis. C : control ; M : soil under maize mulch ; P : soil peanut mulch ; 0 : initial sate ; 1-5 : week 1-week 5 ; a-c : triplicate number.*

The genetic structure of the soil nitrous oxide reductase community also proved to be specifically driven by the type of mulched residue as indicated by the cluster analysis (*Fig. 6B*) : the dendrogram topology displayed 3 monospecific clusters corresponding to the 3 treatments. The peanut treatment triggered the strongest structural shifts within this denitrifiers community as its cluster was much less similar to the control cluster than that of the maize treatment. Again, distribution of intra-cluster branches related to the different sampling dates was inconsistent with a chronological pattern. Additionally and in contrast to the *nirK* community, temporal fluctuations of the nitrous oxide reductase community composition were very weak as indicated by the very short lengths of the dendrogram branches. Co-inertia analysis revealed that the mulch type effect on soil *nosZ* fingerprints was highly correlated to that on soil spectral signatures ($P<0.0001$).

Discussion

Mulch effects on direct N_2O emissions

Combined organic and mineral fertilizers are known to favour soil denitrification and N_2O emissions (IPCC, 2007). In particular, the amplitude of these gaseous N losses has been characterized as plant residue quality and placement driven. Little is known however about the ecology of the denitrifiers community in the detritusphere of mulched crop residues, especially whether soil organic matter quality and denitrifiers community composition hold a functional role in these N_2O releases. Besides a strong initial N_2O emission peak under maize residues, the average emission rate associated to maize treatment over the last four stages was close to 3 μg N_2O-N kg^{-1} soil h^{-1} (corresponding to ca. 0.38 g N ha^{-1} h^{-1} owing to the microcosm size) which was almost three times higher than that of the control and peanut treatments (1.3 and 1.2 μg N_2O-N kg^{-1} soil h^{-1}, respectively or ca. 0.16 g N ha^{-1} h^{-1} equivalent). These emission levels were far below ranges estimated from field experiments. For instance, a 2.5 kg N_2O-N ha^{-1} loss was emitted over 23 days (namely 4.5 g N ha^{-1} h^{-1}) in response to field application of ammonitrate fertilizer and rye residues under zero tillage (Baggs et al., 2003). Similarly, the incorporation of wheat green manure in combination with ammonitrate fertilizer led to a maximum N_2O emission rate of 1.5 kg N ha^{-1} over 55 days (1.1 g N ha^{-1} h^{-1}) (Sarkodie-Addo et al., 2003). Under laboratory conditions, soil N_2O emissions did not exceed 3 mg N_2O-N kg^{-1} soil over 144 h (ca. 21 μg N kg^{-1} soil h^{-1}) in response to red clover or barley straw residues incorporation (Miller et al., 2008). The emissions detected within control microcosms were very likely associated with further consumption of the native soil organic C that was favoured by the initial nitrate supply. N_2O emission from bare soil was also noticed in other studies using mineral N fertilizers (Seneviratne et al., 1998; Miller et al., 2008). Here, the compositional differences between maize and peanut residues significantly impacted the direct N_2O emission patterns. Unexpectedly, the highest losses were not associated to the N rich peanut residues. This result contrasted with most studies reporting that the largest increases in direct N_2O emissions were obtained with low C/N plant debris (Larsson et al., 1998; Velthof et al., 2002; Huang et al., 2004). Here, the poor N content of maize residues was probably counterbalanced by the initial nitrate supply that provided enough mineral N to efficiently mineralize these residues (Jingguo and Bakken, 1997; Cheshire et al., 1999), leading to the build-up of a reducing power involved in denitrification of the excess nitrate. As differences

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in N_2O losses between control and mulch treatments were salient when measured in presence of acetylene but faint when directly measured in the microcosm atmosphere, it can be postulated that the N_2O reductase activity was also favoured in mulch microcosms leading to direct N_2O losses close to those of the control. Unfortunately, the denitrification activity protocol was not performed in the absence of acetylene which prevented us from estimating the soil N_2O reductase activity. However, the increased PCR rates registered for *nosZ* gene in organic treatments could indirectly indicate that this community was also stimulated, and in turn that all denitrification steps were favoured by the residues application. The N_2O reductase activity could also have been directly favoured whithin mulches as already observed with mulched tree leaves and rice debris (Seneviratne et al., 1998). Here at all samplings and mostly with peanut residues, we additionally observed a strong N_2O reductase activity within decaying mulches that were enriched with a glucose-succinate solution and incubated in presence of 0.3% exogenous N_2O that was completely converted to N_2 within 22 hours (personal data). Interestingly, the result from this complementary observation is in line with the lower direct N_2O values measured in peanut microcosms as compared to maize ones (*Table 1*). Moreover, the strongest alterations of the genetic structure of the *nosZ* community occurred in soil under peanut residues.

Mulch effects on the denitrifiers community

The soil denitrification activity increased and the denitrifiers community composition shifted in response to soil amendment with plant materials as compared to unamended soil. Moreover, the extent of these effects varied according to the quality of mulched residues and the incubation stage. Field manipulations of mineral and organic inputs were also shown to impact on these denitrifiers community traits, as exemplified with sewage sludge (Enwall et al., 2005) and composted pig manure (Dambreville et al., 2006) on *narG* and *nosZ* genes diversity. In other ecosystem types or in soil microcosms, denitrifiers were also susceptible to the variation in organic substrates availability and diversity (Hallin et al., 2006; Dodla et al., 2008; Gibert et al., 2008; Henderson et al., 2010). Here, the compositional shifts that affected the nitrite and the nitrous oxide reductase communities in amended soils were community specific in so far as the different clusters and sub-clusters of both dendrograms (see *Figs. 5B and 6B*) did not gather the same experimental units. Additionally, the dissimilarity levels between samples of a given mulch treatment were more pronounced within the *nirK* community. In line with this observation, some studies have also pointed out that the *nirK, nirS* and *nosZ* genes density or diversity could be differently impacted by disturbances like simple and complex substrates addition (Hallin et al., 2006; Henderson et al., 2010), cattle grazing (Philippot et al., 2009) or soil restoration (Smith and Ogram, 2008). This has been attributed to a niche differentiation between the *nirK* and *nirS* denitrifiers (Hallin et al., 2006; Philippot et al., 2009; Braker et al., 2012). Our data suggest that such a niche differentiation among denitrifiers is physically possible within the detritusphere that is known to house a sharp gradient of biotic and abiotic factors (Gaillard et al., 1999; Kandeler et al., 1999). In this background, our denitrification activity values and *nirK* gene profiles did not evolve in the same way. Functional attributes of the denitrifiers community composition have already been investigated in various ecosystems as exemplified recently in rhizosphere (Hamonts et al., 2013), earthworm guts (Depkat-Jakob et al., 2013) or casts (Giannopoulos et al., 2011; Majeed et al., 2013) environments. While positive relationships between *nosZ* gene diversity and denitrification rates were evidenced in forest soils (Rich et al., 2003) and in a wetland mitigation bank (Peralta et al., 2010), most of the time no such correlation could be detected with the *nosZ* gene in soil (Mounier et al., 2004; Rich and Myrold, 2004; Enwall et al., 2005; Boyle et al., 2006), the *nirS* gene in a salt marsh (Cao et al., 2008) and the *nirK* gene in soil (Wertz et al., 2009). More recently, the composition and the abundance of the *nirK*, *nirS* and *nosZ* denitrification genes were clearly connected to functional differences (i.e. *ex situ* N_2O/N_2 ratios) of contrasting organic soils (Braker et al., 2012). These opposite conclusions could mean that the strength of the link between denitrifiers diversity and denitrification rate is ecosystem specific, depending on local selection processes driven by long-term variations in environmental factors. The degree to which the denitrification activity is mainly governed by the denitrifiers diversity may also vary along the year, given that environmental parameters and denitrifiers community attributes are subjected to seasonal variation (Wolsing and Priémé, 2004; Dandie et al., 2008; McGill et al., 2010). It has been suggested that denitrifiers density rather than diversity could better correlate with this process rate (Baudoin et al., 2009; Hallin et al., 2009). Yet in several studies, no or poor correlations between denitrifiers density and activity were observed (Čuel et al., 2010; Dandie et al., 2008; Miller et al., 2008). A possible limitation of using both approaches independently is that overall activity could result from the interaction between density and composition of the denitrifiers community as overall activity can be regarded as the combination of all activities per cell unit that probably vary from one denitrifier to the next owing to a wide range of sensitivity to abiotic factors (e.g. oxygen, pH, temperature), even between closely related strains (Chèneby et al., 2000; Jones et al., 2011). Other methodological considerations restricting data interpretation were discussed by Philippot and Hallin (2005). Eventually, the recurrent difficulty to correlate denitrification activity to denitrifiers community structure could stem from methodological bottlenecks that are still limiting a routine identification of denitrifiers actively contributing to N_2O emissions. Indeed, it is not possible to confirm that all PCR detected marker genes, as depicted in DNA-based fingerprints, are expressed during the denitrification assay or during *in situ* gas sampling, as evidenced along a comparison of DNA and cDNA-derived banding patterns targeting the *nirK* and *nirS* genes (Sharma et al., 2006). However, Wertz et al. (2009) failed to evidence a strong link between denitrification activity and community composition of the active *nirK* denitrifiers determined by DGGE of *nirK* transcripts in an agricultural soil. In our case, the genetic structure of the *nirS* community, which was probably involved in the N_2O emissions, could not be determined and integrated in the functional study of the nitrite reducer community. Such unsatisfactory amplification of the *nirS* gene has also been reported in several publications using the same primers set (Dandie et al., 2008) or a distinct one (Sharma et al., 2005).

Mulch effects on soil organic matter

Besides its capacity to quantitatively predict soil or litter chemical properties such as C-N-P contents, the NIRS technique has also been shown to be sensitive enough to sort soils according to their origins on the basis of qualitative comparisons of their spectral fingerprints (Hedde et al., 2005; Velasquez et al., 2005; Chapuis-Lardy et al., 2010). Here, NIRS was also a valuable tool to discriminate soils according to their mulch cover thus highlighting its usefulness to fingerprint soil organic matter quality. Soil spectral signatures and denitrification activity were both highly susceptible to the biochemical quality of mulched residues and their decay stages but no correlation was found between both variables suggesting that the extent of N_2O emissions was uncoupled to the dynamics of soil organic matter quality. The soil DOM, as influenced by surface decaying plant debris, proved to act as a relevant electrons donor for denitrifiers and its functional value was dependant on the residue type. It is generally recognised that the more DOM is available, the more N_2O is produced (Burford and Bremner, 1975; Murray et al., 2004). Most of the substrates readily available for microorganisms are very likely to be released from plant debris into the soil solution at early decay stages which is congruent here with the strongest N_2O scores recorded when using DOM extracts collected under peanut residues during the first three weeks of their mineralisation. DOM-induced N_2O emissions can also be inflected by the chemical composition of the soil DOM (e.g. C/N ratio) as exemplified with agroforestry residues (Millar and Baggs, 2005). However with DOM originating from maize microcosms, N_2O emissions could not be differentiated from that of the control. If one assume that DOM is proportional to the total soil C content, this result contradicts the similar soil C contents observed between organic treatments that were higher than those measured in control soil. Possibly, soluble compounds released by peanut residues were more easily assimilable than those collected under maize residues. Moreover, it cannot be excluded that mineralization of maize residues generated more microparticles (size >0.45 µm) than the peanut mulch that were located in the soil poral network but discarded from this denitrification assay by the 0.2 µm filtration, resulting in less concentrated soil extracts. Such a preponderant effect of DOM concentration over DOM quality was observed on mineralization and microbial biomass with different types of forest stands (Smolander and Kitunen, 2002). Overall, as N_2O production sustained by all soil extracts were several folds inferior to that obtained with the glucose-succinate solution, organic load of these DOM extracts was a limiting factor for denitrification in this assay.

Conclusions

This study evidenced that the original biochemical quality of mulched residues is potentially a strong driver of the temporal patterns of soil organic matter quality, composition and activity of the denitrifiers community inhabiting the detritusphere. In particular, the quality of the mulched residue was shown to condition the ability of the soil DOM sampled from the soil-litter interface to fuel the N_2O production. Modifications of the *nirK* and *nosZ* community structure were shown to be correlated to the evolution of the soil organic matter quality during the residue decomposition. Against that background and in line with other reports encompassing diverse agroecosystems, our results highlighted however that neither changes in the denitrifiers community composition nor in the soil organic matter were coupled to the denitrification activity pattern during the decomposition of plant debris. These data also suggested the importance to integrate the denitrifiers community within the mulched residues in addition to that of the detritusphere when microbiological determinants of N_2O emissions of mulch-based systems are to be studied.

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MULTI-TEMPORAL DYNAMICS OF LAND USE PATTERNS IN A SITE OF COMMUNITY IMPORTANCE IN SOUTHERN ITALY

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Abstract. The importance of surveying a Site of Community Importance (SIC) lies in ensuring the maintenance of natural habitats of threatened or rare species of flora and fauna. This paper addresses the importance of gathering information on landscape changes for improved estimates of the development and dynamics within a European Union SIC in southern Italy. The main objective is to study spatial and temporal change over the last 50 years, in order to identify the characteristics of change and development and find potentially more sustainable systems of land use. The spatio-temporal changes of agro-forestry and urban conditions were investigated by comparing aerial photographs and through GIS spatial analysis. Landscape heterogeneity obviously changed from 1954 to 2001. The changing patterns in the landscape are the results of long-term interaction between people and nature. The unprecedented growth in the human population in the last centuries translates into increased resource consumption, as manifested in high rates of agriculture and food production, and urbanization, which increased from about 0.9 hectares in 1954-55, to 120 hectares in 2001. Conversely, important elements of the landscape ecology declined by 115 hectares. The proposed study will allow landscape planners to access ecological information through a readily accessible visual format.

Keywords: *land use change; landscape index; sustainable development*

Introduction

Landscape ecology has become one of the most rapidly developing ecological fields worldwide (Forman and Godron, 1986; Huang et al., 2009; Jorgensen, 2011; Turner, 1989; Urban et al., 1987) due to importance to recognize and understand land-cover (biophysical attributes of the earth's surface) and land use (human purpose or intervention applied to these attributes) changes as the major ecological processes affect and are affected by the dynamic interaction of ecosystems (Wilson and Weng, 2011). In the last few decades there has been a degeneration of natural environments both quantitatively and qualitatively, due to continuous urban development (Guagliardi et al., 2012). Thus the need to colonize areas which previously were functional to the environment and ecosystems has expanded. Urbanization, industrialization, and intensive agriculture result, therefore, in rapid landscape change (Muñoz-Rojas et al., 2011; Xie et al., 2012), in loss of ecological capacities; moreover they threaten biodiversity through habitat destruction, and lead to the destruction of natural beauty and historically valuable cultural landscapes, through fragmentation and degradation

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(Kim and Pauleit, 2005). It is well known that changing land use affects onsite landscape properties, for example soil degradation, and increased erosion after deforestation (Schoorl and Veldkamp, 2001). Progressive urban development has led to the loss of the natural environment (Guagliardi et al., 2013c; Zhao et al., 2010), up to the point where man's survival is brought into question. Indeed, by destroying natural habitats man, without realizing it, brings about an obvious decline in the quality of life. For instance, in Calabria, the poor socio-economic management of mountain areas has resulted in the depopulation of these territories with serious environmental consequences. The growing demands of society for productive, ecologically healthy and attractive multifunctional landscapes require new directions and rethinking of landscape planning and management (Bastian et al., 2006).

Recognizing and understanding landscape dynamics as a historical legacy of disturbance is necessary for the sustainable management of ecosystems (Kadioğullari and Başkent, 2008; Yin and Qing, 2010). Using the lens of sustainability science, landscape researchers and practitioners are breaking new ground regarding how people's values, behaviors, and actions influence the structure, function, and change of designed landscapes in an urbanizing world (Musacchio, 2009). For this reason, this surge in interest in landscape ecology is also shown in recent efforts to include a landscape perspective in policies and guidelines for managing public lands (McGarigal and Marks, 1995). Therefore, the study of the temporal changes of spatial patterns in landscapes is important not only to understand how a community has been able to evolve and adapt to new scenarios, which have an impact on the stability of a biocenosis but, more importantly, to allow a better understanding of how human activities can be decisive in arresting the regressive processes of ecosystems, and to improve sustainable management, through laws, regulations, the establishment of National Parks and Biogenetic Reserves, areas of protection and so on. This is important especially in Calabria, which represents an active area in a typical upland Mediterranean environment, in continuous changing due to its geological, geomorphological, climatic, and human pressure characteristics that are common to these of the whole Mediterranean areas (Guagliardi et al., 2013a).

Landscape change analyses focus on the identification of long-term dynamics which, as well represented in Calabria, are rarely just simple conversion from one type to another, but rather continuous transitions of land characteristics (Buttafuoco et al., 2010) through cyclical, linear, secular and reversible processes with variable time-lags (Antrop, 1998; Bürgi and Russell, 2001; Coppin et al., 2004). These evolving dynamics can be known by using of multi-temporal analysis, which can illustrate the change in land cover and consequently the compositional variation; therefore, it becomes possible to open up important scenarios of study related to the planning of the development of human activities such as industries, transportation networks, urban fabric, without penalizing the agricultural and forestry resources and ecological elements. In addition, it becomes possible to create gradient and exposure maps which allow us to consider also the physical and topographic variables, and therefore to determine the factors which influence the ecological dynamics, i.e., how a biocenosis can be influenced by low or abrupt gradients, slope exposure, closeness to urban centers or water sources.

Hence, the use of new and increasingly powerful software allows us to create maps which can be overlaid (Map Overlay) in order to generate matrices showing the changes in land use over time, i.e. the gains or the losses of each land use class. The metrics of landscape represent a very important factor in landscape ecology and spatial planning.

The objective of this study is to analyze and interpret the landscape dynamics in agro-forestry and urban systems of the lower Trionto catchment, in southern Italy. This site was selected for the Mediterranean bio-geographical region, in the Sites of Community Importance (SCI), defined in the European Commission Habitats Directive 92/43/EEC (CEC, 1992) as “a site which, in the bio-geographical region or regions to which it belongs, contributes significantly to the maintenance or restoration at a favorable conservation status of a natural habitat type in Annex I or of a species in Annex II and may also contribute significantly to the coherence of Nature 2000 referred to in Article 3, and/or contributes significantly to the maintenance of biological diversity within the biogeographic region or regions concerned”. The study involves a multi-temporal analysis of land cover and use changes over a period of 47 years (1954-2001) through patterns of landscape diversity in response to urbanization and their correlations with other parameters of landscape aspects, such as patch density, edge density and area weight. This study, supported by quantitative assessments, will lead to a deeper and more robust understanding of land-use and land-cover change and to more appropriate policy intervention. Improved understanding is also required to assess and project the future role of land-use and land-cover change in the functioning of the Earth System.

Materials and Methods

Study area

The study area, selected for the temporal analysis of landscape dynamics, is located in the Sila Massif,north-western Calabria, between the estuary of the river Trionto to the north, close the built-up area of Mirto Crosia, and its confluence with the stream Laurenzana to the south, in the villages of Caloveto to the east and of Cropalati to the west: this area delineates the natural limits of the basin (*Fig. 1*). Its SCI name is “Fiumara Trionto” and its SCI code is “IT9310047” (Decreto 7 Marzo 2012).

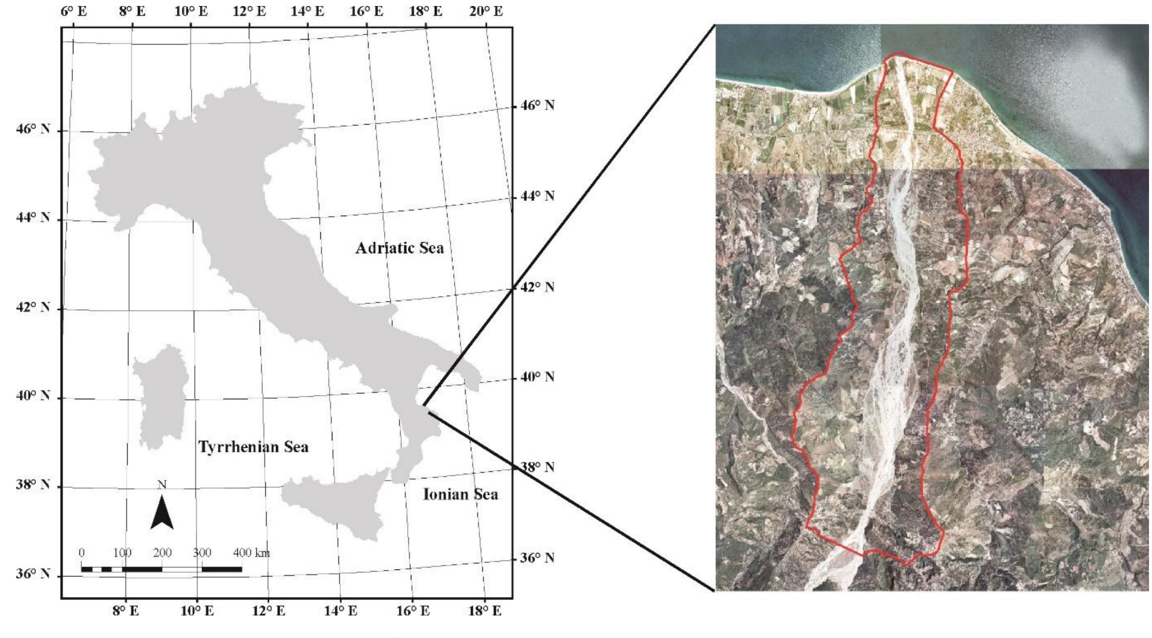

Figure 1. *Location of the study area*

In the study area, the lower part of the Trionto catchment from the confluence of the River Laurenzana to the mouth of the estuary was examined; its maximum altitude (Hmax) is 407 meters and its medium altitude (Hmed) is 133.8 meters. The area is included in sheets of Italian Institute of Military Geography n. 545 "Trionto" and n. 553 "Caloveto".

Soils and stream sediments of the study area are developed over Sila batholith made up of granitoid and gneissic rocks (Upper Paleozoic and Paloezoic), phyllites, schists and ophiolitic rocks (Cretaceous to Paleozoic) (Guagliardi et al., 2013b) overlapped by sedimentary clastics (Recent to Tortonian).

The river bed consists of alluvial deposits, cobblestones and sand. The Mirto Crosia village and the area close to the estuary are located on artificially fixed alluvial deposits or on vegetation. The innermost part of area consists of conglomerates and slightly consolidated sands, easily disjointed and with high permeability and subordinate clay intervals. Climatically, the area is included in the thermo-Mediterranean range with a hot and sub-humid climate (Bernetti, 1995).

Analysis of multi-temporal land use patterns

The analysis of the multi-temporal land use variations of the Trionto catchment was conducted through the techniques of photo interpretation, georeferencing, rectification and mosaicing of aerial photographs. Black and white photographs were studied for the periods 1954-55, 1983 and 2001.

Landscape elements (patches) were digitized as lines and polygons in layers corresponding to different years and categories. The aerial photographs were digitized and analyzed and the visualization and the processing of spatial data were carried out by ArcGIS 9.2 Software (ESRI, 2006), whereas the georeferencing, mosaicing and ortho-rectification of the photos were carried out using ER Mapper 6.4 Software (Earth Resource Mapping, 2003).

The development of GIS (Geographical Information Systems) technology, in particular, has widened the variety of analytical tools available for analyzing and managing landscapes (Hua et al., 2010; Käyhkö et al., 2011; McGarigal and Marks, 1995; Wang et al., 2010). Indeed, the variables which represent the structure and the complexity of the landscape were quantified through a suitable extension for ArcGIS (Patch Analyst).

The integration of the data set with field validation led to the development of an up-to-date land use map.

For the classification of different types of land use, Corine Land Cover (CLC50), with IV level of definition, was applied, in order to obtain a univocal classification, albeit with some changes to create a better fit for the area and the scale studied.

To promote the development of landscape ecology and quantitative research, various indices and landscape pattern models were introduced (Apart et al., 2002; Baker and Cal, 1992; Liding et al., 2008; Pearson, 2002; Raines, 2002). Landscape pattern indices are common tools of landscape ecology that are applied with freely available software (Corry, 2004). These indices express the complexity for each class of land use and can be of assistance in quantifying the fragmentation process.

Among the metrics which characterize the landscape from a structural and functional point of view, those considered in this study are shown in *Table 1*.

Table 1. *List and description of class level metrics used in the study*

Index Class	Index	Description
Patch Density & Metrics	Number of Patches (NP)	Number of patches at the class level
	Mean Patch Size (MPS)	Average patch size for a class
Shape Metrics	Mean Shape Index (MSI)	Deviation of each patch from circular: a circle has a shape index value of 1.0, whereas fragments with irregular shapes will have higher values.
	Area Weighted Mean Shape Index (AWMSI)	Average patch characteristic for a cell selected at random
Edge Metrics	Total Edge (TE)	Total length (m) of edge of a particular patch type (class level)
	Edge Density (ED)	Density (m/ha) of edge of a particular patch type (class level)
	Mean Patch Edge (MPE)	Average amount of edge per patch
Diversity Metrics	Shannon's Diversity Index (SHDI)	Measure of relative patch diversity. It is a relative measure of patch diversity. The index will equal zero when there is only one patch in the landscape and increases as the number of patch types or proportional distribution of patch types increases
	Shannon's Evenness Index (SHEI)	Measure of patch distribution and abundance. It is equal to zero when the observed patch distribution is low and approaches one when the distribution of patch types becomes more even
	Total Landscape Area (TLA)	Sum of areas of all patches in the landscape
	Class Area (CA)	Area (ha) of each patch type (class)

Clearly, patches are the basic building blocks of categorical patch mosaics and, as such, most metrics derive from the spatial character and distribution of the patches themselves. All patch metrics can be grouped loosely according to the level of heterogeneity (patch, class, landscape) and the aspect of landscape pattern represented and can be summarized at the class or landscape level by using the mean and the area weighted mean.

Although each of these attributes plays an individual role in determining ecological function, they may also interact to influence ecological processes (Collinge, 1996).

At the class and landscape level, some of the metrics quantify landscape composition, while others quantify landscape configuration. Landscape composition and configuration can affect ecological processes independently and interactively. Thus, it is especially important to understand, for each metric, which aspect of landscape pattern is being quantified.

Results and Discussion

Based on the research aim and land use status, the study area is divided into 12 landscape types, named according to Corine Land Cover legend: urban fabric, industrial, commercial and transport units, mine, dump and construction sites, arable land, permanent crops, heterogeneous agricultural areas, forests, scrub and/or herbaceous vegetation associations, open space with little or no vegetation, maritime wetlands, inland waters, marine waters. These various landscape types have changed significantly in the last 50 years and the number of landscape patches and patch structure has also changed to different degrees. The land use/cover in 1954-55, 1983 and 2001 are shown in *Fig. 2*,

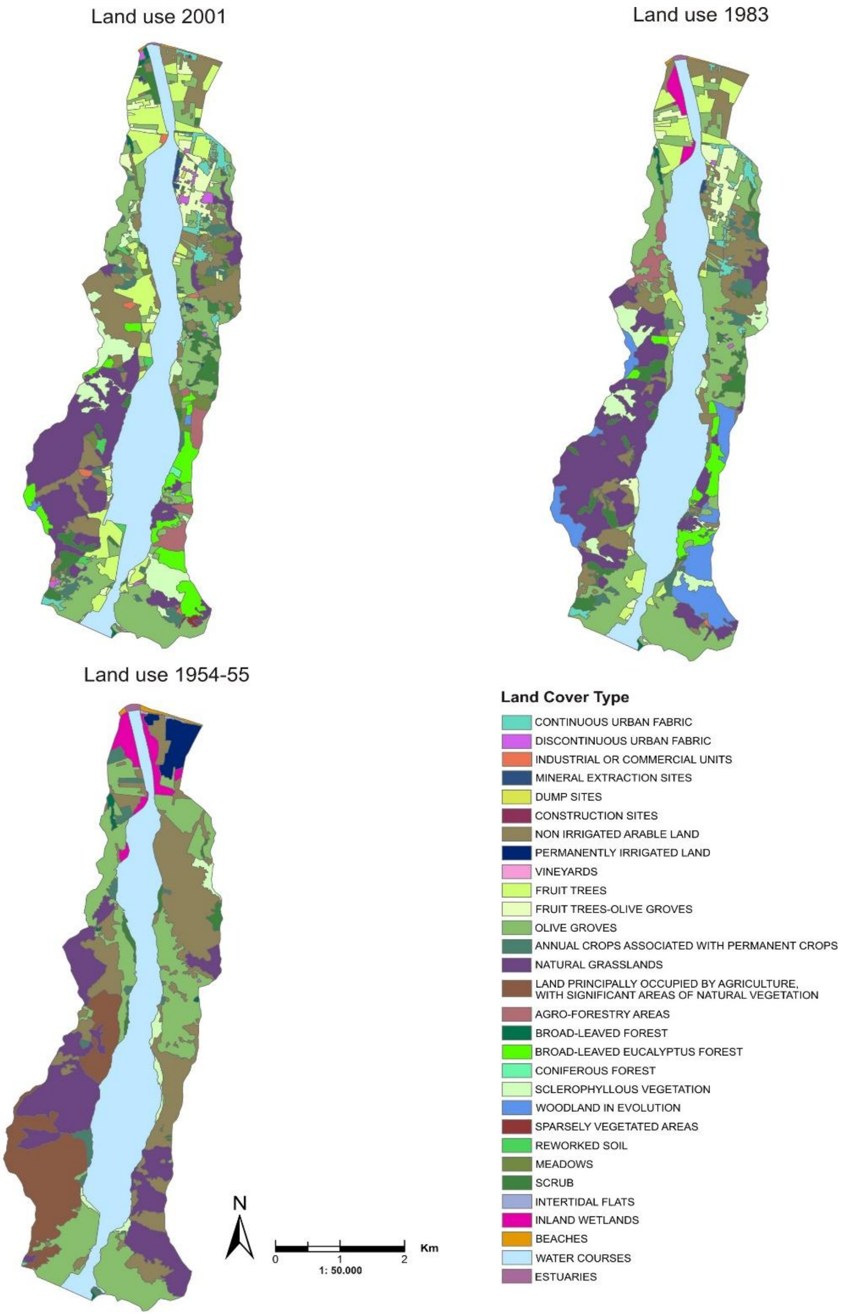

Figure 2. *Maps of land use/cover changes in Trionto catchment, 1954-55 – 2001*

and the quantification of land use change for the analyzed categories is given in *Table 2* (in m^2) and in *Table 3* (percentages of increase or decrease of land cover area in 2001 and 1983 compared to 1954-55). From the analysis of land use/cover images, it is notable that, where today there is the built-up area of Mirto, in 1954-55 there was no urbanization and, moreover, in that period, the only two inhabited areas were Crosia and Cropalati villages.

Table 2. *Comparison of NP and TLA on three periods analysed*

Land Cover Type	**Number of Patch**			**Total Area (m^2)**		
	Land use 2001	Land use 1983	Land use 1954-55	Land use 2001	Land use 1983	Land use 1954-55
Continuous urban fabric	10	17	1	538635	435740	6428
Discontinuous urban fabric	10	2	1	196099	18528	3543
Industrial or commercial units	7	3	0	137586	28394	0
Mineral extraction sites	3	3	0	102371	35278	0
Dump sites	2	0	0	13742	0	0
Construction sites	1	0	0	4023	0	0
Non irrigated land	65	62	39	3807750	3635174	6199311
Permanently irrigated land	0	0	2	0	0	646287
Vineyards	3	0	0	5799	0	0
Fruit trees	40	24	0	2819369	1377036	0
Fruit trees - olive groves	19	18	0	1085777	1036399	0
Olive groves	74	48	18	5243674	6379208	6802746
Annual crop associated with permanent crops	18	22	13	672667	638196	544163
Natural grasslands	13	13	8	4756396	5442707	5026653
Land principally occupied by agriculture, with significant areas of natural vegetation	0	0	2	0	0	3971645
Agro-forestry areas	4	3	0	568954	417986	0
Broad-leaved forest	7	3	4	104523	80813	105932
Broad-leaved eucalyptus forest	10	7	0	1385482	753657	0
Coniferous forest	3	0	0	52552	0	0
Sclerophyllous vegetation	14	10	6	1485277	1084585	432227
Woodland in evolution	2	7	0	59094	1966756	0
Sparsely vegetated areas	1	0	0	53700	0	0
Reworked soil	14	0	0	213623	0	0
Meadows	11	0	0	651282	0	0
Scrub	39	26	9	1292155	1162141	319960
Intertidial flats	0	0	1	0	0	40125
Inland wetlands	0	2	5	0	280226	760526
Beaches	2	2	2	38770	39999	80832
Water courses	1	1	1	7773655	8234730	8094073
Estuaries	1	1	1	11596	26539	39655

Table 3. *Comparison, in percentages of increase or decrease, of land-cover areas on three periods analysed*

Land Cover Type	**Percentages of the Total Area**			**Increase or decrease of land cover area compared to 1954-55 (%)**	
	Land use 2001	Land use 1983	Land use 1954-55	Land use 2001	Land use 1983
Continuous urban fabric	1,63	1,32	0,02	1,61	1,30
Discontinuous urban fabric	0,59	0,06	0,01	0,58	0,05
Industrial or commercial units	0,42	0,09	0,00	0,42	0,09
Mineral extraction sites	0,31	0,11	0,00	0,31	0,11
Dump sites	0,04	0,00	0,00	0,04	0,00
Construction sites	0,01	0,00	0,00	0,01	0,00
Non irrigated land	11,51	10,99	18,74	-7,23	-7,75
Permanently irrigated land	0,00	0,00	1,95	-1,95	-1,95
Vineyards	0,02	0,00	0,00	0,02	0,00
Fruit trees	8,52	4,16	0,00	8,52	4,16
Fruit trees - olive groves	3,28	3,13	0,00	3,28	3,13
Olive groves	15,85	19,29	20,57	-4,71	-1,28
Annual crop associated with permanent crops	2,03	1,93	1,65	0,39	0,28
Natural grasslands	14,38	16,46	15,20	-0,82	1,26
Land principally occupied by agriculture, with significant areas of natural vegetation	0,00	0,00	12,01	-12,01	-12,01
Agro-forestry areas	1,72	1,26	0,00	1,72	1,26
Broad-leaved forest	0,32	0,24	0,32	0,00	-0,08
Broad-leaved eucalyptus forest	4,19	2,28	0,00	4,19	2,28
Coniferous forest	0,16	0,00	0,00	0,16	0,00
Sclerophyllous vegetation	4,49	3,28	1,31	3,18	1,97
Woodland in evolution	0,18	5,95	0,00	0,18	5,95
Sparsely vegetated areas	0,16	0,00	0,00	0,16	0,00
Reworked soil	0,65	0,00	0,00	0,65	0,00
Meadows	1,97	0,00	0,00	1,97	0,00
Scrub	3,91	3,51	0,97	2,94	2,55
Intertidial flats	0,00	0,00	0,12	-0,12	-0,12
Inland wetlands	0,00	0,85	2,30	-2,30	-1,45
Beaches	0,12	0,12	0,24	-0,13	-0,12
Water courses	23,50	24,90	24,47	-0,97	0,43
Estuaries	0,04	0,08	0,12	-0,08	-0,04

Other important findings are: the total absence of industrial areas, mining, olive and fruit orchards and eucalyptus groves, whose reforestation began in the sixties, and the presence of two large surface areas, the first of approximately 64 hectares destined to watering crops and the second of about 1000 hectares of agricultural land-use with significant areas of natural vegetation and non-watered arable land (*Fig. 3*).

The surface of the inland marshes, i.e. areas close to watercourses which were flooded more frequently, was also wide: 76 hectares in 1954-55. In the same period, the scrub areas were lower than 1983 and 2001 because in the fifties, the need for available land to cultivate was greater than in succeeding years.

A radical change in the land use occurred in the seventies when the urban area began to expand rapidly. By 1983 the landscape had completely changed, new categories of land use such as orchards, intercropping between olive groves and fruit orchards as well as Eucalyptus groves appeared. The urban fabric of Mirto occupied part of the surface previously cultivated with olive groves and arable crops. In this scenario industrial and mining areas are also to be found. Crops that require irrigation have been totally replaced by crops that do not; agricultural parts with significant natural areas have been degraded, and a wide area is occupied by forests in evolution, the result of cropping and reforestation of Eucalyptus groves (*Table 2*).

From 1983 to 2001, the continuous and discontinuous urban fabric and all activities connected with the growing population, such as industry and quarrying, landfills, shipyards and reworked soils, increased from about 51 hectares to 120 hectares.

Compared to 1983, in 2001 the surface area occupied by olive groves decreased, while there was an increase in the surface area occupied by the conjunction of fruit orchards and the olive groves, of approximately 149 hectares (*Fig. 3*). Other important changes occurred such as the marked reduction of coppices, or Eucalyptus groves, as well as the decrease of the watercourse and the total disappearance of the inland marshes, currently occupied by the expansion of fruit orchards.

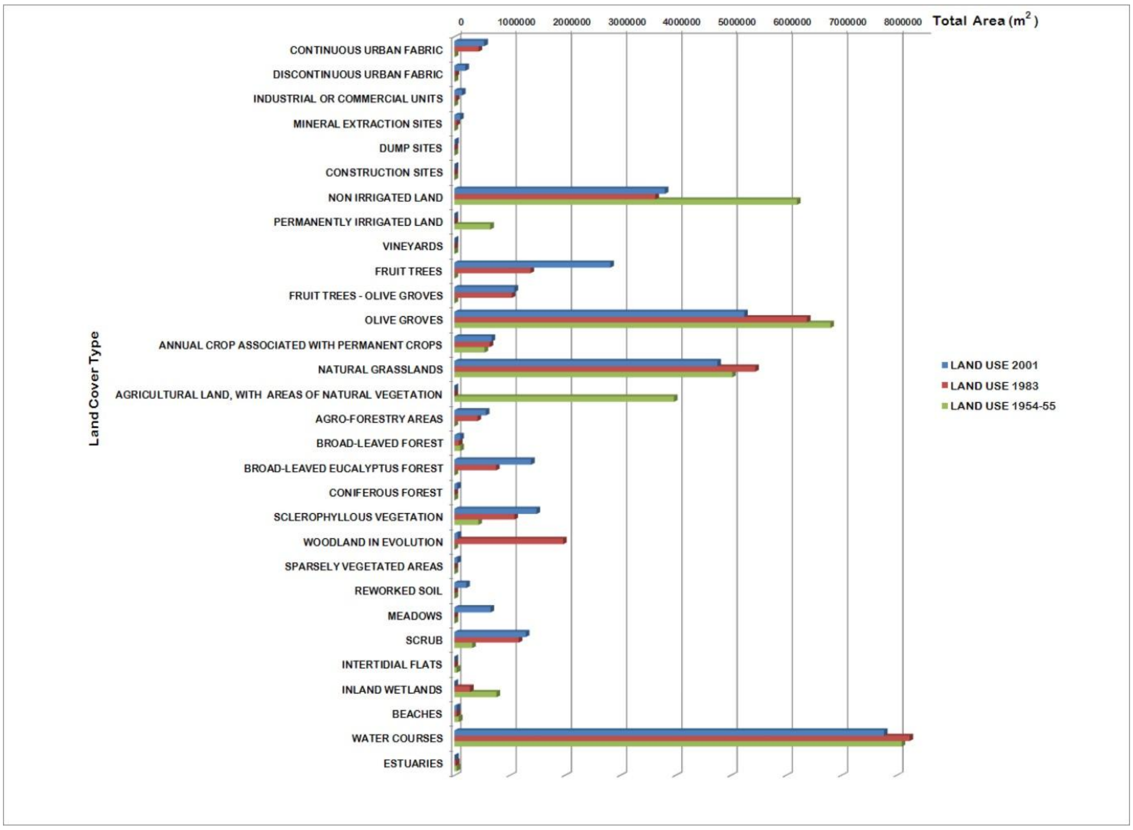

Figure 3. *The change of Total Area (m^2) in land use/cover types over 1954-55 – 2001*

The metric analysis has highlighted the landscape fragmentation, supported by the decrease in the average of the total area of the classes from about 206 hectares in 1954-55 to approximately 127 hectares in 2001 and together with the increase of the number of patches (NP) from 113 hectares in 1954 to 374 hectares in 2001 (*Fig. 4*).

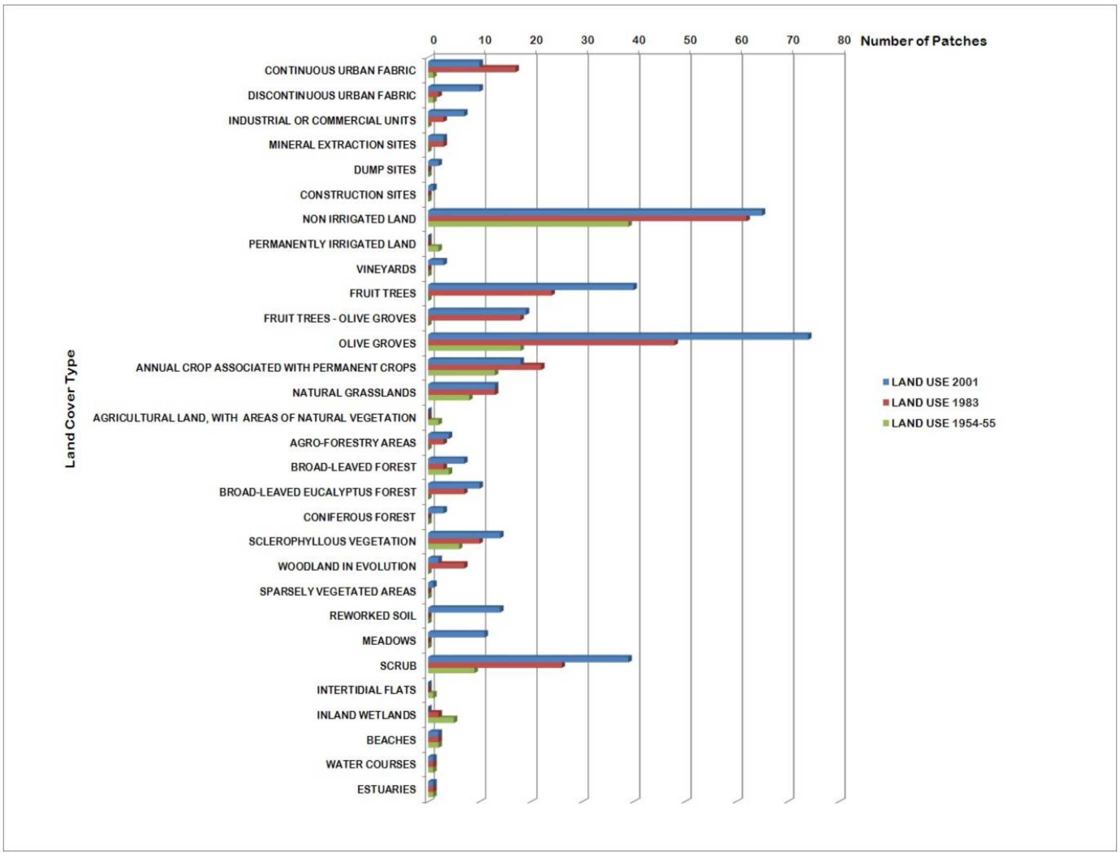

Figure 4. *The change of Number of Patches in land use/cover types over 1954-55 – 2001*

The Mean Patches Size (MPS) for each class also decreased significantly, as can be concluded from *Table 4*. The Mean Shape Index (MSI) and the Area Weighted Mean Shape Index (AWMSI) showed a generally increasing trend in the geometric complexity of the patches, especially concerning the non-agricultural and forestry component of land use; however, their values are close to unity the smaller the size of the patch.

Table 4. *Analysis of landscape metrics for each land use class on three periods analysed*

	Land use 2001								
Land Cover Type	**AWMSI**	**MSI**	**TE**	**ED**	**MPE**	**MPS**	**NP**	**TLA**	**CA**
Continuous urban fabric	3,34	2,14	19048,11	5,76	1904,81	5,39	10	3307,46	53,86
Discontinuous urban fabric	1,89	1,74	8430,41	2,55	843,04	1,96	10	3307,46	19,61
Industrial or commercial units	1,51	1,59	5154,87	1,56	736,41	1,97	7	3307,46	13,76
Mineral extraction sites	2,04	1,76	3431,64	1,04	1143,88	3,41	3	3307,46	10,24
Dump sites	1,37	1,39	780,64	0,24	390,32	0,69	2	3307,46	1,37

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Construction sites	1,80	1,80	405,06	0,12	405,06	0,40	1	3307,46	0,40
Non irrigated land	2,29	1,60	72840,64	22,02	1120,63	5,86	65	3307,46	380,7
Vineyards	1,23	1,26	573,97	0,17	191,32	0,19	3	3307,46	0,58
Fruit trees	1,68	1,45	46426,46	14,04	1160,66	7,05	40	3307,46	281,9
Fruit trees - olive groves	3,35	1,65	27560,78	8,33	1450,57	5,71	19	3307,46	108,5
Olive groves	2,49	1,64	98723,18	29,85	1334,10	7,09	74	3307,46	524,3
Annual crop associated with permanent crops	1,88	1,65	19791,86	5,98	1099,55	3,74	18	3307,46	67,27
Natural grasslands	2,63	2,16	54833,41	16,58	4217,95	36,59	13	3307,46	475,6
Agro-forestry areas	1,57	1,54	7743,86	2,34	1935,97	14,22	4	3307,46	56,90
Broad-leaved forest	1,86	1,74	5053,25	1,53	721,89	1,49	7	3307,46	10,45
Broad-leaved eucalyptus forest	1,98	1,71	22305,28	6,74	2230,53	13,85	10	3307,46	138,5
Coniferous forest	1,42	1,53	1955,22	0,59	651,74	1,75	3	3307,46	5,26
Sclerophyllous vegetation	2,31	2,01	27679,35	8,37	1977,10	10,61	14	3307,46	148,5
Woodland in evolution	1,50	1,50	1827,76	0,55	913,88	2,95	2	3307,46	5,91
Sparsely vegetated areas	2,64	2,64	2169,54	0,66	2169,54	5,37	1	3307,46	5,37
Reworked soil	1,62	1,53	8417,74	2,55	601,27	1,53	14	3307,46	21,36
Meadows	1,93	1,79	15970,49	4,83	1451,86	5,92	11	3307,46	65,13
Scrub	2,38	1,75	39860,48	12,05	1022,06	3,31	39	3307,46	129,2
Beaches	3,80	2,89	3145,00	0,95	1572,50	1,94	2	3307,46	3,88
Water courses	1,93	1,93	738,59	0,22	738,59	1,16	1	3307,46	1,16
Estuaries	3,23	3,23	31889,16	9,64	31889,16	777,3	1	3307,46	777,3

	Land use 1983								
Land Cover Type	**AWMSI**	**MSI**	**TE**	**ED**	**MPE**	**MPS**	**NP**	**TLA**	**CA**
Continuous urban fabric	3,40	1,87	18570,06	5,61	1092,36	2,56	17	3307,41	43,57
Discontinuous urban fabric	1,32	1,31	895,37	0,27	447,68	0,93	2	3307,41	1,85
Industrial or commercial units	1,56	1,60	1622,91	0,49	540,97	0,95	3	3307,41	2,84
Mineral extraction sites	1,44	1,32	1446,35	0,44	482,12	1,18	3	3307,41	3,53
Non irrigated land	2,67	1,65	78841,03	23,84	1271,63	5,86	62	3307,41	363,5
Fruit trees	1,49	1,41	24820,38	7,50	1034,18	5,74	24	3307,41	137,7
Fruit trees - olive groves	2,82	1,51	22880,61	6,92	1271,14	5,76	18	3307,41	103,6
Olive groves	2,70	1,73	94624,08	28,61	1971,34	13,29	48	3307,41	637,9
Annual crop associated with permanent crops	1,61	1,49	18026,99	5,45	819,41	2,90	22	3307,41	63,82
Natural grasslands	3,18	2,02	58195,25	17,60	4476,56	41,87	13	3307,41	544,2
Agro-forestry areas	2,84	2,30	9263,26	2,80	3087,75	13,93	3	3307,41	41,80
Broad-leaved forest	2,48	2,48	4021,37	1,22	1340,46	2,69	3	3307,41	8,08
Broad-leaved eucalyptus forest	1,91	1,61	13221,55	4,00	1888,79	10,77	7	3307,41	75,37
Sclerophyllous vegetation	2,21	2,14	22141,48	6,69	2214,15	10,85	10	3307,41	108,4
Woodland in evolution	1,90	1,84	22989,01	6,95	3284,14	28,10	7	3307,41	196,6
Scrub	2,36	2,12	36833,64	11,14	1416,68	4,47	26	3307,41	116,2

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Inland wetlands	1,73	1,79	4541,40	1,37	2270,70	14,01	2	3307,41	28,02
Beaches	3,68	2,89	3196,67	0,97	1598,34	2,00	2	3307,41	4,00
Water courses	3,01	3,01	30615,54	9,26	30615,54	823,47	1	3307,41	823,4
Estuaries	1,28	1,28	736,74	0,22	736,74	2,65	1	3307,41	2,65

	Land use 1954-55								
Land Cover Type	**AWMSI**	**MSI**	**TE**	**ED**	**MPE**	**MPS**	**NP**	**TLA**	**CA**
Continuous urban fabric	1,30	1,30	370,41	0,11	370,41	0,64	1	3307,41	0,64
Discontinuous urban fabric	1,24	1,24	262,24	0,08	262,24	0,35	1	3307,41	0,35
Non irrigated land	2,49	1,67	75141,73	22,72	1926,71	15,90	39	3307,41	619,9
Permanently irrigated land	1,48	1,37	5131,75	1,55	2565,88	32,31	2	3307,41	64,63
Olive groves	2,61	1,90	70502,16	21,32	3916,79	37,79	18	3307,41	680,2
Annual crop associated with permanent crops	1,49	1,43	12347,21	3,73	949,79	4,19	13	3307,41	54,42
Natural grasslands	2,09	1,99	41746,78	12,62	5218,35	62,83	8	3307,41	502,6
Land principally occupied by agriculture, with significant areas of natural vegetation	2,71	2,51	25129,54	7,60	12564,77	198,5	2	3307,41	397,1
Broad-leaved forest	2,07	1,77	3951,49	1,19	987,87	2,65	4	3307,41	10,59
Sclerophyllous vegetation	2,14	2,10	11680,66	3,53	1946,78	7,20	6	3307,41	43,22
Scrub	2,04	1,98	10569,29	3,20	1174,37	3,56	9	3307,41	32,00
Intertidial flats	4,42	4,42	3138,20	0,95	3138,20	4,01	1	3307,41	4,01
Inland wetlands	1,93	1,79	11723,91	3,54	2344,78	15,21	5	3307,41	76,05
Beaches	2,82	2,19	3419,93	1,03	1709,96	4,04	2	3307,41	8,08
Water courses	2,99	2,99	30113,37	9,10	30113,37	809,4	1	3307,41	809,4
Estuaries	1,22	1,22	858,13	0,26	858,13	3,97	1	3307,41	3,97

Another important element is given by the analysis of indices which quantify the length and the distribution of edges in the landscape Total Edge (TE), Edge Density (ED) and Mean Patch Edge (MPE). The data have revealed that from 1954-55 to 2001, the total perimeter of the patches significantly increased. The ED per hectare increased from 1954-55 to 1983, demonstrating the increase in patch complexity and in the heterogeneity of the mosaic. From 1983 to 2001 the value decreased slightly due to the increasing complexity of the landscape which led to the fusion of the particles and hence a decrease in the value. Considering that the MPE index is the result of the ratio between the total perimeter of each class of land use and the number of patches, it declines with the increase in the number of polygons from 1954-55 to 2001.

The metrics used to describe the whole landscape, namely the Shannon's Diversity Index (SDI) and Shannon's Evenness Index (SEI), showed that, in 1983, there was greater fragmentation and diversification of the types of land use with different extensions compared to 1954-55, whereas from 1983 to 2001, there was a slight decrease in these values, indicating a trend towards simplification, reduction of fragmentation and heterogeneity of territorial spaces (*Table 5*).

Table 5. *Analysis of Shannon's Diversity Index (SHDI) and the Shannon's Evenness Index (SHEI) on three periods analysed*

	2001	**1983**	**1954-55**
Shannon Diversity Index	2,34	2,20	1,91
Shannon Evenness Index	0,72	0,74	0,69

Conclusions

The present study has shown how, through the use of aerial photographs and image processing software tools, it was possible to analyze the evolutionary dynamics of the changes occurring in a geographical area over 50 years, with particular reference to urban development. The expansion of settlements, development of infrastructure, and extension of industrial areas, which increased from about 0.9 hectares, in 1954-55, to 120 hectares in 2001, reduced the land devoted to agriculture and forestry. Similarly, elements playing an important role in the landscape ecology, such as watercourses, beaches, estuaries and marshes, declined by 115 hectares. The proportion of land used for agriculture and forestry is declining, both for the recent area subtracted in an irreversible way, and for the evolutionary tendency of the study area, especially for the on-going urban development and the settlements in the bed of the Trionto river. In fact, new areas of land are made available for agriculture, violating the safeguards of the Site of Community Importance law and creating a possible flooding hazard. The total abandonment of crop requiring irrigation such as vegetables, the reforestation of the Eucalyptus groves (a native of Australia, which led to a halving of the area available for crops such as grain, hay and so on, without however, achieving the objective for which they were introduced), the increase of fruit orchards, demonstrated how man can determine changes in agroforestry ecosystems, taking into account only of short-term economic needs and undermining the balance between human activities and nature, threatening the survival of many rare and endangered animal and plant species, which require a sustainable management of ecosystems for their survival.

Finally, analysis of spatial and temporal changes in landscape pattern-driven factors could provide valuable scientific basis for regional planning and ecological construction.

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COMPARISON OF SPIDER DIVERSITY IN TWO TEMPERATE FORESTS BY A RAPID SURVEY AND ITS POTENTIAL IN NATURE CONSERVATION STUDIES

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Abstract. One of the crucial issues in nature conservation studies refers to the significant investment of time and energy required for a reliable estimation of biodiversity. To overcome this problem we designed a short survey for the estimation the richness of spider species in comparable habitats based on a semi-quantitative approach. Carrying out the survey in protected and unprotected temperate forest in the north-east Slovenia provided sufficient data for evaluation and relative comparison of spider diversity between the forests. High diversity of spiders observed in both forests indicates their importance as refuge habitats in agriculturally degraded landscape. At the same time, the comparison between surveyed forests shows a significantly higher level of spider diversity in the protected one, which supports the current conservation acts and provides a base-line for future monitoring of spider diversity in the forest. Modified set of sampling methods used in the survey revealed high level of efficiency in sampling by hand-held suction device and suggests its potential as an additional method in spider diversity studies in temperate forests with dense undergrowth. As the study is based on one of the most diverse and abundant animal groups in terrestrial ecosystems, it provides a reliable comparison of estimated biodiversity between comparable sites and exhibits the potential to complement the current criteria for assignment and monitoring of the biodiversity required for efficient conservation planning.

Keywords. *species richness, diversity estimation, spiders, protected areas*

Introduction

The data on relative abundance, distribution and richness of taxa provide base-line information in ecological studies and a crucial background for conservation planning (Blackmore, 1996; Humphries et al., 1995; Magurran, 2004, 1988; May, 1988; Raven and Wilson, 1992). As knowing the exact number and identity of each species in the area of interest is close to impossible, evaluations of biodiversity are commonly based on estimation of species richness of a selected group of organisms. In that view the arthropods were neglected until the past decade, when several studies proved their suitability in biodiversity studies over other organisms (e.g. Gregory et al., 2003; Sergio et al., 2006). Apart from the large number of species and an abundance of specimens, the important advantage of the arthropods in biodiversity-assessment studies is their fast growth rate and shorter generation spans, which enables their quick response to anthropogenic and natural changes of environmental conditions (Favila and Halffter, 1997; Kremen et al., 1993).

Spiders are amongst the most diverse, numerous and widespread groups of intermediate-level predators in terrestrial ecosystems, which rapidly colonize available habitats and exploit various niches (Coddington and Levi, 1991; Marc et al., 1999; Wise, 1993). As their distribution is strongly influenced by the habitat structure and the

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vegetation parameters (Buddle et al., 2000; De Souza and Martins, 2004; Greenstone, 1984; Petillon and Garbutt, 2008; Uetz, 1991; Wheater et al., 2000), spiders are recognized as an appropriate indicator group of organisms in biodiversity studies (Coddington et al., 1991; Platnick, 1999).

Although the spiders can be sampled by common methods used for terrestrial arthropods, a combination of methods targeting spiders in different microhabitats is required for objective estimation of biodiversity (Cardoso et al., 2008). On these grounds, a sampling protocol based on a repetitive series of semi-quantitative samplings has been designed for estimating the richness of spider species in tropical forests (Coddington et al., 1991). The protocol and the sampling methods applied have proven adequate for capturing a large number of species and specimens, while the diversity estimators used in the study (Gotelli and Colwell, 2010) enabled comparison of biodiversity between similar habitats (Ryndock et al., 2012). In the following studies, the initial protocol has been modified and successfully applied in various habitats (Cardoso, 2009; Cardoso et al., 2008; Coddington et al., 1996; Dobyns, 1997; Jimenez-Valverde and Lobo, 2006; Muelelwa et al., 2010; Silva et al., 1996; Sørensen et al., 2002; Toti et al., 2000), including European temperate forests (Kuntner and Kostanjšek, 2000; Kuntner, 1999; Scharff et al., 2003).

Determination of priority areas for conservation and assessment of conservation effects, based on biodiversity values, are one of the major issues addressed in conservation studies, which are commonly related to considerable investments of time, energy and expertise (Groves et al., 2002; Humphries and Parenti, 1999; Myers et al., 2000; Tracy and Brussard, 1994). In order to credibly compare the richness of spider species between protected and unprotected temperate forest with minimal effort, we designed a rapid survey based on semi-quantitative approach (Coddington et al., 1991). To increase the sampling efficiency and reduce the sampling time, we expand the set of sampling methods by a hand-held suction device and compare its efficiency to five methods commonly used in spider richness estimation studies. The potential of short surveys based on spider diversity in assessment of conservation priorities and effects is also discussed.

Methods

Study area

The study was carried out in two comparable temperate forests surrounded by agricultural land in Dravsko polje lowland in the north-eastern Slovenia. Both forests are about 250 m above sea levels and share the same climate conditions. The samplings were carried out in a one hectare square plot within each forest. The distance from plots to the nearest non-forest habitat was at least 250 meters in order to avoid the edge effects.

First plot, named “Rače”, was located within forest recognized as *Piceo abietis - Quercetum roboris* in Rački ribniki - Požeg Regional Park, measuring 5 km^2, at N 46°25,8' E 15° 40,7'. The second plot, named “Marjeta”, was selected within a patch of forest recognized as *Galio rotundifolii - Pinetum sylvestris*, measuring 4,5 km^2 and located 5,8 km northeast from the first one, at N 46° 25,8' E 15° 40,7'.

Sampling methods

The methods considered to cover most of the microhabitats include five well-established methods for spider samplings in similar protocols (Cardoso et al., 2008; Coddington et al., 1996). (1) “Ground” - selective spider sampling by hand, forceps or aspirator below the knee-level. (2) “Aerial” - selective sampling by the above mentioned methods above the knee-level, up to the height of the collector’s reach. (3) “Sweep” - sampling of vegetation by round sweeping nets, with a diameter of 30 cm. (4) “Sifter” - sifting of leaf litter by filed sieve with diameter of 30 cm and mesh size of 5 x 5 mm over a white cloth, after which the spiders were collected with an aspirator. (5) “Pitfall” - traps consisting of cups with diameter of 15 cm filled with ethylene glycol were set 1 m apart from each other. Besides these, an additional method was tested for its ability to complement the sampling methods listed above. The method, referred to as “blower” hereafter, includes hand-held suction device - the reversed leaf blower with two-cycle gasoline engine (BVM 250, McCulloch) equipped with a rigid suction tube with diameter of 11 cm. Suction opening was covered by standard-size aerial insect net, which prevented sampled spiders to be sucked into the machine. After two minutes of sampling, the net contents were emptied onto a white sheet. An aspirator was then used to collect the spiders from the sheet.

Design of the study

The samplings were performed between 18th and 24th of July 2013 and include 32 sampling units in each plot. The sampling design was semi-quantitative, with a sampling unit defined as one hour of effective sampling by one person using one sampling method. The sampling units were equally divided between the methods (Coddington et al., 1991), with three hours of sampling per plot of aerial, ground, sweep and sifter by day and three hours by night. Day samples were collected between 9:30 and 13:30 and night samples between 22:00 and 1:00.

Twelve pitfall traps were set in each plot and left during the sampling period, after which three adjacent traps from the same plot were combined to form ‘one sampling unit’. Therefore, effort in a field per sample was comparable with the other sampling techniques (Cardoso, 2009). The duration of sampling unit for blower was arbitrarily determined to be 2 minutes of intense sampling instead of one hour, due to the previously described sampling efficiency of the method (Buffington and Redak, 1998; Samu et al., 1997).

Four collectors worked simultaneously in both plots, rotating the methods between them to reduce the sampling efficiency bias due to collector’s experience (Coddington et al., 1996). In order to avoid the sampling fatigue the number of samples per day was restricted to two samplings in the daytime and two in the nighttime (Dobyns, 1997).

The weather during the samplings was constant, without noticeable precipitation and temperature fluctuations.

Statistical analysis

Only adult specimens were considered for statistical analysis, given that determining of the species in juveniles tends to be difficult and unreliable. The collected spiders were determined to a species level when possible. Otherwise, the specimens were assigned to morphospecies based on their genital morphology. All the collected material is deposited at Department of Biology, Biotechnical Faculty, University of Ljubljana.

To compare the species richness between both plots, the richness was estimated through randomized accumulation curves calculated for each plot by Estimate S program (Win 9.1) (Colwell, 2013). Accumulation curves based on seven commonly used species richness estimators, namely ACE, ICE, Chao1, Chao2, Jack1, Jack2, and Michaelis-Menten (Gotelli and Colwell, 2010), were calculated using one hundred randomizations. To compare the estimated species richness between the plots, an average accumulation curves were calculated for each plot from the mean values of all seven estimators and tested by a two-tailed Student's t-test. The dynamics of the final segments of each accumulation curve was described through the slope values calculated as reported previously (Cardoso et al., 2008). Average of estimators at the end of the survey was used in calculation of inventory completeness, defined as the coefficient between observed and estimated species richness (Coddington et al., 1996). As crude measure of sampling effort the sampling intensity was calculated for each method, as the ratio of collected specimens to species (Coddington et al., 1996).

Results

Over a five day survey, 32 sampling units in each plot yielded 228 adult specimens belonging to 67 species in Rače and 264 specimens belonging to 61 species in Marjeta plot (*Table 1*). List of collected species according to plot and methods are given in *Appendix 1*.

***Table 1**. Estimation of species richness in both sampling plots*

sampling plot	**Rače**	**Marjeta**
observed species richness	**67**	**61**
estimated species richness:		
ACE estimator	**126,1**	**103,1**
ICE estimator	**154,1**	**108,2**
Chao1 estimator	**124,8**	**103,1**
Chao2 estimator	**221,1**	**107,6**
Jack1 estimator	**108,7**	**92**
Jack2 estimator	**143,5**	**112**
Michaelis-Menten estimator	**118,2**	**97,5**
average of estimates	**142,6**	**103,3**
completness (%)	**47**	**64,9**

Higher species richness in Rače comparing to Marjeta plot was indicated by a higher number of observed species and further confirmed by the values of estimated species calculated from all seven estimators (*Table 1*). The latter varied between 92 and 112 species in Marjeta and between 108,7 and 143,5 species in Rače, with averages of 103,3 and 142,6 species for Marjeta and Rače, respectively (*Table 1*). Regardless the plot, the highest estimates were given by Chao2 and the lowest by Jack1 estimator. Average estimated species richness of each plot was used to calculate the inventory completeness, which was just below 65% in Marjeta and 47% in Rače (*Table 1*). The randomized accumulation curves of all seven estimators have not reached the asymptote at the end of the sampling in both plots (*Figure 1*). While the slopes of the accumulation curves for all estimators in Marjeta plot tend to gradually settle around 100 species, the curves continue to rise continuously in Rače plot (*Figure 1b*).

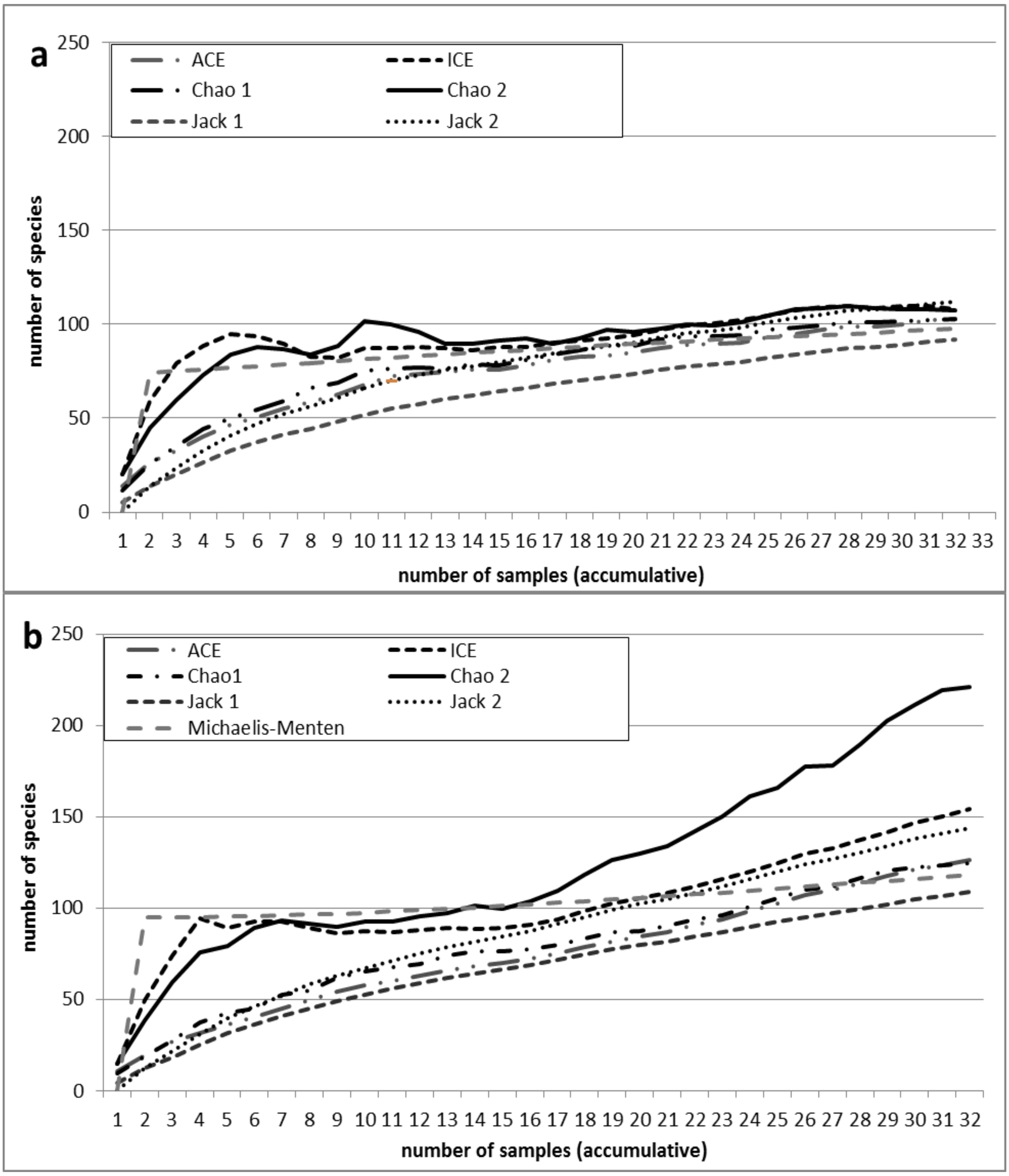

***Figure 1**. Randomized species accumulation curves of seven estimators for Marjeta (a) and Rače plot (b).*

To compare the species richness between the plots, an average accumulation curve was calculated for each plot using all seven estimators (*Figure 2*). The dynamics of average accumulation curves for both plots were almost identical at their beginnings and slowly diverge as the number of summarized samples passes twelve. As indicated by the curved of individual estimators, the average curve from Rače continues to rise steadily,

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with the slope value at the end of the curve of 0,185, while the rise of Marjeta curve tends to slow down, with the slope value of 0,132.

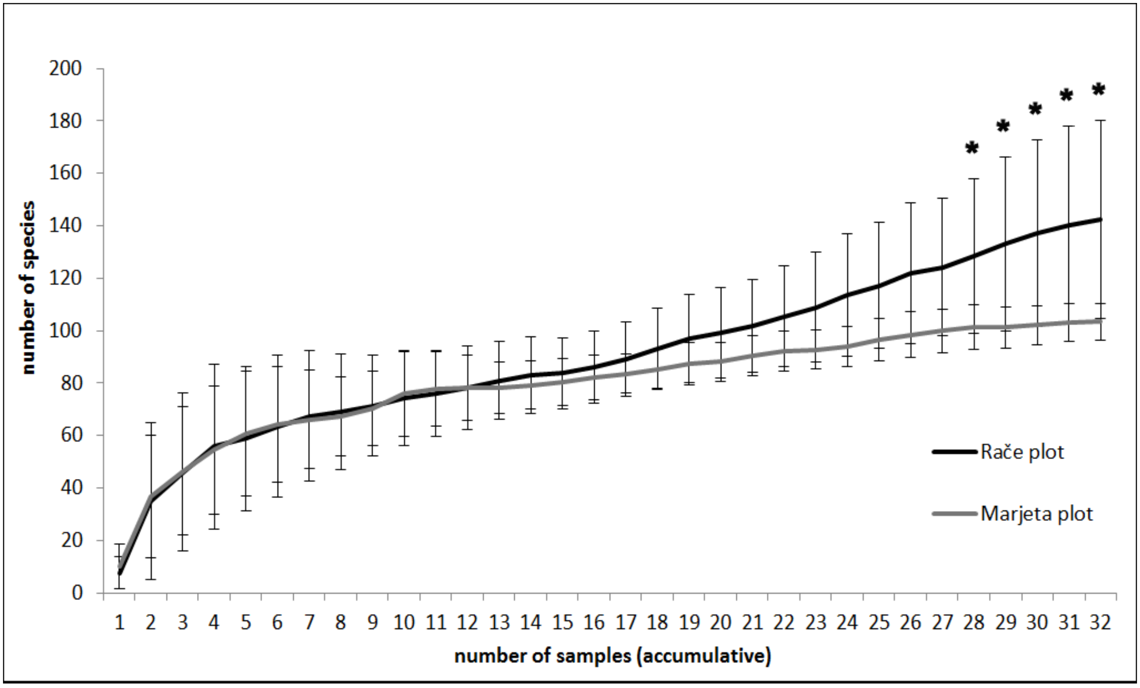

Figure 2. *Average accumulation curves for Rače and Marjeta plot. Curves are calculated as the average of seven estimators (ACE, ICE, Chao 1, Chao 2, Jack 1, Jack 2 and Michaelis-Menten), with standard deviations. Asterisks indicate the statistically significant difference (p>0,05) between the average species richness estimates in the plots.*

Standard deviations depicted on both average curves (*Figure 2*) reflect the variability of the individual estimators shown in *Figure 1*. Gradual approaching of estimators to the asymptote in the Marjeta plot (*Fig, 1a*) attributes in decrease of standard deviation values towards the end of the average Marjeta curve (*Figure 2*). On the other hand, the diverged paths of estimator's curves in Rače (*Figure 1b*), results as gradual increase of the standard deviations in average Rače curve (*Figure 2*). Nevertheless, the differences in estimated species richness between the plots becomes statistically significant after 27 summarized samples, when the 'p values' fall below 0,05 (*Figure 2*).

The efficiency of the used sampling methods was evaluated according to the number of collected specimens, species and unique species per sample, where the latter refers to species collected by one method only (*Table 2, Figure 3*). Among the unselective or 'tool-based' methods, the traps yielded the highest number of individuals per sample. The traps were followed by the sweep net and blower, while sifter provided the lowest numbers of specimens. The number of collected individuals by selective methods (i.e. aerial and ground) was comparable to sifter. The only exception was a ground sampling in Marjeta by night, where the yield of individuals was comparable to the sweep net (*Table 2*). These methods also provided the highest number of species and, together with blower in Marjeta, the highest number of unique species per sample as well (*Table 2*). The sampling intensity varied between 1 and 3 for most of the sampling methods, except for the pitfall traps, for which the values of 4,23 were calculated (*Table 2*).

Table 2. *Efficiency of sampling methods in Rače (R) and Marjeta (M) plots. SIF, SW, GRO, AER, BLO and TRAP stand for sifting, sweep net, ground, aerial, blower and pitfall traps, respectively. D - day samplings, N - night samplings.*

	SIF D		**SIF N**		**SW D**		**SW N**		**GRO D**		**GRO N**		**AER D**		**AER N**		**BLO**		**TRAP**		**TOTAL**	
	R	M	R	M	R	M	R	M	R	M	R	M	R	M	R	M	R	M	R	M	**R**	**M**
samples	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	4	4	4	4	**32**	**32**
individuals	9	19	10	5	38	39	30	34	13	19	18	34	2	17	17	22	37	15	55	60	**228**	**264**
individuals/sample	3	6,3	3,3	1,7	12,7	13	10	11,3	4,3	6,3	6	11,3	0,7	5,7	5,7	7,3	9,3	3,8	13,8	15	**7,1**	**8,3**
species	7	13	7	4	16	18	15	14	8	6	13	18	2	6	6	8	14	11	13	15	**67**	**61**
species/sample	2,3	4,3	2,3	1,3	5,3	6	5	4,7	2,7	2	4,3	6	0,7	2	2	2,7	3,5	2,8	3,3	3,8	**2,1**	**1,9**
unique species	2	3	3	0	6	6	3	2	1	0	4	6	0	2	0	0	8	1	5	3	**32**	**23**
unique species/sample	0,7	1	1	0	2	2	1	0,7	0	0	1,3	2	0	0,7	0	0	2	0,3	1,3	0,8	**1**	**0,7**
sampling intensity	1,3	1,5	1,4	1,3	2,4	2,2	2	2,4	1,6	3	1,4	1,9	1	2,8	2,8	3	2,6	1,4	4,2	4	**3,4**	**4,3**

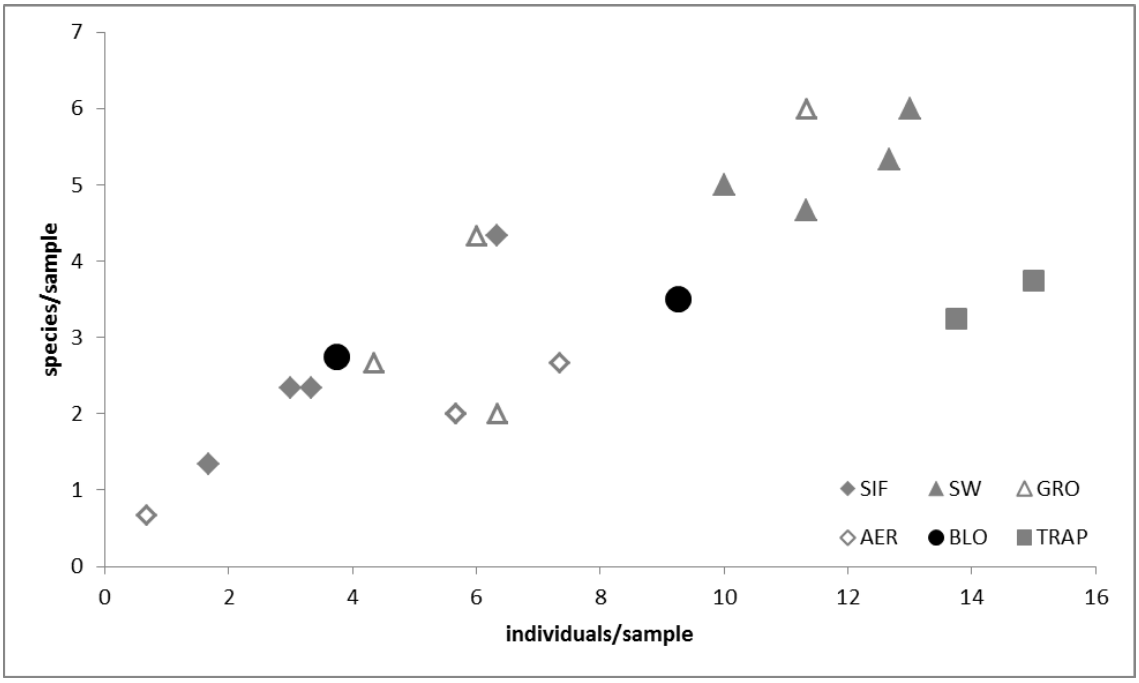

Figure 3. *The effectiveness of the sampling methods shown as the number of collected specimens and species per sample. SIF, SW, GRO, AER, BLO and TRAP stand for sifting, sweep, ground, aerial, blower and pitfall traps, respectively. D - day samplings, N - night samplings.*

Discussion

An increasingly important role of the richness estimation approach in biological inventory for assessment and conservation over the past decades (Barriga et al., 2010; Bonet et al., 2011; Cardoso et al., 2008; de Thoisy et al., 2008; MacLeod et al., 2011; Merlo et al., 2010; Schoeman et al., 2008), and the recognition of spiders as an indicator

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group of organisms in biodiversity studies (Coddington et al., 1991; Platnick, 1999) truggered studies on local spider richness in Slovenian forests. These were performed in several regions (Budja, 2008; Kuntner and Kostanjšek, 2000; Kuntner, 1997, 1996), including the sub-Pannonic region in the north-east of Slovenia (Kuntner, 1999) and generally followed the initial protocol (Coddington et al., 1991), with slight variations in the sampling methods and used estimators.

The estimated species richness in Rače and Marjeta plot exceeds the previous estimation of spider diversity in the north-western Slovenia (Kuntner, 1999) in which the spider diversity was estimated to between 72 to 86 species. With and average diversity estimation of 103 species, the diversity of Marjeta plot is comparable to the forests of sub-Mediterranean region in south-western Slovenia (Kuntner and Kostanjšek, 2000; Kuntner, 1997), while spider richness in Rače plot with 142 estimated species is so far the highest one recorded in Slovenian forests. Compared to similar studies in other regions, the diversity of the surveyed plots is comparable to Appalachian hardwood coves (Coddington et al., 1996; Dobyns, 1997), which is one of the biotically richest regions of temperate North America and considerably exceeds the diversity of the deciduous forests in northern Europe (Scharff et al., 2003).

Regarding that in the inventory still sufficient to accurately estimate the biotic richness, the estimator curves should asymptote (Colwell and Coddington, 1994), most of the previous studies estimating the richness of spider species (Cardoso, 2009; Cardoso et al., 2008; Coddington et al., 2009, 1996; Muelelwa et al., 2010; Scharff et al., 2003), including the above mentioned studies in Slovenia can be considered undersampled. With only 32 sampling units per plot our study is no exception. Climbing accumulation curves described by relatively high slope values at their far ends and low sampling intensity, when compared to similar studies (Coddington et al., 2009, 1996; Scharff et al., 2003; Sørensen et al., 2002), are in accordance with low inventory completeness calculated for the both plots.

With uncompleted inventories, the diversity estimates in both spots are prone to undersampling bias (Coddington et al., 2009), which implies underestimation of already high diversity estimations. However, rather than an accurate estimation of spider diversity by exhaustive sampling, the aim of our survey was to establish a rapid survey method for reliable comparison of species richness between two adjacent and comparable habitats with minimal sampling effort. The latter includes minimization of the sampling units required for distinction of estimated biodiversity, which was achieved by less than thirty sampling units per plot.

Aside from the threat of undersampling bias, our study was designed to minimize previously recognized factors, which may influence the outcome of diversity estimation studies (Cardoso, 2009; Cardoso et al., 2008; Jimenez-Valverde and Lobo, 2006). These include avoidance of the juvenile specimens, minimization of the sampling fatigue and the influence of the collector experience, which were already addressed in the Materials and Methods chapter. As many species of spiders are nocturnal (Green, 1999), our study design also includes a balanced amount of daytime and nighttime samplings (Coddington et al., 1991). In accordance to previous studies in the temperate forests, the selective methods, for instance the aerial and ground methods, provided higher numbers of species and specimens in the night samplings, while the opposite can be observed for tool-based methods sweeping net and sifter, which proved more efficient in the daytime (Budja, 2008; Kuntner and Kostanjšek, 2000; Kuntner, 1996). Regarding the efficiency of the methods, the results were as expected and in accordance with previous works. Non-selective methods, the traps and the sweep net provided the highest number of specimens and species, while the efficiency of the sifter was lower (Coddington et al., 1996; Kuntner and Kostanjšek, 2000). Comparison of selective methods in our study confirmed previous reports on higher efficiency of ground over aerial sampling in temperate forests (Coddington et al., 1996; Dobyns, 1997; Kuntner and Kostanjšek, 2000; Kuntner, 1999, 1997; Scharff et al., 2003).

The sampling approaches used in spider species richness studies, following the initial protocol proposed by Coddington and co-workers (1991), commonly include four to five ‘main’ methods. Although the use of a constant set of methods allows for comparison of studies on similar habitats, regarding the estimated diversity, the adaptation of the methods to specific microhabitats and introduction of new sampling approaches are prerequisite for a reliable estimation of species richness (Cardoso et al., 2008). In that view we considered previous modifications to the sampling sets used in temperate forests (Budja, 2008; Coddington et al., 1996; Dobyns, 1997; Kuntner and Kostanjšek, 2000; Kuntner, 1999; Scharff et al., 2003) and introduced the hand-held reverse leaf blower as an additional method.

With availability of hand-held suction devices (Samu et al., 1997), which replaced the traditional suction devices as the D-vac (Dietrick, 1961) the suction sampling has become widely accessible and frequently used in arthropod surveys (Grootaert et al., 2010). It proved highly efficient and appropriate for spider sampling in various habitats (Bell et al., 2000; Borges and Brown, 2003; Dinter, 1995; Samu et al., 1997)). As an unselective, tool-based method, the blower is suitable for collectors with little or no previous field experience (Buffington and Redak, 1998). At the same time, its ability to retrieve the spiders from microhabitats on or close to the ground (Sanders and Entling, 2011), where spiders dwell regardless the time of the day, eliminates the need for a nighttime sampling.

Regarding the amount of retrieved species and specimens in our study, the blower efficiency is comparable to other unselective methods, which is in accordance with previous studies (Buffington and Redak, 1998). Sampling with the blower also provided a considerable number of unique species from both surveyed plots and therefore efficiently complements other methods in our study. Considering that the blower provided comparable yields of the spiders in two-minute sampling units, in comparison to one-hour samplings of the other methods, the blower may also significantly reduce the duration of similar surveys. By combining the short sampling time and sufficient yields of specimens and species required for reliable statistical analysis (Coddington et al., 1991), the blower has therefore considerable potential as an additional method in spider species richness studies in temperate forests with rich lower vegetation and grass.

As the biodiversity of the Dravsko polje flatland is influenced by the intense agricultural degradation of natural habitats, the observed spider diversity richness in the surveyed forest patches suggests their role as refuge habitats (Pirnat, 2000, 1991), generally recognized by significant biodiversity (Watts et al., 2005). Regardless the background, our study indicates the high biodiversity value and the importance of forest patches in degraded landscape of Dravsko polje, which was (at least to some extent) recognized with the establishment of Rački ribniki - Požeg Regional Park in 1992 (Medobčinski Uradni vestnik, 1992). As a habitat for endangered species listed in Habitats directive (SCI) (Uradni list RS, 2004) the park was assigned to Natura 2000 network.

With the ability to distinguish the estimated spider diversity between protected and unprotected forests in favor of the former, based on only 32 sampling units per plot, our study shows the potential to complement the current criteria for assignment and monitoring of the protected sites. Based on spiders, our study also exploits the terrestrial arthropod diversity, as a rich data source for conservation planning and management (Kremen et al., 1993) and provides a step towards a credible assessment of the biodiversity based on a comprehensive set of criteria, required for efficient conservation planning (Groves et al., 2002; Regan et al., 2007). As a short-termed study providing sufficient amount of data by simple and affordable sampling approach, our survey fulfills most of the requirements of conservation studies (Coddington et al., 1991; Humphries et al., 1995) at one side and avoids the puzzling effect of phenological changes caused by seasons (Coddington et al., 2009). Although the potential of our study as a biodiversity evaluation tool will be evaluated through future studies, the results already support and justify the conservation acts at the Rački ribniki regional park and provide a base-line for future monitoring of spider diversity in the park, based on the comparison to non-protected forests in the region.

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APPENDIX

Appendix 1. *List of species and thier abundance according to sampling methods for Rače (R) and Marjeta (M) plot. SIF, SW, GRO, AER, BLO and TRAP stand for sifting, sweep net, ground, aerial, blower and pitfall traps, respectively. D - day, N - night. Morphospecies indicated by "sp." are followed by consecutive numbers.*

		SIF D		SIF N		SW D		SW N		GRO D		GRO N		AER D		AER N		BLO		TRAP	
		R	M	R	M	R	M	R	M	R	M	R	M	R	M	R	M	R	M	R	M
Abacoproeces	*saltuum*	1																			
Agelena	*labyrinthica*				1		2		3	1	6	1	3			1					
Araneus	*angulatus*								1							1					
Araneus	*diadematus*		1				4	1	2				2		7		4				

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Araniella	*cucurbitina*									1							1				
Aulonia	*albimana*																			5	7
Ballus	*chalybeius*								1												
Bathyphantes	*nigrinus*																	2			
Centromerus	*silvicola*		2																		
Cercidia	*prominens*						2												1		
Micrargus	*subaequalis*							1										1			
Clubiona	*lutescens*					1															
Clubiona	*comta*											1									
Clubiona	*terrestris*								1												
Cyclosa	*conica*														1						
Dendryphantes	*rudis*						1														
Diaea	*dorsata*					4	2														
Diplostyla	*concolor*		1										1							1	
Dipoena	*melanogaster*														1		1				
Drassodex	*sp1.*												2								
Dysdera	*erythrina*		1		1																2
Enoplognatha	*ovata*					8	13	3	8				2	1					1		
Euophrys	*frontalis*	1								1											
Euophrys	*herbigrada*						1														
Euryopis	*flavomaculata*		1																1		1
Euryopis	*laeta*					1															
Evarcha	*falcata*					6	3	5	1												
Floronia	*bucculenta*											1									
Gonatium	*rubellum*	1																			
Hahnia	*nava*	1																			1
Hahnia	*ononidum*			1																	
Hahnia	*sp.*			1																	
Harpactea	*rubicunda*												1								
Heliophanus	*dubius*						1														
Heliophanus	*cf. flavipes*					1															
Hyptiotes	*paradoxus*								1		1		1			2	4				
Larinioides	*sclopetarius*														1						
Linyphia	*hortensis*						1														
Linyphia	*triangularis*					7	2	3	8	2		1						2			
Linyphiidae	*sp.1*					1															
Linyphiidae	*sp.2*	2																			

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Linyphiidae	*sp.3*																	1			
Linyphiidae	*sp.4*					1															
Linyphiidae	*sp.5*																	1			
Linyphiidae	*sp.6*																	1			
Linyphiidae	*sp.7*																	1			
Linyphiidae	*sp.8*																	2			
Linyphiidae	*sp.9*		1																		
Linyphiidae	*sp.10*																		1		
Linyphiidae	*sp.11*																				1
Linyphiidae	*sp.12*																				1
Linyphiidae	*sp.13*																		3		1
Linyphiidae	*sp.14*																			4	
Malthonica	*campestris*											1									
Marpissa	*muscosa*					1						3									
Maso	*sundevalli*	2				2													1		
Microneta	*viaria*		4		2														1		1
Meioneta	*saxatilis*																	13			
Meioneta	*sp.1*																			3	
Meioneta	*sp.2*																			2	
Misumena	*vatia*							1	1												
Neon	*reticulatus*																	2	3		1
Neriene	*clathrata*										2	1									
Neriene	*furtiva*																	1			
Neriene	*radiata*														1					1	
Nuctenea	*umbratica*											3	1			8	3				
Ozyptilla	*praticola*					1															5
Pachygnatha	*listeri*							3										5		2	
Palliduphantes	*pallidus*											1							1		
Parasteatoda	*lunata*												1				2				
Parasteatoda	*tepidariorum*					1	1				2			1	6	3	4		1		
Pardosa	*lugubris*						1			3	7		10							10	27
Pardosa	*sp.1*												1								
Pelecopsis	*elongata*			1																	
Philodromus	*collinus*																				1
Philodromus	*albidus*						1														
Phrurolithus	*festivus*		1	3						2	1	1	2							1	1
Pirata	*uliginosus*									2								2		8	

Platnickina	*tincta*					1	1	1	1				1			2	3				
Robertus	*lividus*		1	1				6	4												2
Sintula	*corniger*																	3			
Styloctetor	*stativus*							1													
Tapinopa	*longidens*												1								
Tegenaria	*ferruginea*												1								
Tenuiphantes	*cristatus*											2									
Tenuiphantes	*flavipes*											1	2						1		
Tenuiphantes	*mengei*				1								1								
Tenuiphantes	*tenuis*		2							1											
Tetragnatha	*nigrita*						1		1												
Theridiidae	*sp. 1*		1																		
Theridion	*pinastri*							1													
Theridiosoma	*gemmosum*					1		1													
Tmarus	*piger*							1													
Tmarus	*stellio*						1														
Trichopterna	*cito*																			7	
Trochosa	*terricola*	1	1	2				1				1								10	8
Xysticus	*kochi*					1															
Xysticus	*sp.1*						1		1												
Zelotes	*apricorum*																			1	
Zelotes	*latreillei*												1								
Zora	*spinimana*		1	1				1													

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LIPID PEROXIDATION AND PROTEIN OXIDATION INDUCED BY DIFFERENT NANOPARTICLES IN ZEBRAFISH ORGANS

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Abstract. The protein oxidation and the lipid peroxidation induced by the presence of five different kinds of nanoparticles were determined in different tissues of zebrafish (*Danio rerio*). Both oxidative stress markers showed to be dose-dependent. At 1 ppm nanoparticle concentration, the five assayed nanoparticles showed higher protein oxidation than the control experiments. Europium-doped yttrium oxide (Y_2O_3:Eu) and carboxylated multi-walled carbon nanotubes (MWCNT-COOH) showed the highest effect, especially in ovaries, testicles and brain, reaching carbonyl content in proteins up to 4-times higher than control, while bismuth germanate (BGO) and MWCNT-COOH nanoparticles induced lipid peroxidation in all analyzed tissues, reaching values up to two-times higher than those from non-exposed control fish. BGO and silver nanoparticles (AgNP) showed less oxidative effect; nevertheless, the protein oxidation still accounted for up to 3-times the control fish values. At 1 ppm, the protein oxidation induced by some nanoparticles reached the values obtained in the experiments with 100 μM H_2O_2. On the other hand, no significant differences were found in gene expression of oxidative stress markers after 48 h treatment. The results from this work clearly indicate that exposure to those nanoparticles may cause significant protein and lipid oxidative damage.

Keywords: *lipid peroxidation, protein oxidation, nanoparticles, oxidative stress, zebrafish, toxicity*

Introduction

Nanotechnology is the origin of a new revolution in material technology with numerous potential applications. The use of nano-structured materials has more than quadrupled in the last 5 years. It is estimated that there are more than 1,000 nano-structured commercial products (Papp et al., 2008; McIntrye, 2012). Nanoparticles (NPs) can be made from almost any kind of materials, and the most used so far are made from carbon, silicon, titanium or other similar elements and materials. Many NPs are also made from metals or metal oxides, containing elements such as cadmium, cerium, copper, germanium, gold, iron, lead, selenium, silver, zinc, or zirconium. In general, the main NPs classes with commercial applications are fullerenes and single-wall/multi-wall carbon nanotubes, quantum dots, metal oxide-based NPs, and polymers (Powell and Kanarek, 2006).

Among the numerous commercial applications of NPs there are antimicrobial preparations, medical materials and devices, medical diagnostics, cosmetics, food packaging, anti-odor textiles, electronics, self-cleaning surfaces, UV-resistant coatings and paints, and lubricants. As the use of NPs increases, it is expected that their presence in aquatic environment will increase. In addition, NPs in aquatic systems can be formed by natural abiotic or biotic processes, induced by human activity (e.g. mining), incidentally introduced to aquatic systems, or intentionally added to these systems

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(Delay and Frimmel, 2012; Wigginton et al., 2007). Specifically, NPs have been proposed as potentially useful for water treatment processes (Wiesner, 2006; Wiesner and Bottero, 2007). The physicochemical behavior of NPs in aquatic environments has been reviewed (Lead and Wilkinson, 2006; Nowack and Bucheli, 2007; Christian et al., 2008; Baalousha et al., 2009). These molecular interactions are essential for the understanding of the mobility and transport, bioavailability, ecotoxicity, and reactivity of NPs in the aqueous phase. It has been found that when nanoparticles are dosed to water columns, they tend to precipitate to the subaquatic sediment, but this doesn't reduce its bioavailability since they accumulated in plant and animal biomass (Lowry et al., 2012).

The ecological importance of the NPs presence in aquatic systems has been discussed (Delay and Frimmel, 2012; Baun et al., 2008) and it is clear that the behavior and role of NPs in aquatic systems is very complex. In addition, the information of NPs effects and toxicity mechanisms in aquatic systems and organisms is still scarce, and the specific toxicity of NPs and their long-term impact on environment and human health remains basically unknown (Papp et al., 2008). These are important issues that need to be assessed in order to understand and even forecast the real risk that nanomaterials may pose to the environment and human health (Wiesner and Botero, 2012).

The current state of knowledge regarding the biological and health effects of NPs has been recently reviewed (Papp et al., 2008; Stark, 2011; Sharifi et al., 2012; Pattan and Kaul, 2012). Several different mechanisms for NPs toxicity have been reported. The increased production of reactive oxygen species (ROS) seems to be an important toxicity mechanism in cells induced by NPs (Nel et al., 2006; Unfried et al., 2007; Moller et al., 2010). These ROS include, among others, hydroxyl radicals, lipidoxyl or peroxyl radicals, singlet oxygen, and peroxinitrite formed from nitrogen oxide (NO). The modulation of several cellular events, including signal transduction, proliferative response, gene expression and protein redox regulation requires moderate amounts of ROS. However, high ROS levels induce oxidative stress which can damage cells by peroxidizing lipids, oxidizing proteins, disrupting DNA, interfering with signaling functions, modulating gene transcription, and finally, inducing cancer, renal disease, neurodegeneration, cardiovascular or pulmonary diseases.

In particular, lipid peroxidation is considered highly dangerous as it alters cell membrane properties (Lam et al., 2006). The chemical composition of nanoparticles seems to be the most decisive factor determining the formation of ROS in exposed cells (Limbach et al., 2007). For example, fullerene and its water-soluble derivative caused membrane disruption in rat liver microsomes by inducing significant oxidative damage (Kamat et al., 2000). Fullerenes are lipophilic and insert into lipid-rich regions (liver and gills) in fish. Other nanomaterials have been shown to selectively translocate into the brain via the olfactory bulb in mammals and fish (Oberdörster, 2004). The toxic effect of ROS could be more pronounced in the central nervous system due to the high content of unsaturated fatty acids in the cells, which are more susceptible to peroxidation (Adibhatla and Hatcher, 2010).

On the other hand, the oxidation of proteins in vertebrates has been the subject of several studies (Stadtman, 1991). Among the various oxidative modifications of amino acids in proteins, carbonyl formation may be an early marker for protein oxidation (Stadtman, 2004). TiO_2 NPs showed a dose-dependent linear increase in generating ROS and the generated ROS correlated with the protein oxidation in both *in vivo* and *in vitro* experiments (Han et al., 2012). The loss of membrane bound enzymes was also

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detected. Finally, TiO_2 NPs intranasally supplied to mice (Wang et al., 2008) induced a significant oxidative damage in brain tissue. The whole brain was affected, and lipid peroxidation, protein oxidation, increased activities of catalase, and an excessive release of glutamic acid and nitric oxide were detected.

There is a consensus that NPs in environmental systems are of ecotoxicological and public health relevance (Oberdörster, 2004; Ray et al., 2009; Stone et al., 2009). Thus, there is an urgent need for the evaluation of NPs biological effects due to the rapid development and commercialization of nano-structured products. In this work, different kinds of NPs are evaluated for oxidative stress in zebrafish. Different tissue targets are studied and the risk of the NPs presence for aquatic organisms is discussed.

Materials and methods

Nanoparticles

Europium-doped hydroxyapatite powder and bismuth germanate ($Bi_4Ge_3O_{12}$, BGO) and Y_2O_3:Eu^{3+} (with silica shell) nanoparticles were synthesized by sol-gel method and subsequent annealing (Taxak et al., 2009; Oviedo et al., 2012). The carboxylated multi-walled carbon nanotubes (MWCNT-COOH) were prepared by chemical vapor deposition and purified by refluxing in an aqueous HNO_3 solution (2.6 M) for 6 h, washed several times with distillated water and dried for 12 h at 80°C (Lin et al., 2002). Silver nanoparticles (AgNP) from Sovereign Silver™ (Russia) were a kind gift of Dr. Nina Bogdanchikova (CNyN-UNAM). The physical properties of the different nanoparticles are shown in *Table 1.*

Table 1. *Physical properties of the different nanoparticles.*

Property	**HA:Eu**	**BGO**	**MWCNT**	**AgNP**	**Y_2O_3:Eu**
Size diameter	50-100 nm	10-15 nm	80-100 nm	10-30 nm	50-100 nm
Zeta potential	-10 mV	ND	-27.3 mV	- 21.2	-25.0 mV
Excitation maximum	280 and 394 nm	284 nm	---	215 nm	237 nm
Emission maximum	575, 613 and 620 nm	485 nm	---	300 nm	611 nm
Melting point	1200 °C	1050 °C	---	810 °C	1600 °C

ND. Not determined

Fish experiments

Adult and healthy zebrafish (*Danio rerio*) of 0.470 ± 0.166 g were obtained from a local fish farm (Granja Acuario Antártico, Cuernavaca, Mexico). Fish were acclimatized in glass tanks at room temperature (25 ±2°C) for 2 days with a photoperiod of 12 h light/12 h dark and they were fed twice a day with a commercial diet. Experiments were carried out in a 20 L aquarium containing 16 L of chlorine-free treated water. Ten fish were treated with 0.5 or 1 ppm of NPs and ten fish were used as control without NPs. Every set of treatments and controls were carried out in three independent experiments. After 48 h of treatment, fish were anesthetized on ice, and then dissected to obtain liver, ovaries, testicles, gills and brain tissues. Positive control experiments for oxidative stress were performed treating fish in the presence

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of 100 µM hydrogen peroxide for 48 h. All tissues were kept in liquid nitrogen and analyzed immediately.

Protein oxidation

The extent of protein oxidation in the different tissues was determined as the carbonyl content in the protein fraction (Levine et al., 1991). Tissues (150-200 mg) from fish treatments with 0.5 or 1 ppm of NPs, or with 100 µM H_2O_2 (positive control) were placed in an eppendorf tube and 4 volumes of 50 mM HEPES buffer (pH 7.2) containing 0.1 mM phenylmethylsulfonyl fluoride were added, and then grinded with pellet pestle (Kimble-Chase 749521-1590). The homogenized mixture was centrifuged at 10,000 g for 5 min at 4°C. Subsequently, 10 µL of streptomycin (20%) were added to 100 µL of supernatant in order to remove the nucleic acids that may erroneously contribute to higher estimation of carbonyls. The mixture was kept at 25°C for 15 min and the precipitated nucleic acids were removed by centrifugation at 10,000 g for 5 min at 4°C. To a 100 µL aliquot of supernatant, 400 µl of 10 mM 2,4-dinitrophenylhydrazine were added to and kept for 1 hour under gentle shaking. Then, the protein was precipitated by adding 500 µL of trichloroacetic acid 20% and recovered by centrifugation at 10,000 g for 5 min at 4°C. The pellet was washed three times with a ethanol:ethylacetate (50:50 v/v) solution. The pellet was dissolved in 500 µL of 6 M guanadine containing 10 mM dithiothreitol at 37°C. An aliquot of 400 µL of this protein solution were diluted with 600 µL of water and spectrometrically read at 370 nm (Oberdörster, 2004).

Lipid peroxidation

The different tissues from fish treatments with 0.5 or 1 ppm of NPs, or with 100 µM H_2O_2 (positive control) were analyzed for lipid peroxidation. The amount of lipid peroxides was estimated by using the Xylenol Orange reagent (Nourooz Zadeh et al., 1994). The FOX reagent contains 2.5 mM ammonium ferrous sulphate and 110 mM perchloric acid in a methanol:water (90:10 v/v) solution. FOX reagent (900 µL) was added to 100 µL of homogenized tissue supernatant (see previous section) and kept for 30 min at 25°C. Then the reaction mixture was read at 560 nm (Gay and Gebicki, 2003). The molar concentration of lipid peroxides was then estimated comparing the absorbance values with those obtained from a standard curve with different concentrations of hydrogen peroxide.

Protein determination

The amount of protein in the tissue homogenates was spectrophotometrically determined with the BioRad protein reagent using BSA as standard.

Isolation of RNA and reverse transcription

Six fish were reared in 6 L containing 1 ppm of the corresponding nanoparticle (BGO, Y_2O_3-Eu^{3+}, MWCNT-COOH and AgNP) in glass flasks at room temperature (25 ± 2°C). Three independent replicates in separated glass flasks were used for each treatment. After 48 h of exposure, fish were anesthetized on ice and frozen in liquid nitrogen. Liver and brain were dissected out and stored at 4°C in RNA later (Qiagen, USA). After 24 h, RNA later was removed and the tissues were stored at -20°C. The rest of the body was frozen and stored at -80°C. The different tissues were grounded in a mortar with liquid nitrogen. The tissue was then recovered in 250 µL TRI Reagent (Sigma, USA), and total RNA was isolated according to the manufacturer's instructions. The absorbance ratio 260/280 nm was measured in a Nanodrop. The banding patterns on a 1% agarose gel were used to verify the quality of the RNA in each sample. One µg of total RNA was treated with DNase I, RNase-free (Fermentas, USA) according to manufacturer's instructions. Reverse transcription of mRNA was carried out with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). Finally, samples were diluted 4-fold and 1 µL was used for the real-time quantitative polymerase chain reaction.

Gene expression analysis

Real-time quantitative PCR was performed using a StepOne Real Time PCR system (Applied Biosystems, USA). The qPCR reactions were carried out in 10 µL of Maxima SYBR Green /ROX qPCR Master Mix (2X) (Fermentas, USA). The following PCR protocol was used: Denaturation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. The correct primer amplification was confirmed by agarose gel electrophoresis. Oligonucleotide primer pairs of oxidative stress genetic expression markers; superoxide dismutase (Sod1), the transcription factor Forkhead box protein O1 (FoxO1) (Subauste and Burant, 2007), and the glutamate-l-cysteine ligase catalytic subunit (Gclc) (Mougiakakos et al., 2012) are described in *Table 2*. β-actin (bact), tubulin alpha I (Tub) and TATA-box binding protein (Tbp) transcripts were used to standardize the results by eliminating variations in mRNA and cDNA quantity. Each experiment was carried out in triplicate.

Table 2*. Oxidative stress genetic expression markers. Oligonucleotide primer pairs of superoxide dismutase (Sod1), the transcription factor Forkhead box protein O1 (FoxO1), and the glutamate-l-cysteine ligase catalytic subunit (Gclc). β-actin (bact), tubulin alpha I (Tub) and TATA-box binding protein (Tbp) transcripts were used to standardize the results by eliminating variations in mRNA and cDNA quantity.*

mRNA	Accesion number	Forward (5'-3')	Reverse (5'-3')	Position
Gclc	NM_199277.2	ACAGCTATCTGGAGAACATGGAGG	TGTTTGGCAACAAACTCCCTCATC	1628-1763
FoxO1a	NM_001077257.2	GACCTCATGCCTTCAGTGGACACT	TGTGTGGGTGAGAAAGAGTGTGAC	1393-1535
Sod1	NM_131294.1	TGAGACACGTCGGAGACCTGGGTA	CCACCCTTCCCCAAGTCATCCTCC	304-460
bact	AF057040	ATGGATGAGGAAATCGCTGCC	CTCCCTGATGTCTGGGTCGTC	54-180
Tbp	NM_200096	CGGTGGATCCTGCGAATTAT	GGTTATGAAGCAAAACAACA	51-146
Tub	AF029250	TCTTTGTAGACCTGGAGCCC	TCAATGAGTTCCTTGCCAAT	197-347

Gene expression statistical analysis

Results of gene expression were normalized using the geometric mean of the three control genes using the Biogazelle qbasePlus software (Gent, Belgium). The relative expression values for each gene were calculated taking as reference the value obtained for the control treatment.

Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA). If the results of the ANOVA indicated significant differences among the mean values, then multiple comparisons of mean values were performed using Tukey's test. Differences were reported as statistically significant at $P < 0.05$. All the statistical analyses were performed using the statistical program SIGMA STAT, 3.1 (Arlington, VA, USA).

Results

Oxidative stress implies a cellular state whereby reactive oxygen species (ROS) production exceeds its metabolism overpassing cellular defenses. The oxidant effect of nanoparticles was estimated in zebrafish (*Danio rerio*) after 48 h exposure to two different NPs concentrations, 0.5 ppm and 1.0 ppm. Five different kinds of nanoparticles were studied including europium-doped hydroxyapatite (HA:Eu), bismuth germanate (BGO), europium-doped yttrium oxide (Y_2O_3:Eu), silver nanoparticles (AgNP), and carboxylated multi-walled carbon nanotubes (MWCNT-COOH). In addition, a positive control was performed in which fish were treated with 100 µM of hydrogen peroxide. The H_2O_2 concentration of this positive control was chosen after preliminary experiments with 10, 50 and 100 µM of H_2O_2, the latter being the one that showed important protein oxidation and lipid peroxidation in the different tissues.

The formation of carbonyl groups is an early marker for protein oxidation (Stadtman, 2004) and was determined in five different tissues of zebrafish after exposure to different NPs. Carbonyl content in liver, ovaries, testicles, gills and brain values were compared with those of a positive control for oxidative stress induced by the presence of 100 µM of hydrogen peroxide (*Fig. 1*). At 0.5 ppm concentration, Y_2O_3:Eu and MWCNT-COOH induced, by far, the highest oxidative stress followed by AgNP, while no protein oxidation was found with HA:Eu and BGO NPs when compared to control experiments (*Fig. 1*). Nevertheless, this increase in carbonyl content in proteins is lower than those found in the positive control with 100 µM H_2O_2, in which liver, ovaries, testicles and brain are especially prone to protein oxidation. At low NPs concentration all tissues seemed to be equally sensible.

On the other hand, in all cases, when NPs concentration was increased from 0.5 ppm to 1 ppm, an increase was observed on protein oxidation. At high NPs concentration (1 ppm), the five assayed NPs showed higher concentration of carbonyl groups than the control experiments (*Fig. 1*). Here, again the Y_2O_3:Eu and MWCNT-COOH nanoparticles showed the higher effect, specially in ovaries, testicles and brain. The carbonyl group content in these tissues reached up to 4-times higher than in control fish after 48 h treatment. AgNP showed lower but significant oxidative effect, nevertheless, the protein oxidation still accounted for up to 3-times the control fish values. At 1 ppm, the induced protein oxidation by NPs reached the values obtained in the experiments with 100 µM H_2O_2. These results clearly indicate that exposure to 1 ppm concentration of these NPs for 48 h is able to induce an important oxidative stress on fish demonstrated by the carbonyl content in protein in the different fish tissues.

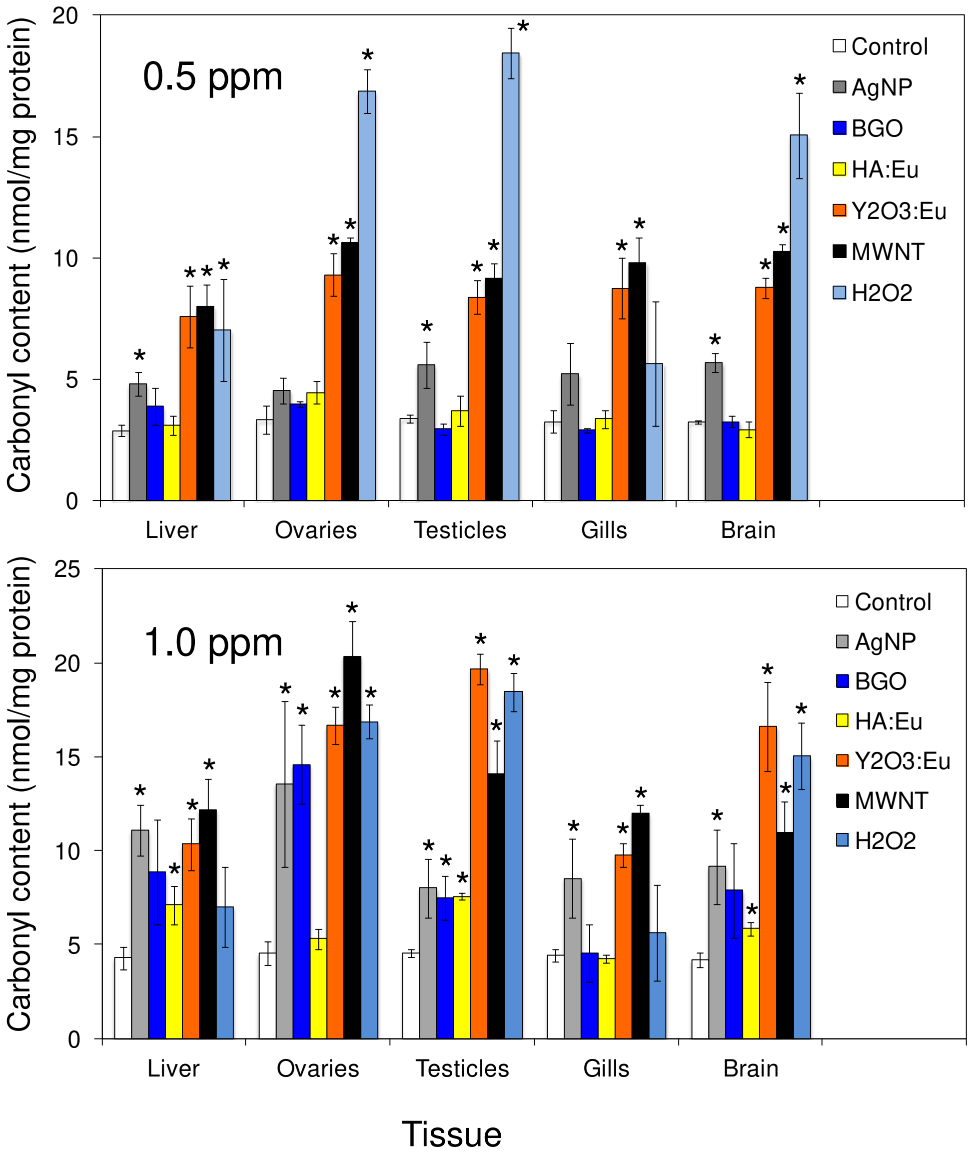

***Figure 1**. Protein oxidation of different zebrafish tissues estimated as carbonyl content in proteins. a) Fish exposed to 0.5 ppm of nanoparticles for 48 hrs. b) Fish exposed to 1 ppm of nanoparticles for 48 hrs. (*)Values significantly different at $P < 0.05$ with respect to the control.*

The concentration of lipid peroxides in the different tissues was estimated after 48 h fish exposure to NPs (*Fig. 2*). At 0.5 ppm of NPs there is low effect, except with MWCNT-COOH nanoparticles in ovaries, testicles and brain. Lipid peroxidation also showed to be dose-dependent. At high NPs concentration (1 ppm) MWCNT-COOH nanoparticles induced higher lipid peroxides content in all analyzed tissues, reaching values up to two-times higher than those from non-exposed control fish.

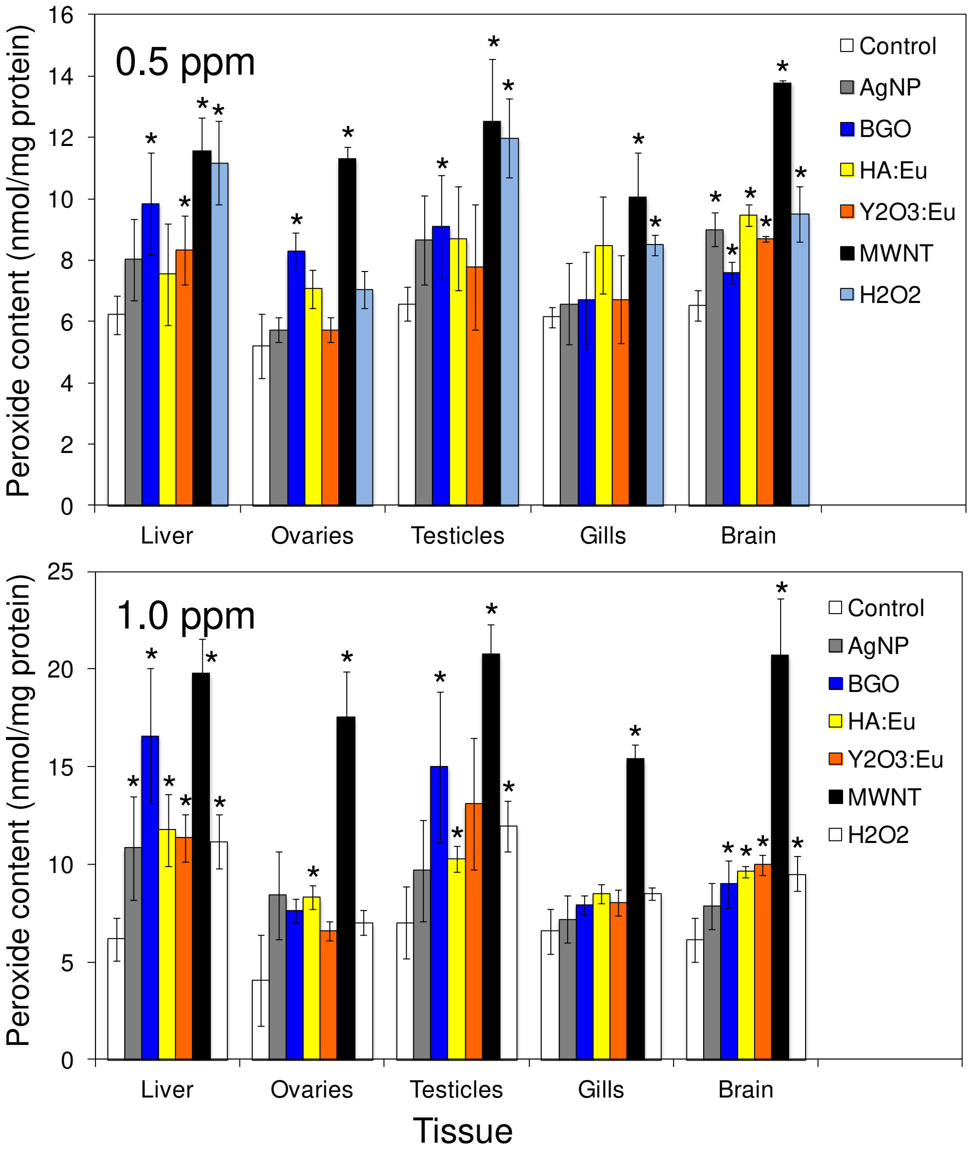

Figure 2*. Lipid proxidation of different zebrafish tissues estimated as peroxyl content in lipids. a) Fish exposed to 0.5 ppm of nanoparticles for 48 hrs. b) Fish exposed to 1 ppm of nanoparticles for 48 hrs. (*)Values significantly different at $P < 0.05$ with respect to the control.*

The genetic expression of oxidative stress markers was measured in treated and untreated fish with 1 ppm of NPs for 48 h (*Table 3*). Expression of superoxide dismutase (Sod1), the transcription factor Forkhead box protein O1 (FoxO1), and the glutamate-l-cysteine ligase catalytic subunit (Gclc) was quantified. β-actin (bact), tubulin alpha I (Tub) and TATA-box binding protein (Tbp) transcripts were used to standardize the results by eliminating variations in mRNA and cDNA quantity. No significant differences were found in the gene expression of any marker gene in liver, brain nor whole fish body (*Table 3*). These results may indicate that 48 h is not enough time for fish to metabolically compensate the accumulation of ROS caused by NPs

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exposure by modifying the expression of these genes involved in contending with oxidative stress.

Table 3. *Relative mRNA levels of oxidative stress related genes in zebrafish exposed to nanoparticles (mean ± standard deviation). Values are relative to control fish.*

AgNP

Gene	Brain	Liver	Whole body
FoxO1a	0.97 (±0.41)	0.86 (±0.13)	1.04 (±0.45)
Gclc	0.78 (±0.07)	1.05 (±0.09)	1.38 (±0.31)
Sod1	0.68 (±0.17)	0.62 (±0.07)	0.94 (±0.19)

BGO

Gene	Brain	Liver	Whole body
FoxO1a	1.18 (±0.08)	0.64 (±0.43)	0.68 (±0.28)
Gclc	0.83 (±0.06)	1.08 (±0.67)	1.24 (±0.06)
Sod1	0.72 (±0.10)	1.65 (±0.63)	1.04 (±0.26)

MWNT-COOH

Gene	Brain	Liver	Whole body
FoxO1a	0.95 (±0.03)	1.28 (±0.21)	1.29 (±0.41)
Gclc	0.63 (±0.16)	1.02 (±0.13)	1.04 (±0.23)
Sod1	0.95 (±0.27)	1.44 (±0.29)	1.38 (±0.28)

Y_2O_3-Eu^{3+}

Gene	Brain	Liver	Whole body
FoxO1a	0.73 (±0.06)	0.70 (±0.18)	0.71 (±0.13)
Gclc	0.94 (±0.08)	0.74 (±0.14)	0.88 (±0.07)
Sod1	0.73 (±0.14)	1.02 (±0.62)	0.82 (±0.07)

Discussion

Our results demonstrated that NPs, after 48 h exposure and independently of its chemical nature, induce protein oxidation and lipid peroxidation of different zebrafish tissues (*Figs. 1 and 2*). Lipid peroxidation is also one of the earliest indicators of oxidative stress (Hwang and Kim, 2007). Reactive oxygen species (ROS) are able to oxidize unsaturated fatty acids producing lipid peroxides. Lipid peroxidation may play an important role in carcinogenesis (Cai et al., 2012), in the molecular mechanisms of multiple sclerosis (Ferreti and Bacchetti, 2011), in Parkinson's disease (Jenner, 1991), and there is increasing evidence of its role in diabetes (Piconi et al., 2003), among other diseases. In comparison with other nanoparticles, HA:Eu showed the lower oxidative stress levels in the different tissues. However, HA:Eu nanoparticles, depending on the shape and concentration, could induce hatching inhibition in fish and a reduction of metabolic activity of the cells (Zhao et al., 2013). Hydroxyapatite NPs could be aggregated into bigger particles around the membrane protein, inducing low toxicity to development of zebrafish embryos, when compared to SiO_2 and TiO_2 nanoparticles (Xu et al., 2012). Apoptosis could be the possible mechanism of hydroxyapatite NPs toxicity, as reported from *in vitro* cell experiments (Wang et al., 2012).

BGO and AgNP induced moderate protein oxidation and lipid peroxidation. AgNPs have potent antibacterial activity making them interesting for several biomedical applications and they are extensively used in the healthcare, food industries, many domestic applications and as additive in surface materials and textiles. Although there is abundant literature on their toxic effects, there is little understanding of the AgNPs interactions with microorganisms (Maillard and Hartemann, 2012). Oxidative stress induced by AgNP has been reported from *in vitro* cell experiments (Arora et al., 2008; Carlson et al, 2008; AshaRani et al., 2009; Piao et al., 2011) and *in vivo* experiments including fish (Choi et al., 2010; Gagné et al., 2012; Wu and Zhou, 2012), but the toxicity mechanism is still not fully elucidated. In these studies at AgNP concentrations of 1 to 100 ppm, the hepatic lipid peroxidation, measured as the malondialdehyde generation, increased from 1.5 to 2-fold, results that are in agreement with those obtained in this work.

Bismuth germanate (BGO) has been used as a scintillator in detectors, industry and medicine for the past forty years (Weber and Monchamp, 1973; Chung and Chan, 1994; Macedo et al., 2004). On the contrary to the controversial AgNP, there is not information on BGO toxicity. As far as we know, this is the first report on the oxidative effects of BGO nanoparticles. BGO nanoparticles showed high protein oxidation at 1 ppm in liver, ovaries and brain, while lipid peroxidation was found only in liver and testicles (*Figs. 1 and 2*).

Yttrium oxide nanoparticles activated with europium (Y_2O_3:Eu) showed high oxidative stress, especially in proteins (*Figs. 1 and 2*). Y_2O_3:Eu is the most red-emitting phosphor widely used in color TV displays and fluorescent lamps. A few studies of the effect of Y_2O_3 nanoparticles are available in literature. Human aortic endothelial cells exposed to Y_2O_3 nanoparticles showed that the NPs were often present within cytoplasmic vesicles, where a transformation is suspected, and their density increased with nanoparticle concentration (Gojova et al., 2007). In addition, Y_2O_3 nanoparticles induced the up-regulation of inflammatory markers observed at the translational level. This effect was concentration dependent, with a statistically significant increase from concentrations of 10 ppm to 50 ppm. As mentioned before, the suggested pathway for nanoparticle-induced inflammation in human aortic endothelial cells is the production of reactive oxygen species (ROS). However, Schubert et al. (2006) suggest that Y_2O_3 nanoparticles could act as antioxidants in neurons.

The carboxylated multi-walled carbon nanotubes (MWCNT-COOH) showed the highest induction of both protein oxidation and lipid peroxidation at the two tested concentrations. The toxicity of MWCNT and their functionalized derivatives has been extensively reviewed (Lam et al., 2006; Schubert et al., 2006; Lacerda et al., 2006; Sargent et al., 2010; Shvedova et al., 2012). Carbon nanotubes (CNT) represent an special case, because relatively high content of transition metals, particularly Fe, Cu, Cr, and Ni (Tessonnier and Su, 2011; Valko et al., 2005), are present in some preparations. It is well known that the presence of these metals in single-walled carbon nanotubes (SWCNT) may be important in determining redox-dependent responses of macrophages (Kagan et al., 2006; Pulskamp et al., 2007). Non-purified iron-rich SWCNT induced higher concentrations of intracellular ROS, specifically hydroxyl radicals, determined by EPR spin-trapping analysis, and decreasing the mitochondrial membrane potential, than purified SWCNT. Two main consequences of ROS production induced by the presence of carbon nanotubes have been proposed: Inflammation, since ROS production is thought to be an essential requirement for inflammasome activation, and the activation of the NADPH oxidase. The recognition of MWCNT-COOH by phagocytes could lead to their internalization via endocytosis with subsequent fusion with lysosomes within the cell. Then, the lysosomes disruption may lead to the activation of the inflammasome. However, the contribution of each

ROS generation mechanism (mitochondria-generated and NADPH oxidase-generated ROS) to the activation of the inflammasome induced by carbon nanotubes requires further investigation (Valko et al., 2005).

A state of oxidative stress is created when the generation of ROS is higher than the rate of their elimination. Jones (2006) redefined oxidative stress as “a disruption of redox signaling and control” bringing antioxidant enzymes into the picture and making the point that the deleterious effect is observed when the accumulation of ROS surpasses the cell's defense capacity. In addition to non-enzymatic defenses mediated by some molecules, enzymatic detoxification of ROS constitutes a major component of oxidative stress defense systems and involves enzymes such as superoxide dismutases, catalases and peroxidases. Basal levels of these enzymes work in concert to remove endogenously produced ROS and keep their levels within physiologically safe limits. In our experiments, the expression of superoxide dismutase (Sod1), the transcription factor Forkhead box protein O1 (FoxO1), and the glutamate-l-cysteine ligase catalytic subunit (Gclc) were quantified in different fish tissues. No significant differences were found in the gene expression of any marker gene in liver, brain and fish body (*Table 3*). These results suggest that after only two days of NPs exposure, the fish is not able to metabolically compensate, by the expression of these genes, the oxidative stress caused by the presence of nanoparticles, leading to protein oxidation and lipid peroxidation in the different fish tissues. The exposure of cultured human cells to concentrations 40-times higher than those used in this work of cerium oxide and titanium dioxide NPs of different sizes, induced the expression of oxidative stress-related genes such as heme oxygenase-1, catalase, glutathione S-transferase, and thioredoxin reductase (Park et al., 2008). After prolonged exposures to relatively high concentrations, low cytotoxicity has been seen in cultured cells owing to increases in oxidative stress, most likely due to the increase of antioxidant defenses (Thakor et al., 2011). Recently, Gagné et al. (2012) performed a DNA microarray study of rainbow trout (*Oncorhynchus mykiss*) in order to identify the fundamental mode of action of AgNPs using the gene expression level. They found that 31% of the genes (from 270 studied genes) responded to either nano- or dissolved-silver, while 9.6% were specifically affected by AgNPs. The authors conclude that the increased lipid peroxidation and the reduced level of DNA strand breaks induced by AgNPs could not be explained by the presence of dissolved silver.

It seems that ROS generation by NPs is generated through several mechanisms including redox-cycling pathways, inflammation and protein denaturation. In addition, the activation of lysosomes may trigger a mitochondrial apoptosis, with the subsequent ROS production. Recently it has been demonstrated that the presence of antioxidant (vitamin E) protects cultured cells from the injury induced by SWCNT through the down-regulation of oxidative stress and prevention of mitochondria mediated apoptosis (Wang et al., 2012).

Thus, the oxidative stress is not the sole origin of the toxic effects of NPs. A better understanding of the nature and significance of such oxidative signaling and metabolic responses will offer new opportunities to control the effects of nanoparticles, including their toxicity. The cell could be considered as a collection of “nanomachines” (van den Heuvel and Dekker, 2007) to which engineered nanomaterials may interfere directly with cellular functions. A priority challenge of nanotechnology is to understand the mechanism leading to oxidative stress and its metabolic consequences in order to emphasize in a better nanomaterials design to prevent toxic effects that would put in risk animal health and environmental equilibrium.

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HABITAT DESIRABILITY MODELING OF CHEETAH (*ACINONYX JUBATUS VENATICUS*) USING MAXIMUM ENTROPY MODEL IN CENTRAL IRAN (A CASE STUDY: YAZD PROVINCE- DAREH ANJIR WILDLIFE REFUGE)

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Abstract. The Asiatic cheetah (*Acinaryx jubatus venaticus*) is an endangered species that only remains in the wild in Iran. Dareh Anjir wildlife refuge in central Iran is one of the best and the most secure habitats for this species. This study used the *MAXENT* method to study data from 2011 to 2013 to determine the suitability of habitat and specify factors affecting the presence of cheetahs. Environmental variables used in modeling were: height layers, slope, prey distribution (*Gazella bennettii, Ovis orientalis, Capra aegagrus*), distance to water resources, vegetation, distance to villages, distance to roads (asphalt, unpaved), distance to mines, and distance to railways. The results show that the most important factor affecting the presence of the cheetah is distance-to-water-resources. The parameters of prey distribution and level of vegetation were identified as the most effective factors for determining a plan to preserve and predict desirable areas for the presence of cheetahs. These predictions indicate that central and southeastern portions of Dareh Anjir wildlife refuge are the most suitable for the cheetah. The results of this study can be used to develop protective and managerial strategies to increase desirable habitats in Yazd province.

Keywords: *Acinonyx jubatus venaticu, Habitat Desirability, Dareh Anjir Wildlife Refuge, MaxEnt, Maximum Entropy*

Introduction

The Asiatic cheetah (acinonyx jubatus venaticus) is a rare and endangered Iranian carnivore. This creature has disappeared from former habitats in other Asian countries and remains in the wild only in Iran. For this reason, it is commonly known as the Iranian cheetah. The Iranian cheetah is the second most-endangered cat in the world after the Amour panther, of which only 30 to 40 remain in the wild (Zamani, 2010). Harrington (1971) states that the number of Iranian cheetahs before WWII was about

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400 and they were spread across the deserts and step regions of eastern Iran and some western areas near the Iraqi border.

In recent years, poaching, habitat degradation agriculture development, and a decrease in prey have caused its numbers to strongly decline to the point where an accurate estimation of the current population is not available.

Understanding major factors affecting the decline of the cheetah population is critical and requires an examination of current cheetah habitats to identify the biological and non-biological factors affecting them. Identification of potential habitats and how to improve and revive the remaining habitats require investigation of the factors effecting species distribution and habitat selection.

Researchers and wildlife managers often map probable and present distributions of an endangered species to establish preserves (Baldwin, 2009). Habitat modeling methods were first developed in the 1970s and were quickly employed in wildlife management (Mack et al., 1997; Anderson et al., 2000). The use of these methods allow large-scale estimation of habitat desirability without the need to gather details of physiological characteristics and species behavior (Morrison et al., 1992). One way to identify environmental parameters and their interactions is to apply ecological modeling to species distribution (Treves et al., 2002; Naturalium, 2009). Such methods are used to: (1) determine species habitat desirability (Vantoor et al., 2011); (2) predict species development trends in a region (Giovanelli et al., 2010); and (3) predict high-risk regions from competition between wildlife species and humans (Leung et al., 2002). Among the methods of modeling wildlife habitats (Levins, 1966), maximum entropy modeling has been shown to perform as well as or better than other methods. This algorithm is a common machine-learning algorithm supported by the thermodynamic regulations of ecological processes (Phillips et al., 2006). It is useful for mapping species distribution and habitat density (Baldwin, 2009). It has high prediction power, even for areas of low species presence and is widely used by researchers because it saves time and is cost effective (Hoffman et al., 2008; Wilting et al., 2010).

The present study used MAXENT maximum entropy for modeling geographical species distribution to model the suitability of the current habitat of the Asiatic cheetah in the Dareh Anjir wildlife refuge in Yazd, Iran. It examined how the presence of the cheetah is affected by environmental variables and developed probabilistic habitat models for the cheetah in the refuge. The model was used to determine desirable habitats and factors affecting the desirability of cheetah habitat using the maximum entropy method.

Materials and Methods

Materials

Study area and population

Dareh Anjir Wildlife Refuge is located in Yazd province in Iran, has an area of 1753.2 ha, and is located at 32°10′N to 32°36′N longitude and 54°48′E to 55°32′E latitude (*Fig. 1*).

The tallest peaks in this region are the Dareh Anjir, Chah Joleh, Adhero, Saghand, and Sobusi mountains. Altitude ranges from 830 m to 2293 m. Plains and flatlands are spread across the whole region. The mean annual precipitation is 74.9 mm and the mean daily temperature is 19.1°C. Based on the Ambrezh identification, Dareh Anjir has a dry and cold climate; and based on the Domarten correlation, the area has a dry climate (Consulting Engineer Corp. of Iran, 2009).

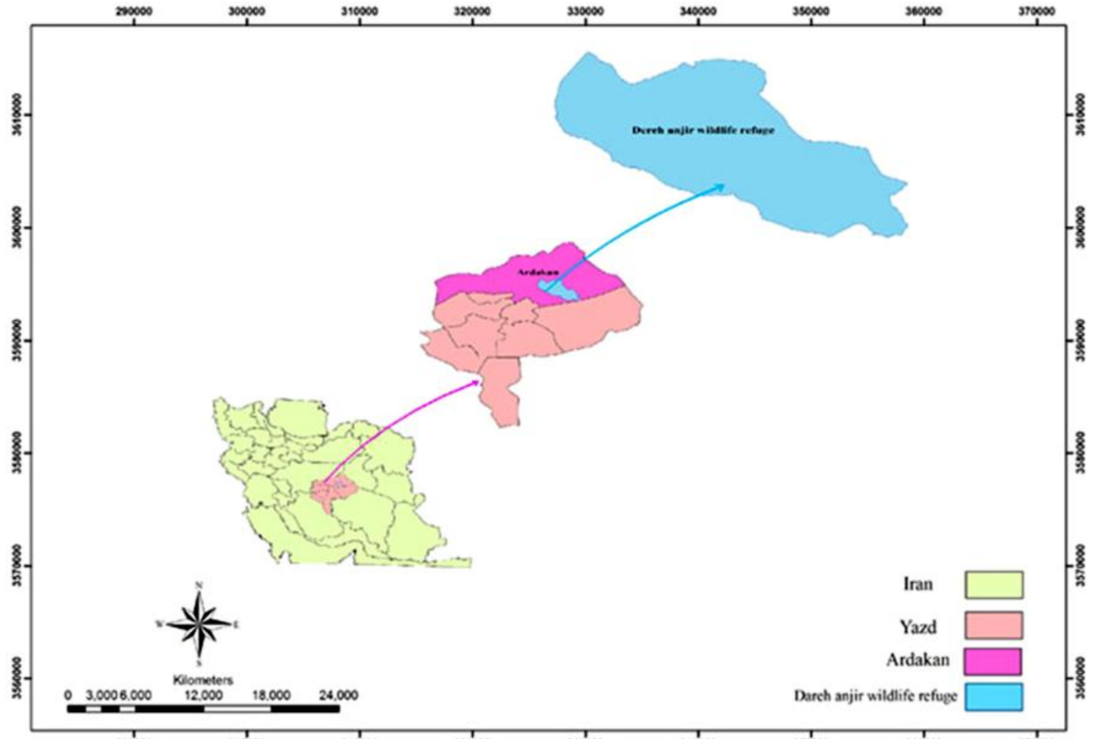

Figure 1. *Map of Dare-Anjir Wildlife Refuge and its location in Iran.*

Dareh Anjir wildlife refuge has no continuously-flowing river. Water reservoirs in this region are often brackish. The few freshwater sources are mainly wells, springs and stone channels (*qanat*) (Abedini, 2009). The wildlife refuge is located in the central Iran zone and exhibits the typical geologic, petrologic, and tectonic specifications of the region (Consulting Engineer Corporation of Iran, 2009). There are 138 species from 36 families and 112 genera in the area, including 13 protected species. The main plants of the area include: Artemisia sieberi، Zygophyllum eurypterum ، Astragalus sp ، Pteropyrum aucheri، Calligonum bungei، Acantholimon sp ، Seidlitzia rosmarinus، Tamarix sp، Lxiolirion tataricum، Ephedra strobilacea ammoniacu (Consulting engineer Corporation of Iran-Vegetation, 2009). Aside from Acinonyx jubatus venaticus, other valueable species such as wild sheep Ovis orientalis and jebeer Gazella bennettii and wild goat Capra aegagrus and wolf Canis lupus, golden jackal Canis aureus, fox Vulpes vulpes, wild cat Felis silvestris, leopard Panthera pardus and golden eagle Aquila chrysaetos, Aquila rapax, Falco peregrines, live in this region(Abedini, 2009). The use of trap cameras and a capture program in 2010 confirmed the presence of about 5 cheetahs in Dareh Anjir wildlife refuge (Zamani, 2010).

Methods

This study used the maximum entropy method and MAXENT software to produce a habitat desirability model and Arc GIS software to determine correlations and information layers for MAXENT. MAXENT combines environmental variables and

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species presence points and produces random points for the foreground, then presents a species distribution model with accompanying statistics and diagrams as output. Indirect variables such as slope, direction, elevation, slopes, type of habitat, and geology were collected across the desert regions and used to produce good indications of species dispersion patterns. Guisam and Zimmermann (2000) believe that these variables are suitable substitutions for a combination of species resources, and direct and indirect variables. Since the present study was performed across 1753.2 ha, indirect variables that present a better estimation about species presence were used for modeling. Information needed for MAXENT can be divided into two groups that include species presence register points and information layers of predictive environmental variables:

- Species presence points: these points are used as dependent variables and include species presence points on the regional level. Cheetah presence points were determined based on direct observation, photography by trap cameras, and traces such as feces, paw prints, and resting places for 2011-2012. The coordinates of these points were registered using GPS units at 39 points. They were then saved in CSV format in Excel and prepared for entry into MAXENT.
- Predictive environmental variables: These included behavioral and ecological features of the cheetahs, interviews with experts and wildlife rangers, and 11 independent habitat variables (elevation, slope, direction, water resources, vegetation, prey distribution, unpaved roads, asphalt roads, railways, mines, villages) that affect species presence were used for modeling. Raster plans were created in ASCII format in Arc GIS9.3 for use as environmental variables in MAXENT.

Information layers for elevation, slope and geographical direction were obtained from regional line maps prepared using a digital elevation model (DEM). Freshwater sources were wells, springs and stone channels (*qanat*) that provide water required for wildlife. The results of previous studies and the present study identified three species as preferential prey for the cheetah: Gazella bennettii, ovis orientalis and capra aegagrus. To prepare the information layer for prey, presence maps for the three species were produced separately in a polygonal vector shape and then the maps were combined into one map with the number of prey specified in each pixel. Studies have shown that the Dareh Anjir wildlife refuge provides good shelter and good vegetation for the herbivore cheetah prey.Predictive variables of human interaction in the Dareh Anjir wildlife refuge and were villages, roads (unpaved, asphalt), mines, and railroads (*Figure 2*). The villages include Saghand village on the eastern boundary and the Moghestan villages (Olia, Sofla) near the northeastern boundary. Additional residential compounds exist to the southeast for the major mineral and industrial center at Chadormalu. The access roads into Dareh Anjir wildlife refuge are the Yazd-Tabas asphalt highway, and the Chadormalu-Saghand and Kharanaq asphalt roads. Human activity on the region level includes mines, railroads, and railroad stations that decrease habitat security and have a degrading effect on wildlife and their habitats. A uranium mine is located in the region of Chah Joleh mountain and is under the jurisdiction of the Atomic Energy Organization. Chadormalu iron ore mine is also located in this region. The railroads in the region are the Ardakan-Chadormalu, Chadormalu-Ardakan, and the Ardakan-Bafq railways.

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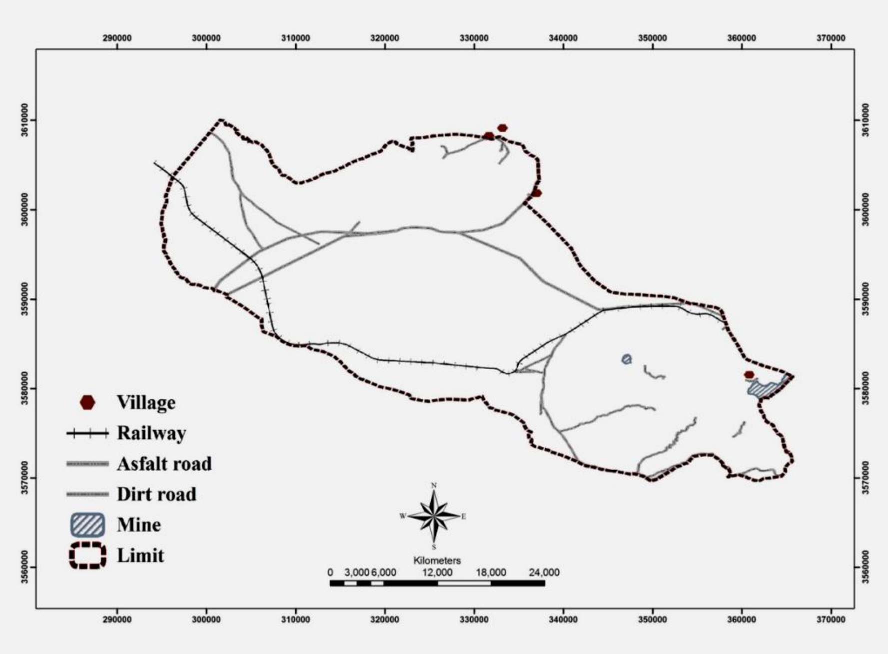

Figure 2*.Map of human-made factor in Dareh Anjir wildlife refuge*

MAXENT information layers for elevation, slope, direction, distance from water resources, and distance from human activity (villages, roads, mines, railroads) are continuous predictive variables and information layers on prey and vegetation are categorical predictive variables. Cell size was 30×30 mm for all Raster maps; since the maps should overlap, a digital elevation map was used as a reference to make the layers isometric. before MAXENT analysis, it was necessary to prepare the Raster maps for successive analyses and overlapping. These processes include:

- Normality of data: The maximum entropy method is sensitive to normality and equality of primary data (coordinates, line and column numbers, resolution). If this principle is not considered, calculations deviate and produce unreliable output, thus, all plans were equalized into one form in Arc GIS using the spatial analyst tool multivariate command.
- Data correlation: MAXENT requires variables that do not correlate; when two variables are highly dependent, both appear in the final model with one coefficient. When two or more variables have a correlation of more than 0.8, it is necessary to omit one variable from MAXENT. This done in Arc GIS using the spatial analyst tool multivariate command. Since the correlation between all environmental variables was less than 0.8, none were omitted.
- Since prey and water reservoirs attract carnivores, and villages, mines, roads and railroads deter wildlife, the distance command in Arc GIS was used to enter these factor maps into analysis.

MAXENT, version 3.3.3, was used to model maximum entropy of the geographical distribution of species to predict potential regions for the presence of cheetahs. Species presence data and predictive variables were entered into analysis. Two groups of presence data (test and training) were included in the random sampling to properly evaluate the model. Studies show that test data should consider 25% to 30% of presence data and that training data should consider 70% to 75% of presence data: 70% of presence points were randomly used to build a model and the remaining 30% was used to evaluate the results of the model. For random sampling, cross validation was repeated 10 times. Output parameters produced by the model were:

- **Cheetah.html:** The main output file which contains statistical calculations, maps, model's image and other links of the file.
- **Cheetah s.asc:** including the plan predicted with ascii format.
- **Cheetah.png:** including predicted distribution image.
- **Plot:** including different maps and graphs to print and use in reports.

In MAXENT, it is possible to calculate the area under the receiver operating characteristic (ROC) curve to evaluate model performance. This curve is represented as a graph where the vertical axis represents sensitivity (true positive) and the horizontal axis represents specificity (false positives). The area under curve (AUC) is created from the sensitivity and feature values and is a quantitative indicator of the efficiency and predictive power of the model. The range of values for the AUC is the lowest permitted value of 0.5 (random prediction) to 1.0 (completely accurate prediction) (Fielding and Bell 1997, Phillips et al. 2006).

Results

The model was created using MAXENT and the 39 presence records for one year and environmental records for different areas.

AUC

A value of 0.5 is the lowest value allowed for AUC. When AUC approaches 1.0, it means that the blue and green lines are far from the black line. *Figure 3* shows the maximum entropy algorithm used to model performance using the ROC curve. As shown, the AUC is 0.95 for the training data and 0.89 for the test data. The maximum entropy algorithm was very good and significant at the $p < 0.001$ level for predicting the presence of cheetahs.

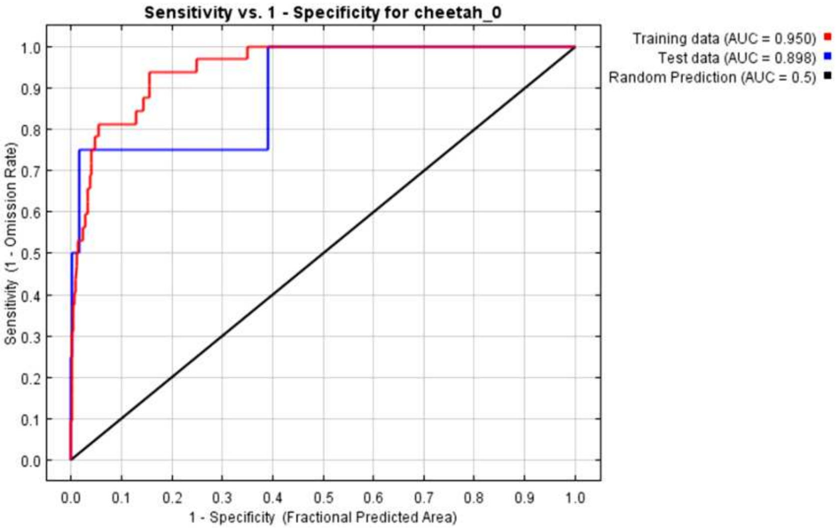

***Figure 3**. ROC curve calculated for the presence of Cheetah in Dareh Anjir*

The jackknife feature in MAXENT shows the importance of an environmental variable and the impact on model efficacy of the a variable that is removed or stands alone. Jackknife for AUC was greater for the gradient variable, perhaps because cheetah presence points declined at high gradients (*Fig. 4*).

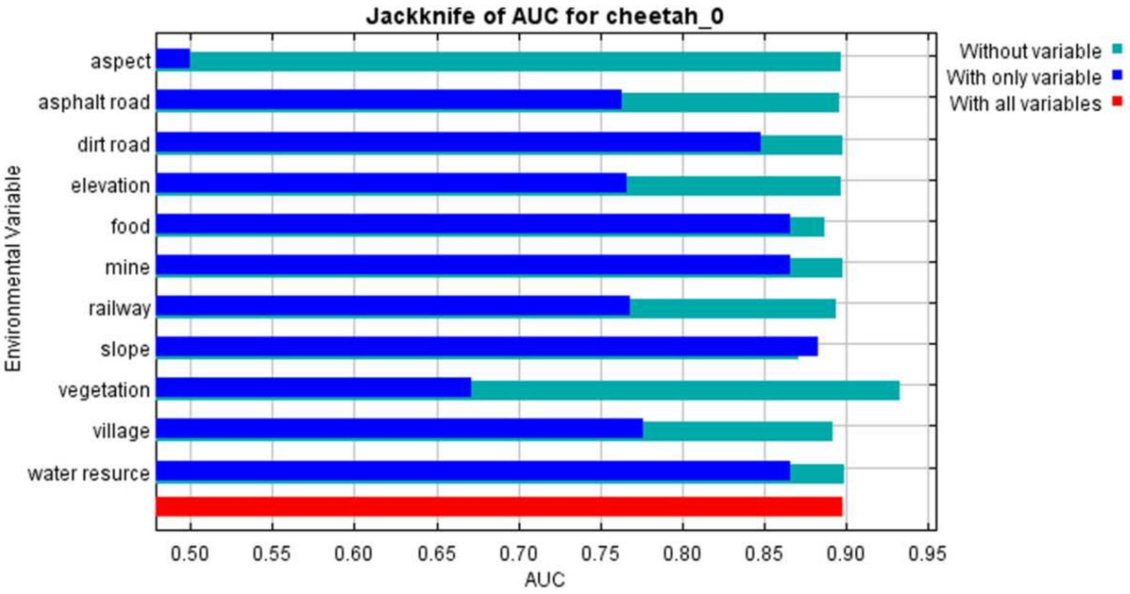

Figure 4.*Test of Jackknife, the area under the curve for environmental variables in Dareh Anjir*

MAXENT specifies the relative contribution of each variable. The results of the maximum entropy algorithm indicates that the distance-to-water-reservoir variable had the greatest relative contribution (45%) and permutation importance (40.2%) and was the most effective non-biological parameter for species distribution and prediction map creation. Distance-to-water-reservoir was assigned 45% of total change, indicating the usefulness of this variable. The second most effective parameter was prey distribution (13.4% of total change), and the third most effective parameter was vegetation (12% of total change); other variables contributed from 0.0% to 12% (*Table 1*).

Table1*. Relative contribution of the independent variables to the MaxEnt model*

Variables	Percent contribution	Permutation Importance
Water resource	45	40.2
Food	13.4	15.9
Vegetation	12	10.1
Slope	11	3.3
Dirt road	10.8	4.8
Railway	3.8	12.6
Asphalt road	2.7	11.5
Village	1	0.9
Mine	0.3	0.7
Elevation	0	0
Aspect	0	0

According to jackknife, for both test and training data, the environmental variable that showed the most value when used separately was distance-to-water-reservoir (*Fig. 5*).

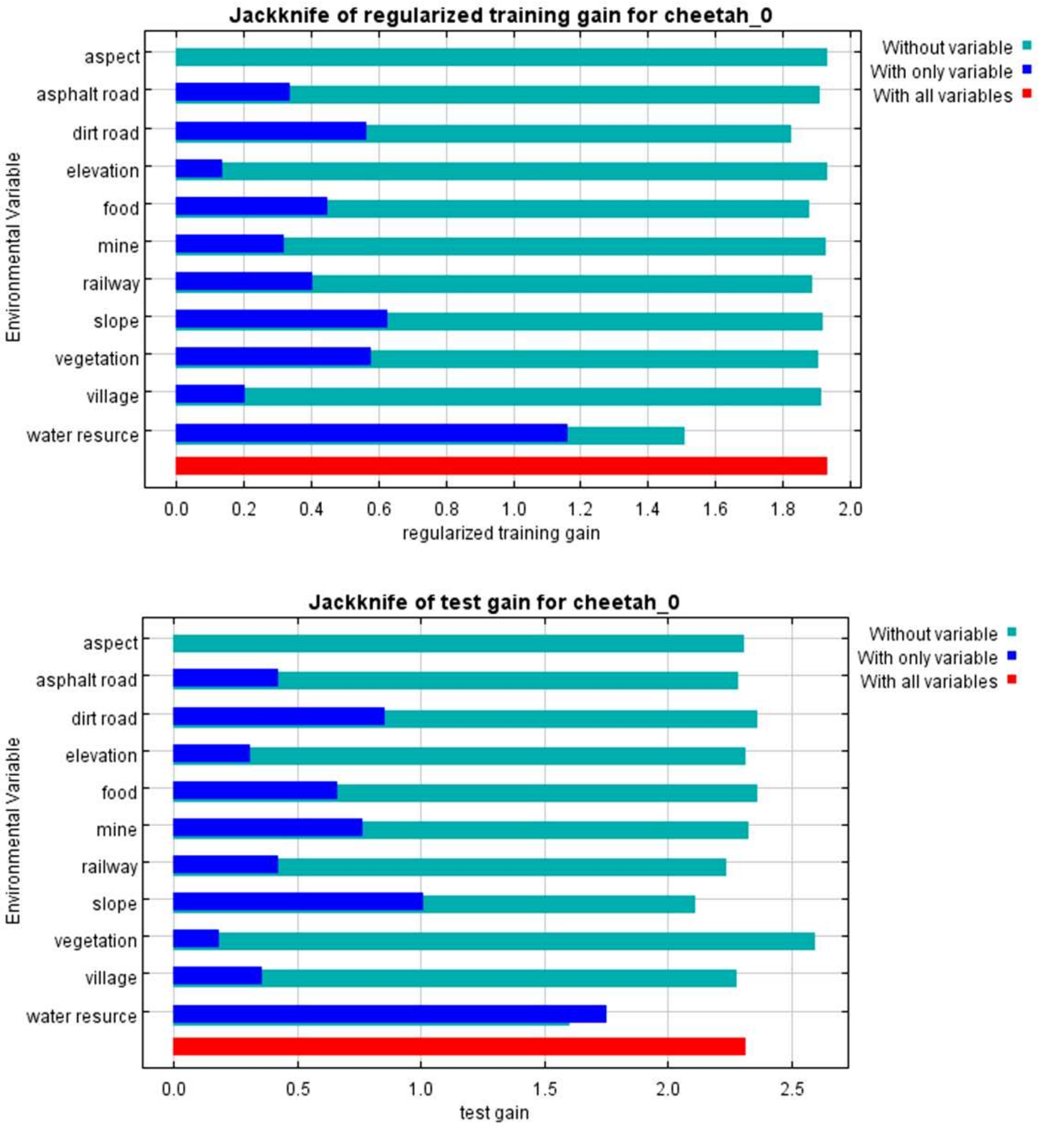

Figure 5.*Jack nife test for test and training presence data*

Analysis of omission/commission

A cumulative diagram shows the omission error for the test and training data.The omission error, or false negative (less than the real value or omission rate), occurs when a species is definitively observed to exist in one place, but the model indicates that the species does not exist in that location. On the graph, the black line is that produced by MAXENT for which the error value is zero. The green line is the error value for test data and the blue line is the error value for training data. When the blue or green line is located under and close to the black line, the error value decreases and the better the result. The value of the omission error for the training data in this study was lower than that for the test data. Analysis of the omission rate and predicted area as a function of the cumulative threshold (Philips et al., 2005) showed that the omission rate was close to the predicted area omission rate, indicating the model is robust for further analysis (*Fig. 6*).

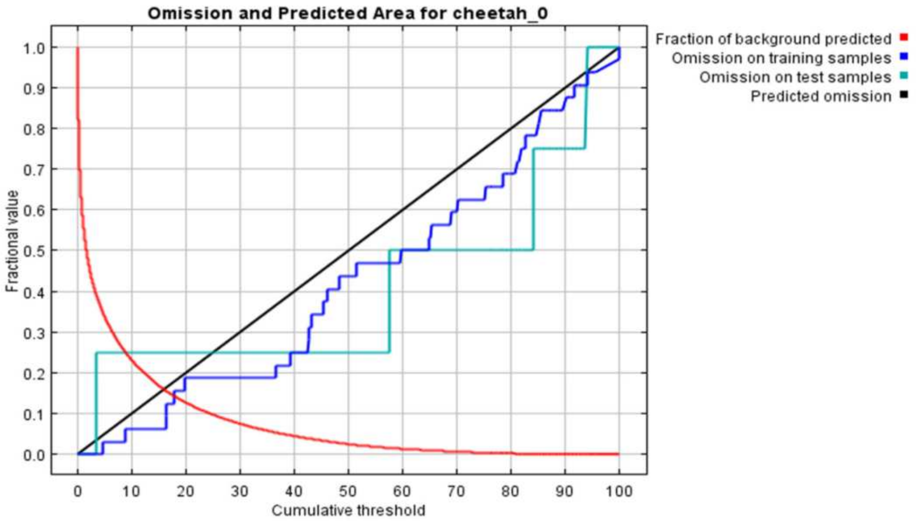

Figure 6.*Omission rate and predicted area as a function of t he cumulative threshold*

Cheetah.png the map of habitat desirability

The map of habitat desirability in Dareh Anjir wildlife refuge varied from zero to one (*Fig.7*). Very desirable areas with a high probability of cheetah presence are shown in red; locations where the probable presence of cheetahs is zero are shown in blue, and places where the probable presence cheetahs varies from 38% to 69% are shown in green.

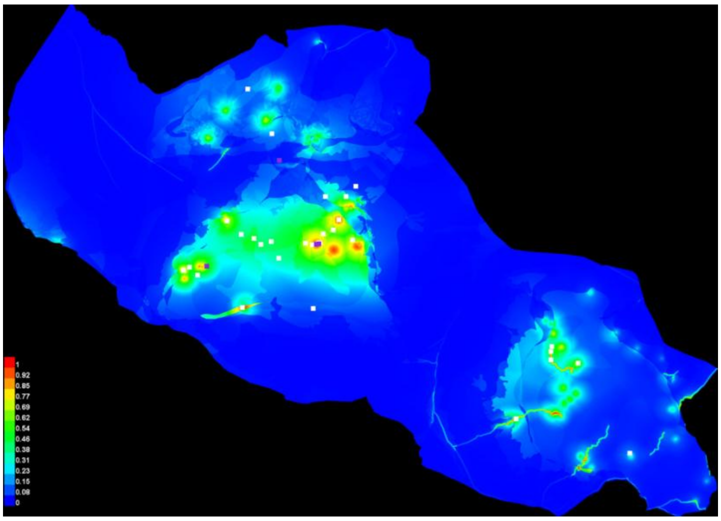

Figure 7*.MaxEnt habitat suitability map (Dareh Anjir Wildlife Refuge)*

The maps were then classified again according to the threshold of possible observation of the species under the study. This was is based on investigation and expert opinion and the desirability of habitat was classified as desirable or undesirable.

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Locations with a probability of 0% to 14% observation of cheetahs was classified as undesirable and the regions with a probability of 14% to 100% was desirable (*Fig 8*).

Desirable areas are those areas known to be used by cheetahs or that have the potential of being occupied by cheetahs. This classification indicated that 27837 ha of Dareh Anjir wildlife refuge is desirable and the remaining 147465 ha were undesirable areas for the presence of cheetahs.

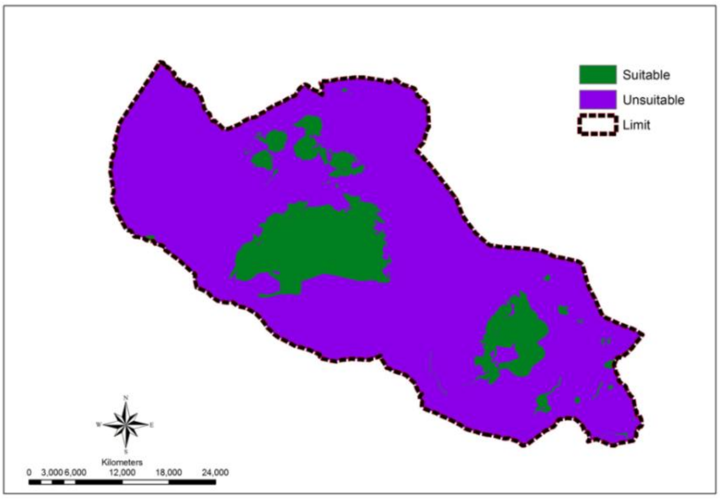

Figure 8. *Suitable and Unsuitable habitats for Cheetah in Dareh Anjir Wildlife Refuge*

Discussion

Identification of potentially desirable areas for the existence of Asiatic cheetahs is an important step toward preserving these animals. Dareh Anjir wildlife refuge is a major habitat for the Asiatic cheetah in Iran, but this model can also be used for other habitats in this region. The results of modeling with the maximum entropy algorithm for the species in Dareh Anjir wildlife refuge made it clear that the most important factor in determining and increasing desirable habitats for Asiatic cheetah is distance-to-water-reservoirs.

Omidi et al. (2010) estimated the appropriate altitude and gradient for a desirable habitat for the pardus saxicolor panther in Esfahan's Kola Ghazi national park was 1800 m to 2400 m in altitude at 20% to70% gradients using the ENFA method. The interpretation of the habitat desirability map show that the most desirable cheetah habitat is located in Dareh Anjir wildlife refuge between 1000 m and 2200 m with gradients of 0%-65%. The maximum entropy algorithm predictions indicate that the central, north and southeastern portions of Dareh Anjir wildlife refuge have highest probability of cheetah presence. *Table 1* shows that three parameters (distance-to-water-reservoir, prey distribution, vegetation) were most effective for increasing habitat fit and predicting desirable locations for the presence of cheetahs in Dareh Anjir wildlife refuge. Of the three, distance-to-water-reservoir was the most significant factor for prediction of desirable regions for the presence of cheetahs. There are 166 ha of water reservoirs in desirable areas and 23 ha in undesirable areas. Since this variable increases the probability of cheetah presence and in recognition of the droughts of recent years in this area, undesirable areas can be prioritized for maintaining good conditions for the water reservoirs using managerial strategies.

When the desirable habitat map is compared to that showing water reservoir distribution points, it is clear that areas for the highest desirability for cheetahs have the most water reservoirs, indicating the correspondence of the model with actual conditions for habitat. The continuous variable response curve for distance-to-water-reservoir indicates that the greater the distance-to-water-reservoir for an area, the lower the possibility of observing cheetahs in that area (*Fig. 9*).

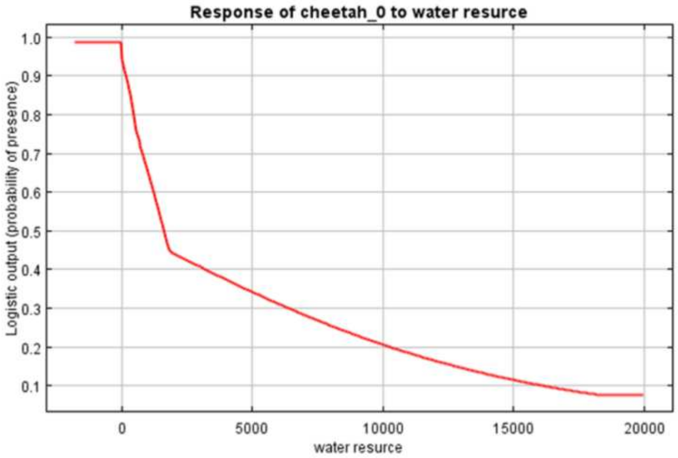

Figure 9. *Continuous environmental variable response curve for distance-to-water-reservoir for the probability of observing cheetahs in Dareh Anjir area.*

Recent studies show three herbivore species are major and preferential cheetah prey (Zamani, 2010; Conservation of Asiatic Cheetah and its Habitats, 2008). Unfortunately, the degradation and decrease in their natural habitats and poaching has decreased their number which, in turn, decreased the likelihood of the presence of cheetahs. In the areas of Dareh Anjir refuge with abundant populations of prey, the likelihood of observing cheetahs and potential habitat increased. The response curve for the categorical variable of prey shows that locations where all three types of prey exist have an increased probability of observing cheetahs (*Fig. 10*)

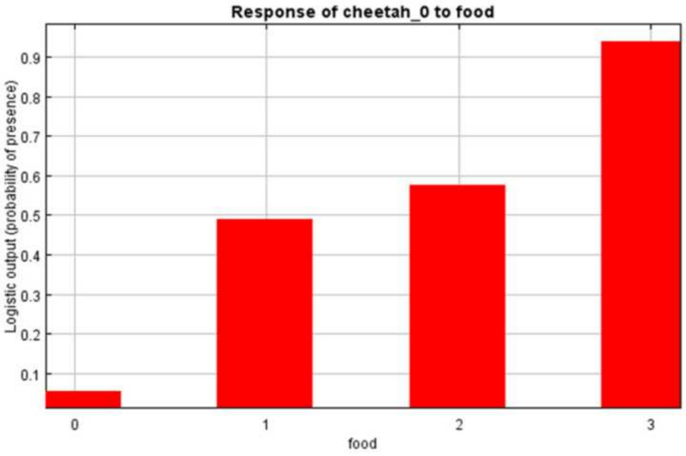

Figure 10.*Categorical environmental variable response curves for prey distribution and predicted observation of cheetahs in Dareh Anjir area.*

Vegetation is one important factor that increases habitat desirability and the probability of the presence of cheetahs in an area. Vegetation is a food for cheetah prey and can shelter cheetahs, thus, any area having suitable vegetation is a desirable habitat. In regions with vegetation, areas with the presence of Artemisia sieberi-Zygophyllum are the most desirable habitats for cheetahs and the probability of cheetah presence is high in such areas.

The conformity of this model was confirmed by comparing observed conditions for cheetahs in the past 10 years with the most desirable modeled classes of habitat. Most of these observations are located in the areas where there is an overlap of desirable classes of habitat. It can be concluded that the existing habitat for cheetahs in Dareh Anjir wildlife refuge is greater than what currently used by this species. If this area is well-managed, it should be possible to increase the number of cheetahs existing in this area (*Fig. 11*).

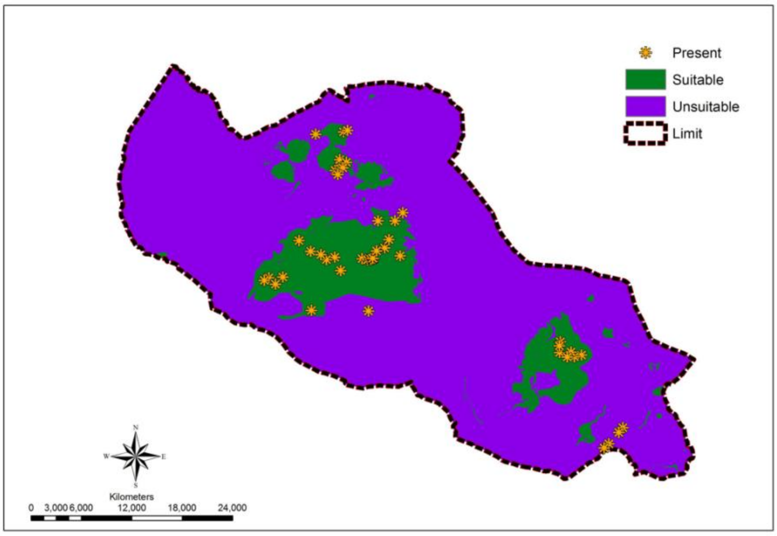

***Figure 11**. Suitable and Unsuitable habitats and presence points for Cheetah in Dareh Anjir Wildlife Refuge*

Conclusion

Evaluating potential habitats for cheetahs is one of the most important steps toward the conservation of the cheetah and helps managers and authorities identify and conserve more suitable areas. The value of the proposed model and its conclusion are a result of the maps that were formulated. Each pixel of these maps has a special value by which similar modeling of other area can be performed to design good conservation management for many species and their habitats. The large industrial and mineral iron facility at Chadormalu is located inside Dareh Anjir wildlife refuge and is near known desirable habitats. It is important to control the activities of this center to mitigate its bad effects on these desirable habitats.

Reports from the general environment conservation office indicate that the existence of roads is a major factor threatening cheetahs, as proven by the number of cheetahs killed by automobiles in Dareh Anjir wildlife refuge. The best way to resolve this problem is to relocate roads from inside the park to outside the area and deploy warning signs along the roadways. Since many alternate routes exist around and inside Dareh

Anjir wildlife refuge, the general environment conservation office points to the control of the entry of non-residents, particularly hunters, into the area. This requires an increase in the number of guards, especially in busy areas and the provision of facilities for this task.

The area has very hot and dry summers; this variable can be controlled by assuring adequate water resources in desirable cheetah habitats. The simplest way to increase desirable habitats for the cheetah in the study region is to improve management of water sources and create more sources of water sources in these habitats. It is recommended that modeling desirable habitats should be used in all projects designed to increase and protect Iranian wildlife species. Using these models, managers can identify controllable and important environmental variables that have the greatest effect on desirable habitats for these species. The identification of desirable habitats allows the creation of new and increasing populations.

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TRACE ELEMENTS IN WHITING *MERLANGIUS MERLANGUS* AND ITS PARASITE *HYSTEROTHYLACIUM ADUNCUM*: ROLE OF AGE, SIZE AND PARASITISM ON THE HOST

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Abstract. We measured the concentrations of the trace elements B, Mn, V, Co, Cr, Ni, Cu, Zn, As, Se, Mo, Pb and Cd in whiting *Merlangius merlangus* infested or non-infested with the intestinal helminth *Hysterothylacium aduncum,* as well as the concentrations in *H. aduncum* itself using inductively coupled plasma mass spectrometry (ICP-MS). The levels of most trace elements did not differ between the tissues of infested and non-infested fish. The tissues and parasites of younger fish (<2.1 year old), however, carried higher amounts of trace elements than those of older individuals. *H. aduncum* accumulated all trace elements in greater amounts, up 255 folds, than any fish tissues tested. These results support the idea that the higher metabolic activity of younger fish may lead to higher amounts of trace element accumulation in their tissues. Moreover, trace element accumulation is not cumulative as fish grow, and fluctuates during various life cycle of fish.

Keywords: *trace elements, Merlangius merlangus, Black Sea, parasite, fish.*

Introduction

Trace element accumulation in seafood is a major environmental concern (Gunkel, 1994), particularly in closed seas under heavy anthropogenic pollution pressure such as the Black Sea. Trace elements, required by the organisms in minute amounts, are considered potentially environmental toxic substances if their levels are higher than biologically acceptable levels. While the majority of trace element pollution comes from parent rocks and metallic minerals, accumulation of trace elements from effluents in contact with agricultural and industrial activities, as well as with shoreline urban development has intensified in the last decade (Adriano, 2001). Therefore, an assessment of trace elements accumulating in ailments through seafood is needed to be able to initiate and implement proper management strategies.

Bioaccumulation of trace elements in the indicator model organisms may provide an insight into the health of the ecosystem. Since not all bioindicator organisms behave biologically the same for a given trace element, an urgent evaluation is required to determine their robustness as models for trace elements accumulation in an ecosystem. Though various fish tissues have been proposed for pollution monitoring in aquatic ecosystems (Eira et al., 2009), the bioamplified trace element levels in parasites could make these organisms a better trace element pollution indicator than their hosts. Several studies strongly indicate higher levels of trace element accumulation in intestinal

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acanthocephalans in comparison to the levels in their fish hosts (Gabrashanska and Nedeva., 1996; Galli et al*.,* 1998; Sures et al*.,* 1999). However, many questions regarding the accumulation of trace elements in a host-pathogen interaction remain to be answered. *Anguilla carassus*, found in the intestines of the European eel *Anguilla anguilla*, did not accumulate lead as the acanthocephalan *Paratenuisentis ambiguous* (Sures et al*.,* 1999; Sures et al*.,* 1994; Zimmermann et al*.,* 2004). As accumulating evidence suggests, the bioaccumulation of trace element dynamics in parasites likely depends on the host, trace element type, and environments. Gabrashanska and Nedeva (1996) interestingly reported that the trace elements Cu and Zn, are higher in, parasitized fish tissues compared to unparasitized fish, assessing that trace elements accumulation in fish is biased by parasite infection. Furthermore, because infested fish are more prone to trace element toxicity (Boyce and Yamada, 1977; Pascoe and Cram, 1977), other such factors as tissue types, age or sex of fish are to be evaluated to understand their influence on the bioaccumulation of trace elements, both in infested and non-infested fish. Moreover, it is still not clear whether high accumulation of trace elements in parasites indicates lower retrospective exposure for the host in comparison to non-infested fish.

The whiting *Merlangius merlangus*-nematode *Hysterothylacium aduncum* host-parasite constitutes an excellent candidate model system for monitoring the accumulation of trace elements in the Black Sea. The fish is a non-migratory species in the Black Sea, allowing for its easy sampling in its local environment at any time of the year. In addition, the whiting is a bottom dwelling fish and therefore possibly exposed to trace elements more than pelagic fish. Moreover, being a carnivor species preying on all fish species including its own, there is a high prevalence of intestinal parasitic nematodes in adult individuals enabling satisfactory levels of sampling adult nematodes from each fish.

Here, we aimed to assess whether trace element concentrations in whiting infested with *H. aduncum* or free of the parasite differed. Moreover, we also evaluated the effects of size and sex of the fish host as a factor for bioaccumulation. As we hypothesized that parasite- infested fish accumulated more trace elements due to parasite induced enhancement of the metabolic activity, we compared trace element between sexes and in various age classes of parasitized and non-parasitized fish, as well as with the intestinal parasites themselves. This study stand as a model for monitoring and evaluating trace element pollution in the Black Sea.

Material and Methods

Fish and sampling

Whiting *Merlangius merlangus* both infested and non-infested with the nematode *Hysterothylacium aduncum* (nematoda: anisakidae) were collected (n = 288) using dip-nets during December and January, 2011 in the Surmene Bay, Trabzon Province, Turkey, in the south-eastern Black Sea (40°55' N; 40°11' E) (*Table 1*). The sampling was carried out at the same place in two consecutive months as the whiting is a local, non migratory species. After measuring length (cm) and weight (g), the samples were sorted by sexes and three length (total length) classes: 1) smaller than 15, 2) between 15-17 cm and 3) bigger than 17 cm. Subsequently, these length classes were transformed, by the von Bertalanffy equation, to age classes as previously described

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(Altuntas and Ogut., 2010) using the data referring to the same species, in the same region of Turkey (Ciloglu et al., 2001).

Table 1. *Sampling scheme of whiting* M. merlangus *for age groups, sexes, and tissues.*

Age Class	Sex	Tissue	Number of Pools	Fish/pool	n
< 2.1	Male	Sperm Duct	3	10	93
		Liver	3	9	
		Muscle	3	10	
		Gills	3	11	
	Female	Gonad	3	8	84
		Liver	3	7	
		Muscle	3	7	
		Gills	3	7	
2.1-2.9	Male	Sperm Duct	3	8	36
		Liver	3	7	
		Muscle	3	8	
		Gills	3	8	
	Female	Gonad	3	13	52
		Liver	3	13	
		Muscle	3	13	
		Gills	3	13	
>2.9	Male	Sperm Duct	2	1	5
		Liver	2	1	
		Muscle	2	1	
		Gills	2	1	
	Female	Gonad	3	5	18
		Liver	3	5	
		Muscle	3	5	
		Gills	3	5	

Tissue and intestinal parasite sampling

All whiting sampled in the study was dissected to remove the viscera and to obtain tissue samples for trace element analysis (*Table 2*). About 0.5 g of muscle, liver, gills, gonad, refers to ovaries (female) or sperm duct of fish from each stratification were pooled (five to eight of the same tissue/pool) and analysed for metal content. The digestive tracts were then cut longitudinally to determine the number of adult nematode. Note that all nematodes present in the digestive truck are adults, L5 stage nematodes, therefore "infested fish" refers to fish having adult nematodes in their intestines. Generally, infested fish have one to five adult nematodes in their digestive tract. In a parallel sampling, analyses of the 15 individual digestive tract nematodes cleared by glycerin and photomicrographed all were identified as *H. aduncum* according to the keys created by Skryabın (1969).

***Table 2**. Sampling scheme of the intestinal parasite H. aduncum from its host M. merlangus.*

Age Class	Infested	Sex	Number of Pools	Fish/pool	n
< 2.9	Y	Male	3	4	265
		Female	3	4	
	N	Male	4	29	
		Female	4	31	
>2.9	Y	Male	0	0	23
		Female	1	1	
	N	Male	2	3	
		Female	3	6	

Measurement of trace elements

All fish tissues and parasites were freeze-dried until constant weights were acquired. After homogenization, about 0.5 g of dry tissue was digested in 7 mL nitric acid+1 mL hydrogen peroxide in a microwave digestion unit (Milestone Ethos 1, HPR-FO-17). Then, the samples were further diluted to 25 mL using deionized water. The same procedures were also applied to the blank samples. The amounts of trace elements B, Mn, V, Co, Cr, Ni, Cu, Zn, As, Se, Mo, Pb and Cd were measured using a high performance inductively coupled plasma mass spectrometer (Bruker 820 ICP-MS) operated in collision reaction interface (CRI) mode to overcome polyatomic interferences. The accuracy and reliability of the analyses were assured using certificated reference material: DORM 3 (Fish protein). Internal standards having Li, Sc, Y, In, and Tb (50 ppb (ug/L) were used as reference. The calibration standards and the samples were matrix-matched. Reference material and their recovery are listed in *Table 3*.

***Table 3.** Metal concentrations (µg g-1 dry wt) in certified reference material (DORM-3).*

Metals	Certified value	Observed value	Recovery (%)
Cr	1.89±0.17	[a]1.85±0.19	97.88
Ni	1.28±0.24	1.24±0.22	96.88
Cu	15.5±0.63	14.8±0.43	95.48
Zn	51.3±3.10	49.18±0.89	95.87
As	6.88±0.30	6.70±0.36	97.38
Cd	0.29±0.02	0.28±0.04	96.55
Hg	0.38±0.06	0.35±0.06	91.62
Pb	0.40±0.05	0.38±0.05	95.00

Values indicate the average value ± SE

Parasites belonging to the oldest age group (>2.9 year old), and a combined sample of parasites from the smallest age group and middle age group fish were assayed to determine the rates of trace elements in a unit of sample, using the same procedures described above except for the digestion, which was carried out in 1mL nitric acid at room temperature because of the small pool size of parasites.

Statistical analysis

Metal concentrations in parasitized and unparasitized fish were compared using a two-way ANOVA test by taking tissues and age classes, or tissues and sexes, into consideration as variables. Moreover, using the same two-way ANOVA approach, we also compared the amounts of trace elements in different tissues of male and female or age groups ignoring the infection status. The levels of trace elements in parasites were compared using chi-square test. P-values less than 0.05 were considered statistically significant.

Results

Influence of age and sex on accumulation of trace elements in M. merlangus

To assess whether different whiting tissues accumulated trace element in a similar fashion, we quantified B, Mn, V, Co, Cr, Ni, Cu, Zn, As, Se, Mo, Pb and Cd in muscle, liver, gills and gonad (ovaries) or sperm ducts by ICP-MS. Tissues had statistically different levels of trace elements, with the exception of Cr and Ni (One-way ANOVA, $P<0.05$, *Figs. 1 and 2*). Accumulation of Pb, B, V, Mn, Co and Se in the gills, Mo and Cd in the liver, and Cu and As in the gonad/sperm duct and liver were significantly higher compared to the other tissues tested ($P<0.05$). Therefore, our analysis of these thirteen trace elements showed that fish tissues accumulated trace elements in different quantities.

To determine whether age was a factor influencing trace element accumulation, we divided our fish samples into three age classes. The youngest age class (<2.1 year old) had significantly ($P<0.05$) higher amounts of trace elements than the oldest fish (>2.9 year old) (*Figs. 1 and 2*). The age effect was mainly visible for the amounts of Mn, V, Ni, Mo and Cd in the youngest fish group, whereas Pb was the only trace element highly concentrated in the gills of the oldest age class ($P<0.05$).

Sexes of fish also had an impact on the accumulation of some of trace elements in whiting (*Fig. 2*). Co, Zn, As and Se, were present at higher levels in gonads (ovaries) than in other tissues, whereas the concentrations of trace elements in the tissues of male and female whiting were not significantly different from each other ($P>0.05$).

Influence of parasite infestation on accumulation of trace elements in the host

Twenty-four out of 288 of the whiting sampled for this study were infested with the adult nematode, *H. aduncum*. We therefore assessed whether the presence of parasite influenced accumulation of trace elements in the whiting host. The amounts of the trace elements Ni, Pb, Mn, Co and Mo were significantly higher in the infested fish than that in non-infested fish ($P<0.05$; *Figs. 1 and 2*). Furthermore, the younger infested whiting carried higher amounts of trace elements compared to individuals of the other two age classes.

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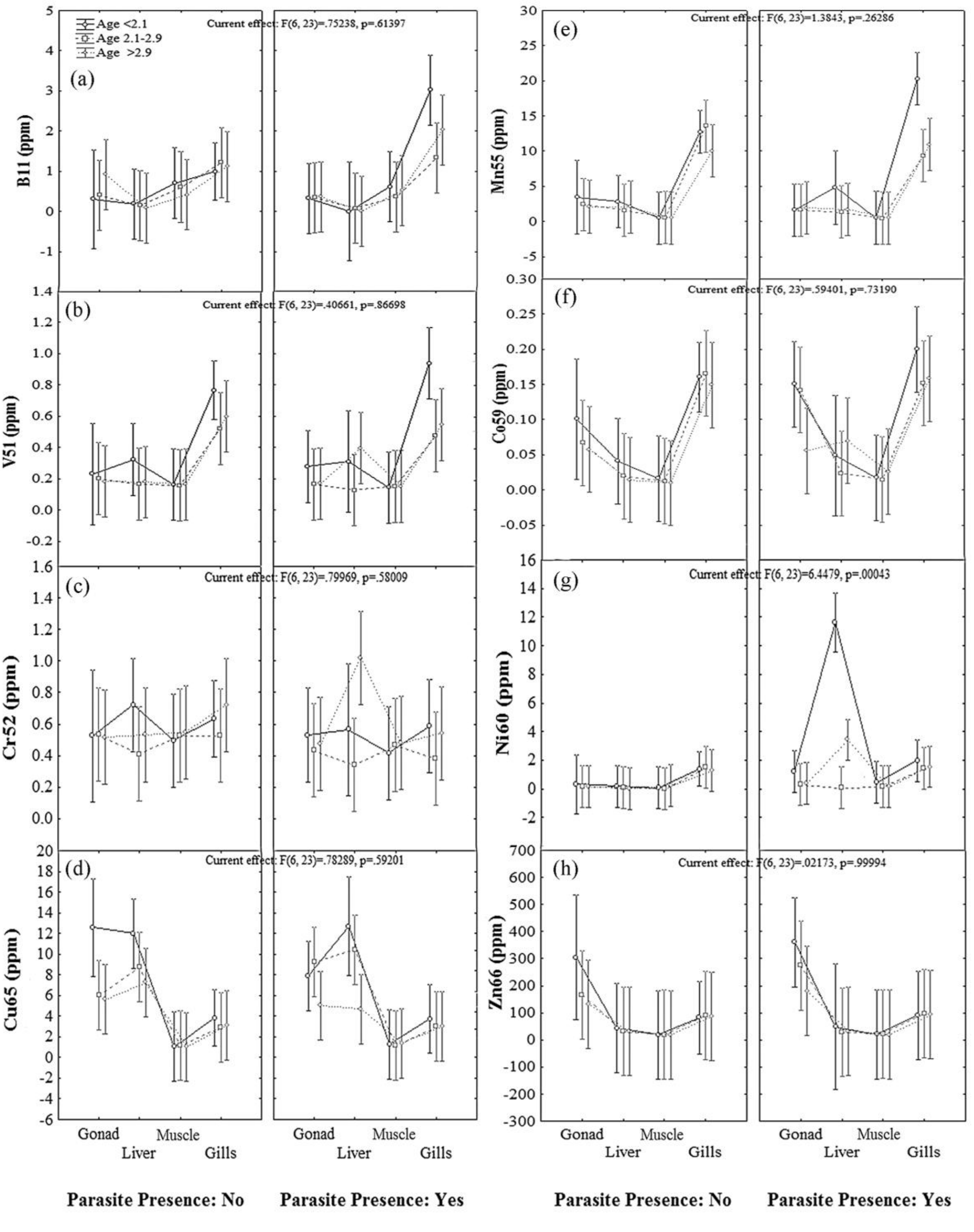
This was most evident for the accumulation of the trace element Ni: the younger age class presented significantly higher amounts of Ni in the kidney (P=0.0043; *Fig. 1g*) compared to the other two classes. Moreover, the levels of Ni in the kidneys of mid-age class whiting were higher than that of the oldest age class fish (P<0.05).



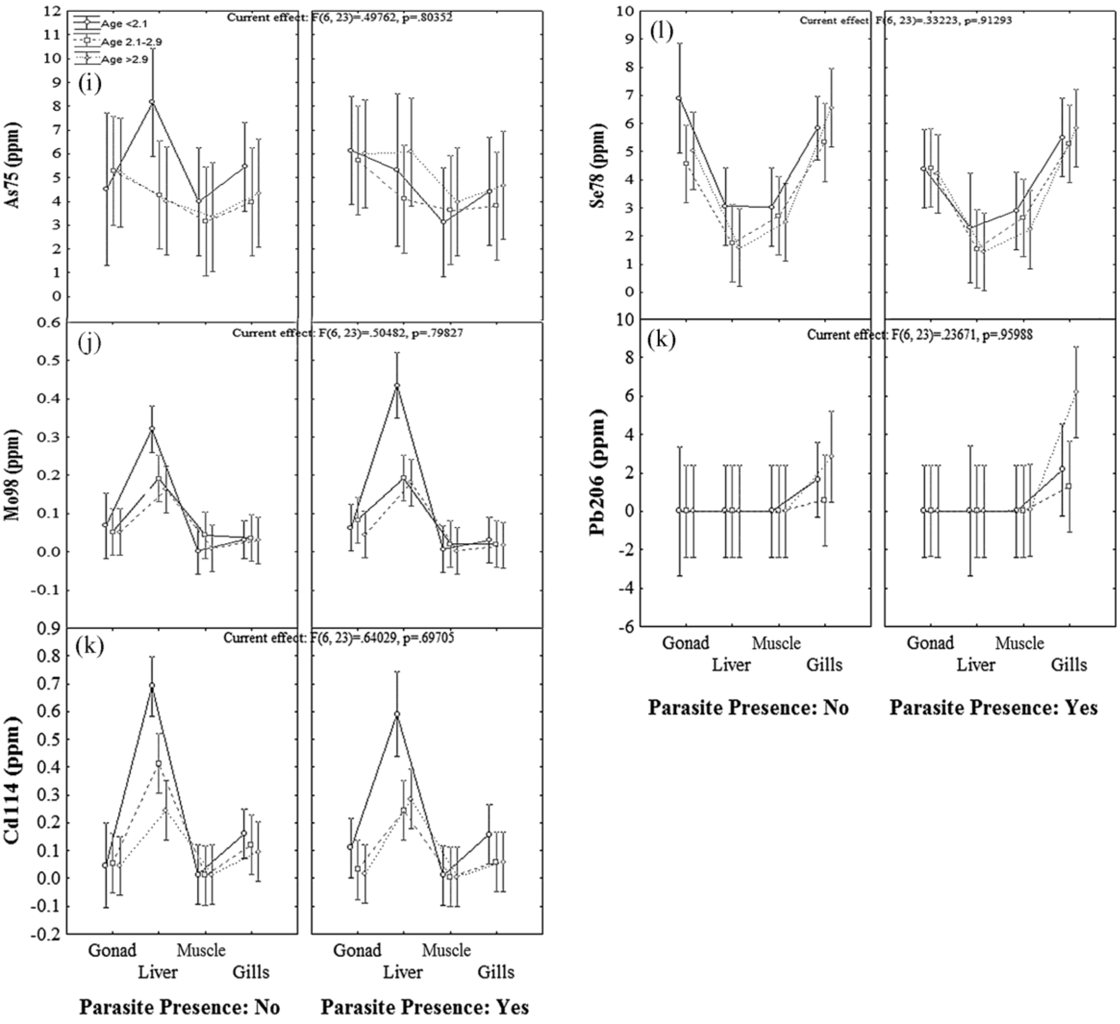


Figure 1. *Concentrations of trace Elements in various tissues of whiting M. merlangus in three age classes (<2.1, 2.1-2.9, and >2.9) with and without the endoparasite H. aduncum sampled in the south-eastern Black Sea. Bars denote 95% confidence intervals.*

The concentration of B was similar in both infested and non-infested fish in the two older classes (P>0.05), whereas they were higher in the gills of young whiting (P<0.05; *Fig. 1a*). We measured a similar trend for Mn and V (*Fig. 1e*,*1b*). Pb amounts in infested fish were also higher than those of non-infested fish. However, the amounts of Pb in gills were significantly higher than Pb in other tissues in old whiting (P<0.05; *Fig. 1k*).

The concentrations of Co and Zn were significantly higher in the gonad (ovaries) of infested fish than that of non-infested fish (P<0.05; *Fig. 2b*). Both As and Mo concentrations were higher in the kidneys of male fish than that of female fish (*Fig. 2c*). The rate of As was significantly higher in the sperm duct of infested fish than that in the ovary of infested whiting (P<0.05).

Trace elements in the endoparasite H. aduncum

Parasites carried higher amounts of trace elements than any host tissue tested. For example, Cu amounts in the parasites of the younger group whiting were 3 times, 2.5 times times, 19 times and 7 times higher than the average trace element rates found in host tissues; gonad, liver, muscle and gills respectively (*Table 4*). Moreover, various tissues presented different odds of the level in the parasite to the level in the tissue. The amount Cd was 255 times higher in the same group of parasites than that in the muscle of the respective hosts, whereas it was 49 times, 6 times and 22 times of that from ovaries, liver and gills respectively.. Analysis of intestinal parasites from young (<2.9 year old) and older (>2.9 year old) whiting also showed that the trace element amounts in parasites were higher in the nematodes found in younger fish, except for the levels of Cd and Co (*Table 4*). The differences of average trace elements in mid-age class, younger, and older fish were statistically higher for the eight trace elements (B,V, Cr, Mn, Cu, Zn, As and Se) tested ($P<0.05$; *Figures 1 and 2*). Moreover, parasites in the younger fish always presented higher rates of trace elements.

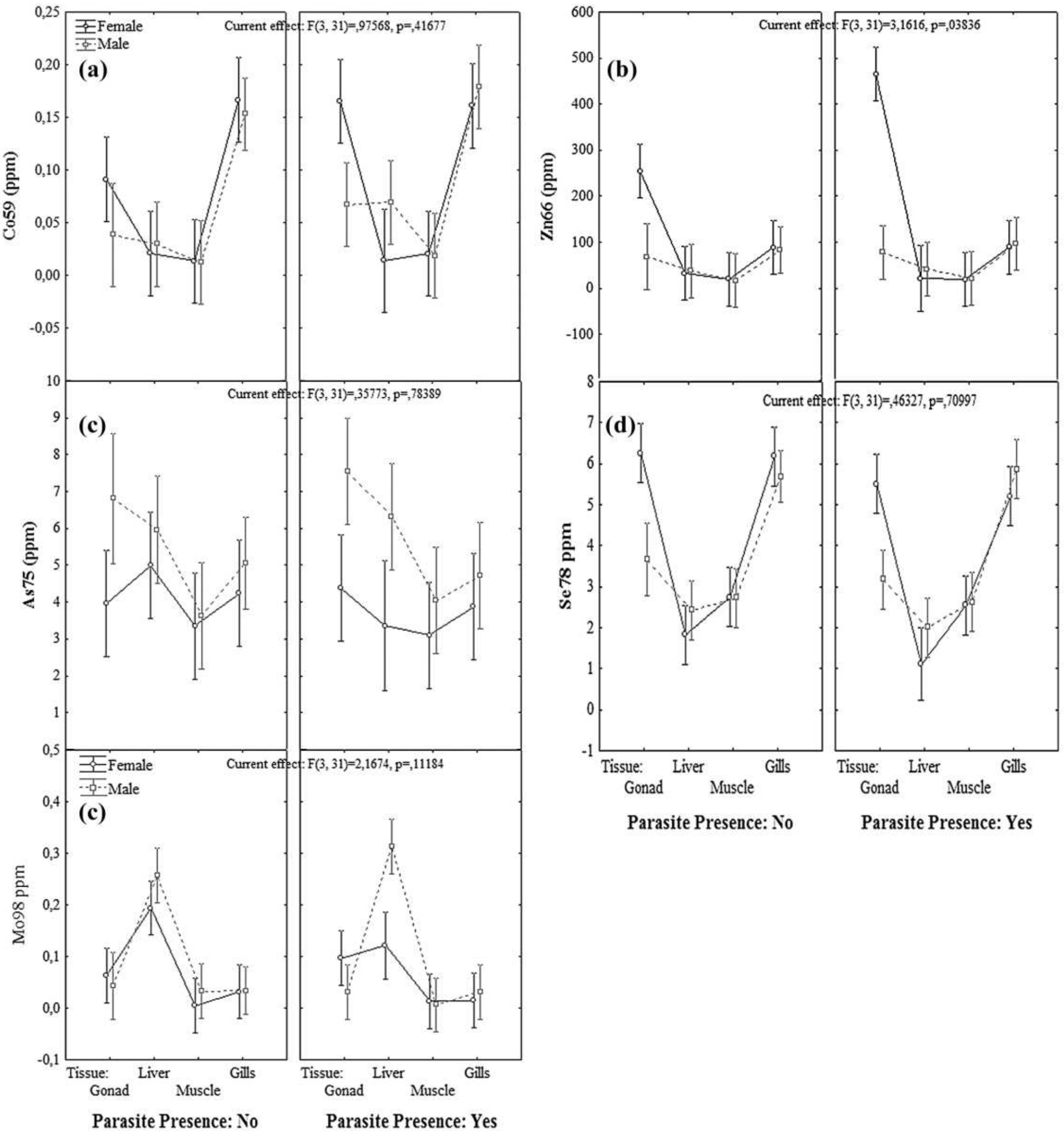


Figure 2. *Concentrations of trace Elements in various tissues of male and female whiting M. merlangus with and without the endoparasite H. aduncum in the south-eastern Black Sea. Bars denote 95% confidence intervals.*

Table 4. *Trace element levels measured in endoparasite H. aduncum harvested from whiting M. merlangus aged less than 2.9 year old or more than 2.9 year old. Chi-square tests were used to compare the levels of trace elements in parasites collected from different age classes. The parasites from these two age classes were tested separately. . All parasites were grouped according the host's age and sexes due to considerable small size of the parasite. Values for amount of trace elements in host tissues are represent as average values ± standard The same letters refer to the groups statistically the same deviation*

	Amount of Trace Element in Parasites (micrograms per g dwt)			Amount of Trace Element in Host Tissues (micrograms per g dwt)			
Trace Element	Age class < 2.9	Age class > 2.9	P-Value	Gonad	Liver	Muscle	Gills
B	8.3	5.6	0.001	0.46 ± 0.11^{a}	0.09 ± 0.02^{a}	0.54 ± 0.05^{a}	1.57 ± 0.29^{b}
V	3.25	2.75	0.361	0.20 ± 0.01^{a}	0.25 ± 0.05^{a}	0.16 ± 0.01^{a}	0.65 ± 0.07^{b}
Cr	10.1	8.05	0.030	0.50 ± 0.02	0.60 ± 0.11	0.49 ± 0.02	0.57 ± 0.03
Mn	20.75	14.95	0.000	2.07 ± 0.83^{a}	2.17 ± 0.83^{a}	0.51 ± 0.79^{a}	12.82 ± 0.76^{b}
Co	2.4	2.6	0.689	0.10 ± 0.01^{a}	0.04 ± 0.01^{b}	0.02 ± 0.01^{b}	0.16 ± 0.01^{c}
Ni	2.9	1.95	0.054	0.43 ± 0.56	1.75 ± 0.56	0.16 ± 0.54	1.50 ± 0.52
Cu	22.55	16.9	0.000	7.28 ± 0.74^{a}	8.99 ± 0.74^{a}	1.16 ± 0.71^{b}	3.30 ± 0.68^{b}
Zn	384.15	282.9	0.000	230.26 ± 27.75^{a}	34.61 ± 27.75^{b}	19.63 ± 26.57^{b}	88.77 ± 25.52^{b}
As	18.65	16.6	0.122	5.57 ± 0.44^{a}	5.3 ± 0.44^{a}	3.53 ± 0.42^{b}	4.52 ± 0.40^{b}
Se	24.25	11.85	0.000	4.74 ± 0.27^{a}	1.91 ± 0.27^{b}	2.66 ± 0.26^{b}	5.73 ± 0.24^{a}
Mo	0.95	0.8	0.612	0.06 ± 0.02^{a}	0.23 ± 0.02^{b}	0.015 ± 0.06^{b}	0.03 ± 0.015^{a}
Cd	2.45	2.55	0.841	0.05 ± 0.032^{a}	0.40 ± 0.032^{a}	0.01 ± 0.03^{a}	0.11 ± 0.03^{b}

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Discussion

In this study, we investigated whether bioaccumulation of trace elements in whiting *M. merlangus* was influenced by sex, age or by the presence of the intestinal parasite *H. aduncum*. The age of the host appeared to be the principal factor in the bioaccumulation in both tissues of whiting and their respective intestinal parasites. Moreover, the amounts elements were significantly higher in the intestinal parasites than those in the tissues of the host. Our results indicate that the whiting – nematode model could be used for studying dynamics of trace element accumulation in respect to the age, sex and host tissues and be used for biomonitoring of trace element pollution in the Black Sea.

While the amounts of five trace elements (Co, Ni, Mo, As and Cd) in younger and older fish was the same, B, V, Cr, Mn, Cu, Zn and Se levels were significantly higher in younger fish, probably due to the differences in metabolic activity of younger and older fish. Older fish was expected to have higher levels of trace elements indicating retrospective exposure from water. However, our results show that younger fish have higher levels of trace element accumulation. It should be noted here that according to these results surrounding water column seems to be not the biggest source of trace elements. Other reasons such as metabolic rate differences, food quality or foraging shifts of whiting in their life cycle could be responsible for this difference. Younger fish have higher weight specific metabolic rates (mg $O_2 \cdot kg^{-1} \cdot h^{-1}$), such as consumption of oxygen at a given time, than older fish (Sims, 1996). Furthermore, trace elements accumulation increases fish metabolic rate (Vinodhini and Narayanan, 2008). Our result also showed that infested young fish accumulated higher amounts for trace elements that infested old fish: it is possible that parasite induced enhancement of the metabolic activity may make the fish host more prone to environmental toxicity (Boyce and Yamada, 1977; Pascoe and Cram, 1977), another piece of evidence suggesting that the metabolic rate could be related to trace element accumulation. Higher metabolic rate will result in higher energy and oxygen demand, leading to more exposure to trace elements by the elevation in the gill ventilation rates and feeding to comply extra energy costs. The amounts of trace elements in parasites collected from younger fish were also higher from those in older fish, indicating that the rates of accumulation is different during their life history. Accumulation and excretion of trace elements could fluctuate seasonally (Retief et al., 2009). Therefore, further study is necessary to verify potential seasonal effects.

Our study showed that infested fish had higher levels of trace elements comparing to non-infested ones. This is in contrast with previous report showing that infested fish have lower levels of metals compared to non-infested fish from the same area (Azmat et al*.*, 2008; Morsy et al*.*, 2012). It is possible to argue that the amount of trace elements is expected higher in infested fish comparing to non-infested fish: since parasites compete with their hosts for nutrients, this would also lead to sharing of trace elements between host and parasite. Therefore, the amount of metals in the non-infested fish is expected to be higher than that in non-infested fish.

Higher amounts of trace elements in parasites compared to the tissues of its host is reported in sea bream (*Sparus aurata*), a culture fish in the Mediterranean Sea (Dural et al*.*, 2011). In our study, in accordance, we found that Cr and Co were markedly higher in parasites than that in the host tissues. Morsy et al. (2012) reported that the amount of Ni and Mn were significantly higher in parasites compared to the tissues of the host sea bass *Dicentrarchus labrax.* Similarly, the trace metals Pb, Cd, Hg, As, Zn and Fe found higher in parasites comparing to the concentrations in tissues of their host *Liza vaigiensis* (Azmat et al*.,* 2008). Our data therefore confirm that *M. merlangus* and *H. aduncum* have a trace element accumulation pattern similar to other host-parasite models reported (Azmat et al*.,* 2008; Eira et al*.,* 2009; Sures, 2003).

All trace elements accumulated at higher levels in parasites than in tissues of host, in accordance to the findings of some researchers (Azmat et al*.,* 2008; Eira et al*.,* 2009; Sures, 2003). Most studies of parasite bioaccumulation have used acanthocephalans as model organisms. As reviewed by Sures (2003), acanthocephalans cannot synthesize their own fatty acids and cholesterols, therefore they depend on their hosts intestinal lumen to obtain them. Bile acids present in host intestine facilitate formation of organo-metallic complexes, more readily available for absorption by the host and parasites (Sures and Siddall 1999; Zimmermann et al. 2003). Similar studies have been also performed with nematodes as well. Nematode parasites *Echinocephalus* sp. and *Ascaris* sp, presented several folds of trace elements in comparison to tissues of their host harbor porpoise (*Phococena phocoena*) (Azmat et al., 2008; Szefer et al., 1998).

In summary, our study shows that the age of whiting is an important factor for monitoring bioaccumulation of trace elements both in the fish tissues, and in the intestinal parasites. Younger fish infested with nematodes tended to have higher levels of trace elements. The proportion of all trace elements in parasites collected from younger fish was higher than that in the parasites of older fish and from all the host tissues tested. Therefore, the age of fish sampled needs to be recorded and considered while evaluating trace element pollutions. Moreover, intestinal parasites of whiting shoved high difference in terms of trace element levels comparing to fish tissues, and endoparasites could be therefore a more appropriate tool for determining trace element dynamics and pollution in an ecosystem.

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SUSTAINABLE CONSERVATION PERSPECTIVES FOR EPIPHYTIC ORCHIDS IN THE CENTRAL HIMALAYAS, NEPAL

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Abstract. Anthropogenic disturbances are major drivers of biodiversity loss. This is especially true for subtropical and tropical forest ecosystems. Epiphytes are plants that grow upon another plant (often trees) and, thus, fundamentally depend on their hosts. Epiphytic plants are diverse and can create important microcosms for many other organisms, including micro-organisms, insects, birds and mammals, which are rarely encountered on the floor. We identified the main habitat requirements for the conservation of epiphytic orchids and we outline key areas to focus on when designing management strategies for their protection and sustainable utilization. This approach is based on a review of the literature, as well as our own research on habitat requirements and the distribution of epiphytic orchids along a gradient from natural habitats to single trees in urban areas in the Kathmandu Valley, Nepal. Key areas to focus on for the sustainable conservation and utilization of epiphytic orchids are (i) habitat protection, (ii) habitat restoration, and (iii) the socio-economic relevance (utilization to fundraising) of conservation. While remnants of natural habitats are indispensable for the conservation of epiphytic orchid communities, groups of native trees in urban settings can still serve as stepping stones for epiphytic orchids. Therefore, the conservation of even small patches of primary forest within agricultural land areas or single trees within religious park complexes is crucial for the continual existence of epiphytic orchid populations in urban areas. Finally, we propose management concepts for the long-term maintenance of high epiphytic diversity. This framework has been developed for epiphytic orchids in the Himalayas, but can be more generally applied to other groups of epiphytes and other regions in the tropics and subtropics.

Keywords: *biodiversity, epiphytic orchids, disturbance, land-use, protection, Himalayas, Nepal*

Introduction

The loss of biodiversity over the last 100 years is immense, with nearly a third of plant species estimated to be threatened by extinction (GBR, 2010). Tropical and subtropical forests are among the most threatened ecosystems worldwide (Nieder et al., 2001). Land-use change by conversion to agriculture, settlement and infrastructure are considered to be the most important drivers of the loss of these forests and consequently of biodiversity decline (Sala et al., 2000). Epiphytic vascular plant species, such as orchids and ferns, and non-vascular species, such as bryophytes, algae, fungi and lichens, are especially threatened. This is due to their extreme microhabitats, often the trunk or branches of trees; however, some species survive in more anthropogenic habitats, such as on telegraph wires. The orchid family is the second largest family after Asteraceae in the flowering plant kingdom and it is estimated that more than 850

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species of orchids are existing in the Himalayan region (Acharya et al., 2011) with 437 species in Nepal alone (Rokaya et al., 2013).

Forests are an integral part of rural households' livelihood in Nepal (about 80% of the total population), as they provide daily subsistence needs (fuel, building materials, livestock feed and bedding, food and medicines) and generate income. However, they are also the main habitat of epiphytic plants, which thrive in the moist microclimate within the forest canopy. With their very special requirements (e.g. nutrients, light, and water availability), epiphytes are highly vulnerable to anthropogenic influences such as habitat degradation and deforestation (Wolf, 2005). Consequently, the collection, sale and utilization of a broad range of timber and non-timber forest products enhance exploitation, deforestation and degradation of remaining habitats (Bajracharya et al., 1994; Shrestha et al., 1999; Pimm and Raven, 2000; Rajbhandari and Bhattarai, 2001).

Most scientific research on orchids has been largely related to floristic exploration and taxonomic description, with little research addressing ecological and conservational issues (Sparrow, 1996; Zotz et al., 2001; Winkler et al., 2009). Epiphytes are ecologically important as they play a vital role in the ecosystem by producing relatively high amounts of organic matter on the host trees, providing living areas for other organisms and contributing to nutrient cycles, as many can fix atmospheric nitrogen (Nadkarni, 1994). Epiphyte and orchid diversity also appear to be very good indicators of the degree of disturbance suffered by a habitat in the urban environment (Newman, 2009). Both the distribution and abundance of epiphytic orchids, especially their habitat requirements and the human induced environmental changes to their habitats, were still poorly known in the Himalayan region until very recently. Studies of Adhikari and Fischer (2011) and Adhikari et al. (2012a) clearly showed that different orchid species vary in their response to anthropogenic disturbances (i.e. across a gradient from forest to urban area). As expected, most of the orchid species preferred less disturbed areas; however, several species will also grow on individual trees in polluted city centres (e.g. common species like *Rhynchostylis retusa* (L.) Blume and *Vanda cristata* Wall. ex Lindl.).

Knowledge and understanding of species distribution patterns is the basis for species conservation (Hirata et al., 2009). In particular for plants, and especially orchids, the main aspects to understand to design effective conservation strategies are: population ecology and dynamics (Annaselvam and Parthasathy, 2001; Winkler et al., 2009); life-cycle stages (Gomez et al. 2006); seed germination conditions, i.e. the relationship with mycorrhizal fungi and seedling establishment (Ackerman et al., 1996; Goode and Allen, 2009; Gowland et al., 2011; Light et al., 2011); pollination biology (Schiestl, 2005); and, the influence of different human land-use intensities (Adhikari et al., 2012a). Studying the relationship of epiphytic orchids to host tree characteristics and microhabitat quality (Köster et al., 2009) is a prerequisite to develop effective strategies for their conservation (Brooks et al., 2006). This is especially important given the high rate of population depletion and their relatively high vulnerability, e.g. due to highly specialized pollination systems and the requirement of mycorrhiza for germination. Climatic variables (temperature and precipitation) have been found to provide a good potential explanation for orchid species richness along the Himalayan elevational gradient (Acharya et al., 2011); such environmental conditions associated with altitude also exert a large influence on orchid species composition (Jacquemyn et al., 2005).

Nepal's forests are facing severe threats because of increasing demand on agricultural land, timber, fuel wood and fodder, and encroachment of the forest area for settlements. Nepal lost ca. 1.2 million ha of forest land (about 25 % of its total forest cover) between 1990 and 2005 (FAO, 2005). Although overall deforestation rates have decreased since the 1990s, rates of loss of primary forests have increased significantly (FAO, 2005). This loss of primary forest will directly influence epiphytic ecosystems. At the current rate of deforestation, and without any control measures, the country will lose vital forest habitat.

We aim to outline a fundamental management concept for protection and sustainable utilization of epiphytic orchids in the tropics and subtropics. The framework will be based on existing knowledge of epiphytic orchids combined with what we have learned from our own studies on anthropogenic impacts on epiphytic orchid populations in the Kathmandu Valley, Nepal, in the central Himalayas (Adhikari et al., 2012a). Specifically, we ask the questions: How can existing populations of epiphytic orchids be managed under anthropogenic disturbances? What kind of landscape management is needed to ensure long-term protection of epiphytic species?

Materials and methods

Nomenclature of epiphytic orchids WCSP (2011).

Study Area

Our study site is the Kathmandu valley area, Nepal (27° 76'~ 62' N 85° 25'~ 45' E) with the four closely located cities of Kathmandu, Bhaktapur, Lalitpur, and Banepa and represents the most urbanized place in Nepal, and, close to the city agglomeration, has unmanaged forest vegetation (Shivapuri Nagarjun National Park). The annual average rainfall is 1400 mm (Pant and Dangol, 2009) with the absolute maximum during summer time. The temperature in general is lowest 2°C in winter and highest 28°C in summer. Kathmandu valley has Nepal's the largest urban population and hosts 12% of all households of Nepal (Zurick et al., 2005).

The natural vegetation of this valley is subtropical evergreen broad-leaved forest, nowadays remaining in the city area with a few isolated forest patches, several park areas and temple areas with groups of trees (Adhikari and Fischer, 2011). The most common tree species in Kathmandu valley are *Schima wallichii, Alnus nepalensis* and *Ficus religiosa*, while *Castanopsis indica, Myrsine capitellata, Myrica esculenta, Pyrus pashia* and *Pinus roxburghii* are more frequent in the surrounding forest areas. Our study area was defined as up to about 1700 m altitude in the northern part (national park), foot hills of the Kathmandu valley in the west and south, and up to Banepa city in the east (*Fig. 1*).

Sampling

We used a stratified systematic sampling strategy for the selection of sampling points within the study area (*Fig. 1*). We established 156 systematic sampling study points: host trees were selected on a plot less basis (Wolf et al., 2009). The study points were fixed around one tree of a host species having with at least one orchid species that was of ≥10cm diameter breast height (dbh, i.e., diameter at 1.30 m). At each point we studied the ten individual trees (dbh ≥10 cm) closest to the first selected host species in each point within 300 meters. Each tree was examined from different points, thereby assuring a clear view of all tree parts (Migenis and Ackerman, 1993). For larger trees,

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we used single-rope climbing techniques (Mitchell et al., 2002) and binoculars to sample host trees completely for epiphytic orchids. For each point tree sampled, we determined host characteristics, such as dbh, height, species identity, rugosity, sunlight intensity, host bark pH. During field work at each point we cross-checked the geographical information system (GIS) results by estimating the percentage of land use. The time frame of our sampling covered the flowering period of most species, allowing for a high proportion of orchid species to be identified to species level.

For more details see Adhikari and Fischer (2011), and Adhikari et al. (2012a, b).

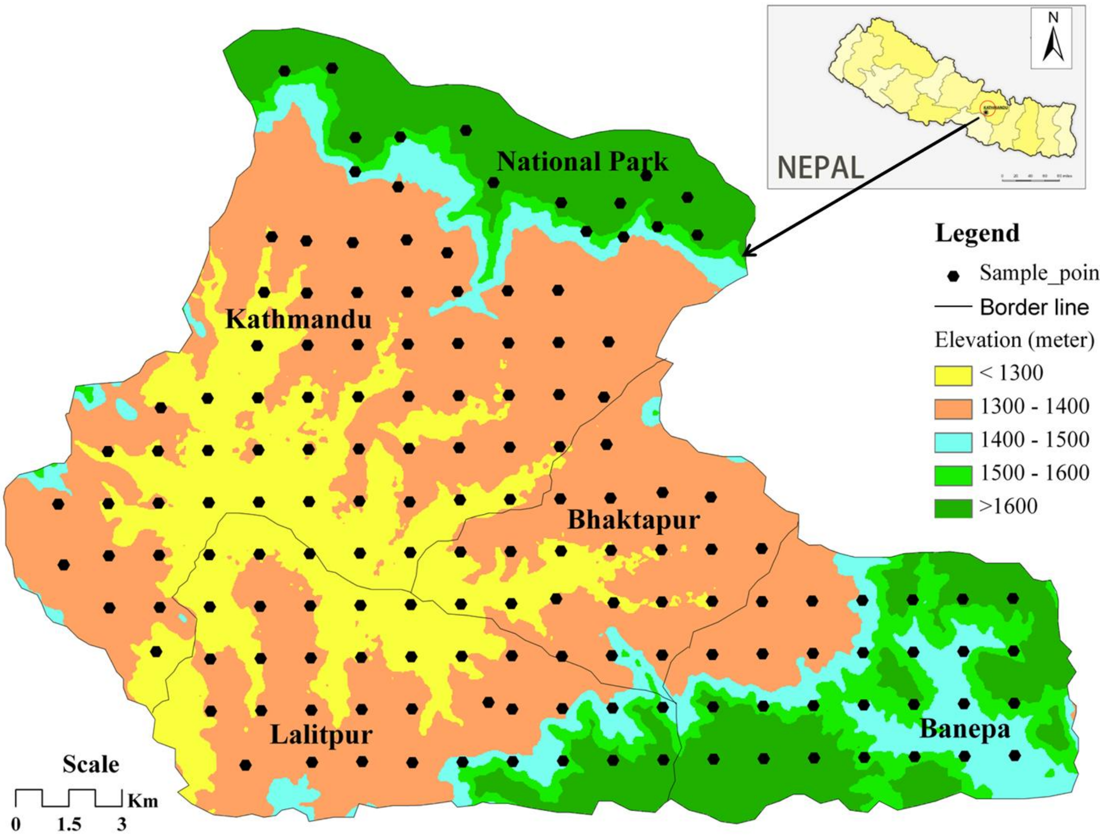

Figure 1. *The study area geography including grid points (1.5×1.5km) in different human impact categories in different cities in the Kathmandu valley and National Park.*

Statistical Analysis

All data were stored in a relational data base system in MS-Access. To analyse relationship between the orchid influencing factors we used spearman rank correlation coefficient. We analysed the similarity structure of the orchid influencing factors with a principle component analysis (PCA) with the function “rda” from the vegan package (Oksanen et al., 2011) in R (R development core team 2.12.1). Variables were standardized to make different physical units comparable. Ellipses enveloping 90 % of the trees with a selected orchid species respectively, without it were draw with the function “ordiellispe”.

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Identifying habitat requirements for conservation of epiphytic orchids

The diversity and abundance of epiphytic orchids in different land use types are influenced by several variables including elevation, precipitation rhythm, bark rugosity, bark pH and sunlight intensity (Callaway et al., 2002; Adhikari et al., 2012a; b). Some orchids are generalists but others have very specific requirements, e.g. *Psychilis monensis* Sauleda frequency high on rough-barked substrates (Tupac et al., 2007). In Nepal, *Rhynchostylis retusa* was found to settle on a wide range of host trees but had higher abundance on some native species such as *Ficus religiosa* L. and *Schima wallichii* (DC.) Korth. (Adhikari and Fischer, 2011). For successful establishment of epiphytic orchids, trees with rugose bark (e.g. *Schima wallichii*) may be preferred hosts because seeds may become more easily lodged and moisture is retained for longer periods of time. Similarly, higher host bark water holding capacity enhances orchid abundance (Adhikari and Fischer, 2011). In addition, older and larger native tree-host species host higher numbers of epiphytic orchids than younger trees due to the time for colonization (Adhikari et al., 2012a). Since older and larger trees support more individuals and species of epiphytes, preserving such trees is an important contribution to maintain biodiversity (Heitz, 2005). Large exotic tree species with rugose bark are also present in the Kathmandu Valley (predominantly *Populus euramericana* Guinier*)*, but Adhikari et al., (2012b) found only two of these trees (from several hundred) hosting epiphytic orchids showing that native trees are preferred habitat over exotic ones. This is not unexpected and shows that there has likely been some degree of coevolution between these epiphytes and their native host-trees. Variation among tree species for different bark chemicals can also influence orchid seed germination and early growth (Frei and Dodson, 1972). Thus, even visually suitable trees may be unsuitable due to other factors such as bark chemistry, which may explain the preference towards native host species. Köster et al. (2011) found that both host species composition (mostly due to native species) of a forest stand and their characteristics as micro-sites influence epiphytic species diversity, this is similar in Kathmandu valley (Adhikari et al., 2012a).

Increased knowledge of host-tree characteristics and other variables, from the example of epiphytic orchids in the Kathmandu Valley, Nepal (*Fig. 1*; Adhikari et al., 2012b) is shows that orchid protection and conservation is not an easy task. Different epiphytic orchid species interact differently with the given set of environmental factors (*Fig. 2*): for *Vanda cristata* there is no single environmental factor of special influence, while for *R. retusa* high bark pH and light availability are important. Similarly, *Acampe rigida* (Buch.-Ham. ex Sm.) Hunt prefers light and a large dbh (diameter at breast height) of the host tree, *Bulbophyllum affine* Wall. ex Lindl. occurs in areas more exposed to wind, while for *Dendrobium bicameratum* Lindl. both exposure to wind and bark rugosity are important. Therefore, in order to protect a diversity of orchid species, it is the diversity of epiphytic habitat conditions (i.e. micro-site conditions) which have to be protected.

The selection of suitable tree species (i.e. native ones) for reforestation purposes in conservation programs in subtropical regions is essential for future epiphytic species protection, in urban areas and elsewhere. Managed and natural forest patches, tree plantations (Hietz, 2005) and even single, old and native host trees in urbanized areas may be considered as important epiphytic habitats (Köster et al., 2009).

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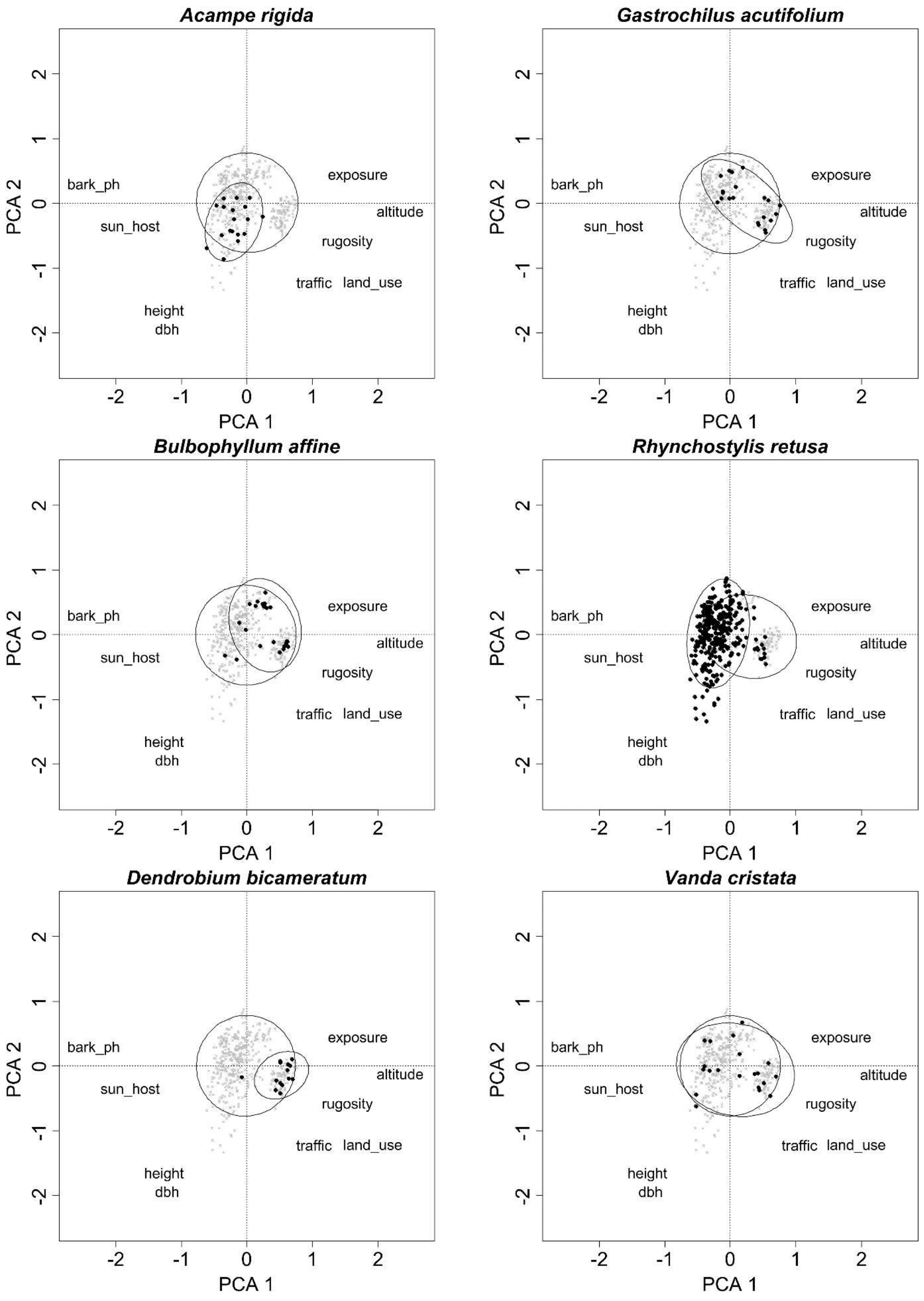
Acampe rigida

Gastrochilus acutifolium

Bulbophyllum affine

Rhynchostylis retusa

Dendrobium bicameratum

Vanda cristata

Figure 2. *Principal component analysis of host tree species in the Kathmandu Valley (for methods and study area see Adhikari et al. 2012b) based on host tree characteristics. For each orchid species the tree individuals which host this orchid are displayed as black dots whereas tree individuals without this orchid are the grey dots. Ellipses envelop 90 % of all tree individuals hosting a selected orchid species, respectively hosting it or not. If the ellipses overlap more or less totally the orchid does not depend on the analyzed site conditions offered by the host (e.g. Vanda cristata). If, however, the ellipses only overlap a bit, the orchid is strongly dependent on the indicated site conditions (e.g. Rhynchostylis retusa). Site conditions are: host bark pH, sunlight intensity, exposure to wind, bark rugosity, land use intensities, altitude, host height and dbh (diameter at breast height), and vehicle traffic*

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Other factors that potentially play a role to develop conservation planning schemes of epiphytic communities include understanding how traditional management practices adopted in commercial monocultures can affect epiphytic communities (Barthlott et al., 2001).

Key elements for conservation planning

Conservation of epiphytic orchids has to address three aspects: (i) the elements that should be protected, (ii) the activities that should be carried out to protect valuable elements, and (iii) the socio-economic background of conservation (*Fig. 3*).

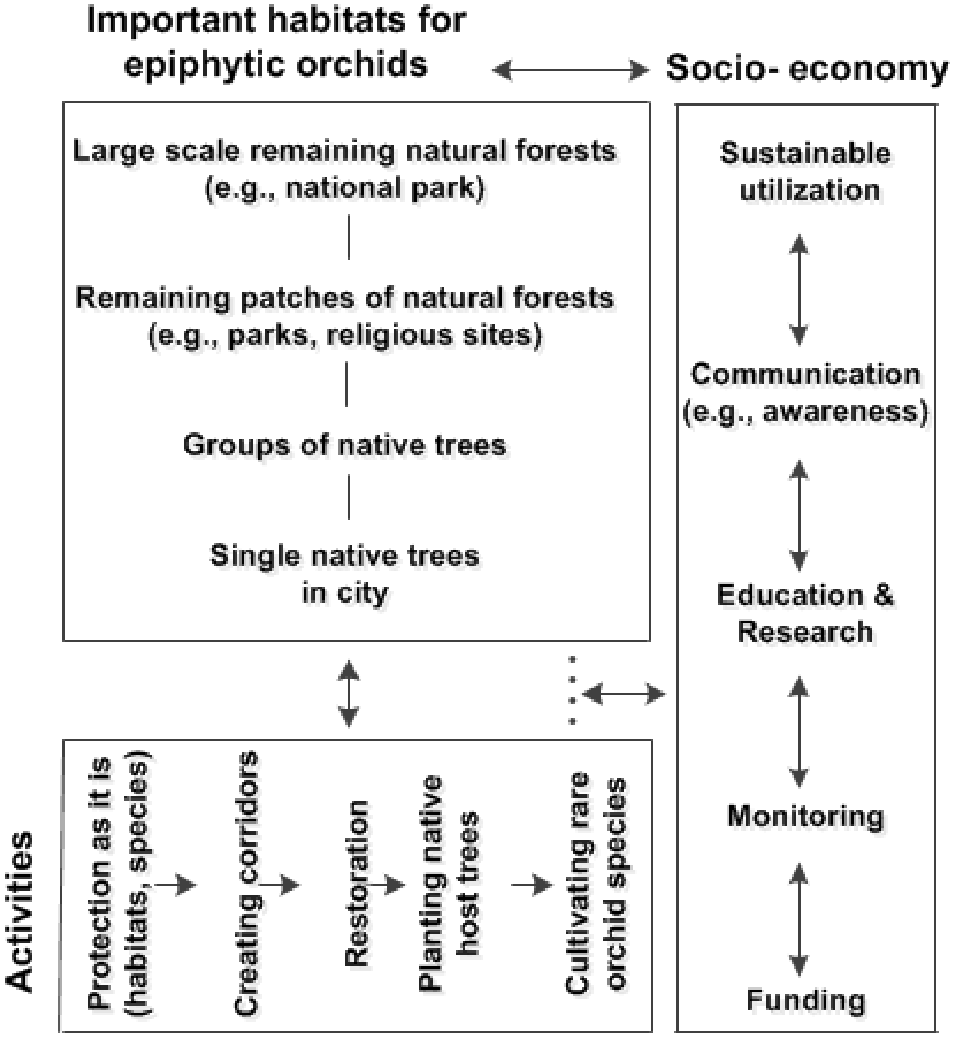

***Figure 3**. Key elements for conservation of epiphytes in subtropics*

To establish acceptance for orchid diversity protection in the community, public awareness has to be increased, in the long run, by educational programs. The success of activities has to be evaluated by monitoring programs. The activities need to be funded, which again requires public acceptance (Cao et al., 2011). In the following we will discuss these aspects and outline a plan for epiphytic orchid protection in settled areas of the subtropics.

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Habitat protection and land use planning

Protection of remaining habitats for epiphytic orchids in the subtropics is essential. Conservation strategies for epiphytic species have to include preservation of the remaining areas of primary and near-natural forests (Sosa and Platas, 1998). While forests are most important for the conservation of epiphytic orchids, also small groups of trees and single trees can make a valuable contribution to conserve epiphytic orchids. In particular, old trees in the Himalayas (e.g. *Ficus religiosa* and *Schima wallichii*) need to be protected because these host trees harbor high numbers of epiphytic plants. Single host trees can often be found in religious temple complexes and groups of trees within remnant forest patches. Both these habitats should therefore be prioritized for protection in city areas as they host the richest communities of epiphytic orchids (Adhikari and Fischer, 2011). Protection of the remnant forests, against further direct anthropogenic activities (e.g. logging, lopping and fuel wood collection) is essential in rural as well as in urbanized areas.

Species protection

Favourable conditions for epiphytic communities in a particular landscape (Adhikari et al., 2012a), the availability of suitable host trees (Callaway et al., 2002; Hirata et al., 2009), and seedling establishment (Werner and Gradstein, 2008) are most important for maintaining epiphytic communities. Orchid seeds are very small and contain limited energy reserves, the relationship between orchids and mycorrhizal fungi allows the orchid seed to germinate and the seedling to establish (Zotz et al., 2001; Dearnaley, 2007; Gowland et al., 2011). Although there are species-specific associations between orchids and mycorrhizal fungi (Gowland et al., 2011), generalist culturable fungi may be useful for propagation of rare orchid species and thus may save some species from extinction (Chen et al., 2012). To enhance the resilience of the epiphyte vegetation to logging it thus appears wise to spare not only single but also a varied species group of trees in order to maintain the available diversity of mychorrizal fungi (Heitz, 2005; Higuera and Wolf, 2010).

Strategies to protect over-collected, endangered and rare ornamental species should also be included in propagation projects as a participation approach (Sosa and Platas, 1998). Maintaining the species in *ex-situ* conservation (e.g. botanical gardens or nurseries) is one of the alternative strategies (Koirala et al., 2010). Orchid species collected from areas which are subject to clear cutting from urban areas may be replaced on a suitable host tree in the *ex-situ* conservation areas by government and research institutes. In 2012, Nepal's Ministry of Forest and Soil Conservation published guidelines ("Nirdeshika") for how to collect and cultivate orchids; around 20 species are allowed to be propagated in tissue culture, including *Acampe papillosa* (Lindl.) Lindl*.*, *Coelogyne cristata* Lindl*.*, *C. flavids* Hook. Fil. Wall. ex*.*, and *Dendrobium amoenum* Wall. ex Lindl.. This will enhance local populations of these particular orchid species and also, through increased awareness, potentially benefit conservation efforts of other orchids.

Restoration and Stepping stones

Human activities alter the structure of landscape and ecosystems worldwide, often drastically by converting forestland into agricultural and urban land. In such areas only isolated forest patches, small groups of trees or single tree individuals remain

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(Nadkarni, 1994) and their removal affects the whole epiphytic community ecosystem. In Kathmandu Valley, habitat modification from natural to high intensity land-use was associated with a decline in the species richness and abundance of epiphytic orchids (Adhikari et al., 2012b). This was mainly driven by the reduced diversity of host species creating a more homogenous microclimate in the disturbed and secondary vegetation compared to the primary forest.

Habitat degradation is one of the major problems associated with over deforestation, exploitation and urbanization for epiphytic orchids in the Himalayas (Rajbhandari and Bhattarai, 2001). The first step for improvement is to restore the degraded habitats so that the native species of the area can be conserved in their natural habitats; a second step is the re-introduction of those species that historically occurred in an area (Shashidhar and Kumar, 2009). A suitable approach would be (i) to protect old trees in rural and urban areas as carriers of epiphytes since old trees are not only crucial for those epiphytes but also may serve as a seed source for the recruitment of orchids in the nearby regenerating forests (Wolf, 2005); (ii) to plant native tree species when replacing the older trees in urban habitats, to ensure these areas are maintained as stepping stones for epiphytic plants. Such stepping stones help link existing patches and host trees, providing opportunities for dispersal, facilitating gene flow between populations, and increasing the likelihood that the patches will be colonised by epiphytic orchids (Barnes and Lowell, 1999). Therefore, protection of existing habitats needs to be complemented by the establishment of such stepping stones so that the fragments can be connected and a network of such areas are formed; this will also benefit the conservation of other native species and, thus, biodiversity in general.

Planting "trees for the future" for epiphytic orchids

The development of a conservation action plan for endangered tree host species, as well as orchid species, is urgently needed. *Ex-situ* propagation of orchids is beneficial but will not lead to long-term population establishment without there being suitable future habitat for these plants. Common native host species usually offer the best micro-habitat for epiphytes (Adhikari et al., 2012a) and even native single individual trees can play a major role in the conservation of biodiversity in tropical and subtropical urban regions (Turner et al., 1994). To find the best sites to plant such "trees for the future" is a task for landscape planning (urban forestry), but should focus on creating a network of native trees and patches across the urban areas. In the long term, conservation management efforts should focus on increasing overall numbers of native trees in the landscape, especially in parks and religious sites of urban areas.

Sustainable utilization

Sustainable conservation of epiphytic orchids in developing countries will only be successful if it can be linked to the socio-economic system (Brooks et al., 2006) or, in other words: if it can provide economic benefits to the local people. In the case of the Himalayan region there are several potential ways to do this. Areas that are rich in epiphytic orchid species could be used for promotion for eco-tourism (Koirala et al., 2010). Then the local stakeholders can earn some income while at the same time increasing awareness for the need to conserve epiphytes. In the Himalayas, up to now, there were no specific or model conservation activities adopted, but some private hotels in Daman (Makawanpur district) and Bandipur (Tanahun district) do use collected orchids for decoration, attracting tourist and conservation awareness.

As the human population increases, activities such as the collection of native flora for medicinal uses puts a strain on the natural populations, e.g. a previously common orchid, *Dendrobium densiflorum* Lindl., around the Kathmandu Valley, is now very rare because the bulbs were collected illegally for medicinal purposes (Shrestha, 2003). Thus, a second possibility could be the promotion of the sustainable harvest of these species, as has been suggested in the Andes (Acebey et al., 2010). Target species, of importance in local culture, should be identified for mass propagation by tissue culture followed by cultivation for ornamental and medicinal uses. Selective collection may conserve and facilitate the restoration of epiphyte numbers and diversity locally. It cannot, however, safeguard epiphyte diversity on a regional scale (Wolf, 2005) and more ecological information would be required for this as well as specific guidelines for sustainable use. Sustainable utilization could be achieved by a number of ways including the collection only of abundant and more widely distributed species or collection could be restricted to harvesting from the lower parts of the trees (Wolf and Konings, 2001). Alternative good strategies for epiphytic conservation in the longer term include, only harvesting some of the pseudobulbs, or just the flowers or, further, collection restricted to fallen orchids (Demetria and Ticktin, 2011). Sustainable harvesting systems may really work, as has been reported from Costa Rica (Brooks et al., 2006); however, monitoring is required to evaluate the concrete consequences of harvesting activities. Nevertheless, sustainable harvest needs to be combined with community-based propagation approaches for target species because of their very low population growth rates. Lastly, awareness has to be raised in the local communities so that the living orchid is more valuable than those collected and sold, e.g. by implementing eco-tourism facilities. In the Himalayas there are many possibilities for orchid conservation in community forests, and these can be achieved, with the help of local communities. In our studies, so far, we have little evidence that local people are aware of the importance of the orchids and the surrounding habitat (Adhikari et al., 2012a). Future work should address this lack of knowledge and how it can be implemented at both the local and the regional level.

Communication and education

There is a need to provide awareness activities, training programs, and information on the habitat requirements of epiphytic orchids to government and stakeholders, so as to develop management strategies for their conservation (Adhikari et al., 2012a). To succeed, wildlife conservation policy will have to be a mix of protectionism, community involvement, public relations, conservation education, and revenue sharing (Hackel, 1999) and this is also true for wild epiphytic community conservation in the Himalayas. Furthermore, educational institutions should promote research on conservation modalities and scientific cultivation of wild and endangered epiphytic orchid species. Thus, a strategy for communication to the public should focus on the beauty, medicinal use and eco-tourism potential of these species and their host trees (e.g., *Ficus religiosa*). The way, however, is still long and educating the younger generation in schools, for example, will be essential to reach this goal.

Monitoring and funding

Without monitoring it is impossible to provide a systematic evaluation of which strategies are best suited for different conservation tasks (Brooks et al., 2006). Monitoring of the species populations should always be part of a management plan for sustainable use (Wolf, 2001). Long-term monitoring of potentially vulnerable sites for epiphytes is also advised, especially of those sites that are outside current protected areas. Under sustainable multifunctional epiphyte management, it is necessary to include specific spatial distributions of species and objectives apply in planning and monitoring programs. To implement all these kinds of activities money is needed. Stake holders, government, decision makers, NGOs and INGOs therefore need to search for funding for conservation activities of epiphytic orchids, in Himalayas as well as in the whole tropics and subtropics.

Planning Scheme

We suggest that effective conservation of epiphytic orchids will require: (i) Protection of existing forests and use of forest management practices that maintain the canopy diversity. (ii) Protection of existing old patches of trees as well as single remaining host tree individuals in agricultural or urban areas as small “ecological island” habitats for epiphytic orchids, and as stepping stones between larger forest patches as carriers of existing epiphytic orchids. (iii) Planting of new (mainly native) trees, as hosts for the future, in groups instead of single isolated individuals; trees should especially be planted in areas where orchids still exist to provide more trees for orchid population growth (e.g. along riparian system). (iv) Planting of trees in urban settings with suitable host characteristics (e.g. older and larger trees, rougher bark, low pH, exposed to wind, and reduced human impact). (v) Planting of religious relevant trees in areas where the orchid species is recently missing because such tree species have a high degree of acceptance among people. (vi) Public education awareness campaign programs organized through various extension methods and through mass media. And finally, (vii) Government and research institutes, including educational institutions, should promote research on conservation modalities and scientific cultivation; especially for endangered orchid species there must be suitable places where recovery management plans can be established (e.g. national parks).

Conclusions

There is a dire need to effectively manage the forest and biodiversity of the Himalayas for the economic, cultural, and political stability of the country as well as to meet international obligations. Decentralization of conservation programs (e.g. community-based) and local institution empowerment are the success for epiphyte conservation. When the life-span of a conservation project expires, the success of any project will depend on the ability of the local communities to manage their resources over the long term (Brooks et al., 2006). Therefore, there is a need for applying conservation practices in the area where epiphytes play ecological roles in the forest canopy of subtropical regions. Species need to be able to migrate when the conditions are not ideal for their sustenance and survival.

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ATTRIBUTES OF FOREST DIVERSITY IN THE YUNMENG MOUNTAIN NATIONAL FOREST PARK IN BEIJING, CHINA

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Abstract. The Yunmeng Mountain National Forest Park is close to Beijing city and is ecologically important for its citizens. Forty-two quadrats of 10 m × 10 m were established along an altitudinal gradient within the forest. Species composition and environmental variables were measured and recorded in each quadrat. TWINSPAN (Two-Way Indicator Species Analysis) and CCA (Canonical Correspondence Analysis) were used to analyze the relationships between forest types and environmental factors, and species diversity indices were used to analyze the pattern of species diversity in this park. The results showed that there were seven forest communities (formations); most of which were secondary natural forests, along with some plantations. Each forest type had its own characteristic composition, structure, function, and environment. The variation in forest communities was most significantly correlated with elevation and slope position but was also related to slope, aspect, soil depth, and litter depth. Species richness and diversity increased significantly along altitudinal and slope position gradients. The highest diversity appeared near the mountain summit. Elevation and slope position were key factors influencing forest distribution and species diversity. Effective management measures should be considered in this park.

Keywords: *forest conservation; forest classification; forest-environment relation; species diversity; mountain development*

Introduction

Variation in forest vegetation and species diversity and their underlying mechanisms form the basis for conservation of natural forests (Fetene et al., 2006, Muhumuza and Byarugaba, 2009; Zilliox and Gosselin, 2014). Mountainous regions are significant for nature conservation because in countries with limited protected areas, such as China, most natural forest communities are centralized in the mountains (Zhang et al., 2012). Beijing is the capital city of China, and its natural forests are very limited (Zhang et al., 2013). Therefore, these natural forests are especially important for providing ecological services to people living in such a large city (Muenchow et al., 2013).

A complex of factors determines the community composition, structure, and related species diversity of mountain forest vegetation (Schmidt et al., 2006; Korner, 2007; Luth et al., 2011). One important factor is altitude, which has a strong influence on the structure of the forest vegetation in most mountains of the world (e.g., Zhang, 2002; Hawkins and Diniz, 2004; Rahbek, 2005). Whether species diversity increases or decreases with increasing elevation or peaks at intermediate elevation depends largely on specific patterns of interactions among forest communities, species, and environmental factors (Lomolino, 2001; Brown, 2001; Korner, 2007). Changes in forest type and species diversity along altitudinal gradients have been studied frequently in plant ecology (Fetene et al., 2006; Otypkova et al., 2011), and most have found a ‘‘humped’’ distribution, with peak species diversity occurring near the middle of the

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gradient (Austrheim, 2002; Zhang et al., 2012). However, there are a number of exceptions to this pattern (Pausas, 1994; Zilliox and Gosselin, 2014).

Yunmeng Mountain, belonging to the western part of the Yan Mountain Range, is one of the most well-known, picturesque mountains in suburban Beijing, and the topography of the forest park is unique. It is the closest national nature-forest park to the city center of Beijing and is a famous eco-tourism site (Xiang and Zhang, 2009). Forest vegetation plays a significant role in local development, and it should be protected and utilized reasonably. A number of studies have been conducted in this geographical area, including studies related to floristic characteristics and plant resources (He et al., 1992), resources and evaluation of eco-tourism (Wang et al., 2004), restoration of degraded scrublands at lower elevations (Dai et al., 1990), and vertical distribution of tree species (Zheng et al., 2007). However, no studies have examined the association between major environmental variables and forest or species diversity in the Yunmeng Mountain Forest Park. This work studies forest and species diversity and their relationship to environmental variables. Our objectives were (1) to analyze the interdependencies among forest characteristics and environmental variables, (2) to identify the key environmental factors influencing forest composition and species diversity, and (3) to test the hypothesis that highest species diversity appears at intermediate elevation.

Materials and methods

Study sites

The Yunmeng Mountain National Forest Park is located at 116°40′- 116°50′E, 40°26′- 40°38′N in the north of Beijing City and is approximately 85 km from the city center. The park occupies a total area of 2,208 ha, and its main peak is 1,414 m.a.s.l. This area is deeply affected by the warm-temperate, subhumid Southeast monsoon. The annual mean temperature is approximately 10ºC, the mean monthly temperature of July is 25ºC, and the mean monthly temperature of January is -7ºC. The annual mean precipitation is 600-700 mm, and it increases with increasing elevation. Most rain (over 76%) falls from June through September. The main soil types are brown forest soil and drab soil.

The forest park has rich scenic resources, thick forests, and significant animal and plant diversity, including 711 species of plants. Vegetation is mainly secondary broad-leaved deciduous forest, with some plantations of coniferous forest. The Yunmeng Mountain Forest Park, with its unique topography, ponds, waterfalls, forests, and vivid landscapes integrates the functions of conservation and sightseeing through eco-tourism.

Data collection

Based on a general investigation, 15 sampling points, each separated by 50 m in elevation, were set up along an altitudinal gradient between 750 and 1401 m.a.s.l. Two to four quadrats around each sampling point were established along the contour line. The quadrat size was 10 m × 10 m. The cover, height, abundance, and basal area for tree species and the height and cover for shrubs and herbs were measured and recorded in each quadrat. Plant height was measured using a clinometer for trees and a steel tape measure for shrubs and herbs. The basal diameter of trees was measured using a caliper and was used to calculate basal area. A total of 102 plant species were recorded in 42 quadrats.

Six environmental variables were measured and recorded for each quadrat: elevation, slope, aspect, slope position, soil depth, and litter depth. Elevation was determined with a GPS, slope and slope aspect were measured using a compass meter, soil depth was measured with a penetrometer, and litter depth was measured using a ruler (Zhang et al., 2013). Elevation, slope, soil depth, and litter thickness were continuous variables. Slope position was classified from 1 to 5, representing hill ridge, upper, middle, lower location, and valley bottom, respectively. Aspect measurements were classified from 1 to 8 in the following way: 1 (337.6°-22.5°), 2 (22.6°-67.5°), 3 (292.6°-337.5°), 4 (67.6°-112.5°), 5 (247.6°-292.5°), 6 (112.6°-157.5°), 7 (202.6°-247.5°), and 8 (157.6°-202.5°).

Data analysis

The Importance Value (IV) of each species was calculated and used in multivariate analyses of forest communities and species diversity. The importance value was calculated by the formula (Zhang et al., 2013):

$$IV_{\text{Tree}} = (\text{Relative abundance} + \text{Relative dominance} + \text{Relative height})/3$$

$$IV_{\text{Scrubs and Herbs}} = (\text{Relative cover} + \text{Relative height})/2$$

Dominance was defined as a species' basal area. In statistical analyses, species were represented by their importance values in the 42 quadrats. The environmental variables were represented by values for elevation, slope, aspect, slope position, soil depth, and litter depth in the 42 quadrats.

The TWINSPAN (Two-Way Indicator Species Analysis) classification and CCA (Canonical Correspondence Analysis) ordination method (ter Braak and Smilauer, 2002) were used to identify forest community types and to analyze their relationship to environmental variables. TWINSPAN and CCA were calculated using the computer programs TWINSPAN (Hill, 1979) and CANOCO (ter Braak and Smilauer 2002), respectively.

Three species diversity indices, one for species richness, one for species diversity, and one for species evenness, were used to assess diversity (Zhang, 2011). These indices were calculated as:

Species number (as an index of richness): $D = S$

Shannon-Wiener index: $H' = -\sum P_i \ln P_i$

Pielou evenness index: $E = \dfrac{H'}{\ln(S)}$

where P_i is the relative importance value of species i, $P_i = N_i/N$, N_i is the importance value of species i, N is the sum of importance values for all species in a quadrat, and S is the species number present in a quadrat.

Regression and correlation analyses were used to assess the relationship between species diversity and environmental variables.

Results

Forest community diversity

Based on importance values, TWINSPAN classified the 42 quadrats into seven clusters, representing seven forest communities (forest formations) (*Fig. 1*). The variation among forest communities was clear and was related to ecological gradients; elevation increased from formation I through VII (*Fig. 1*) (Hill, 1979; Zhang, 2011). The name and major characteristics of the seven communities are described below.

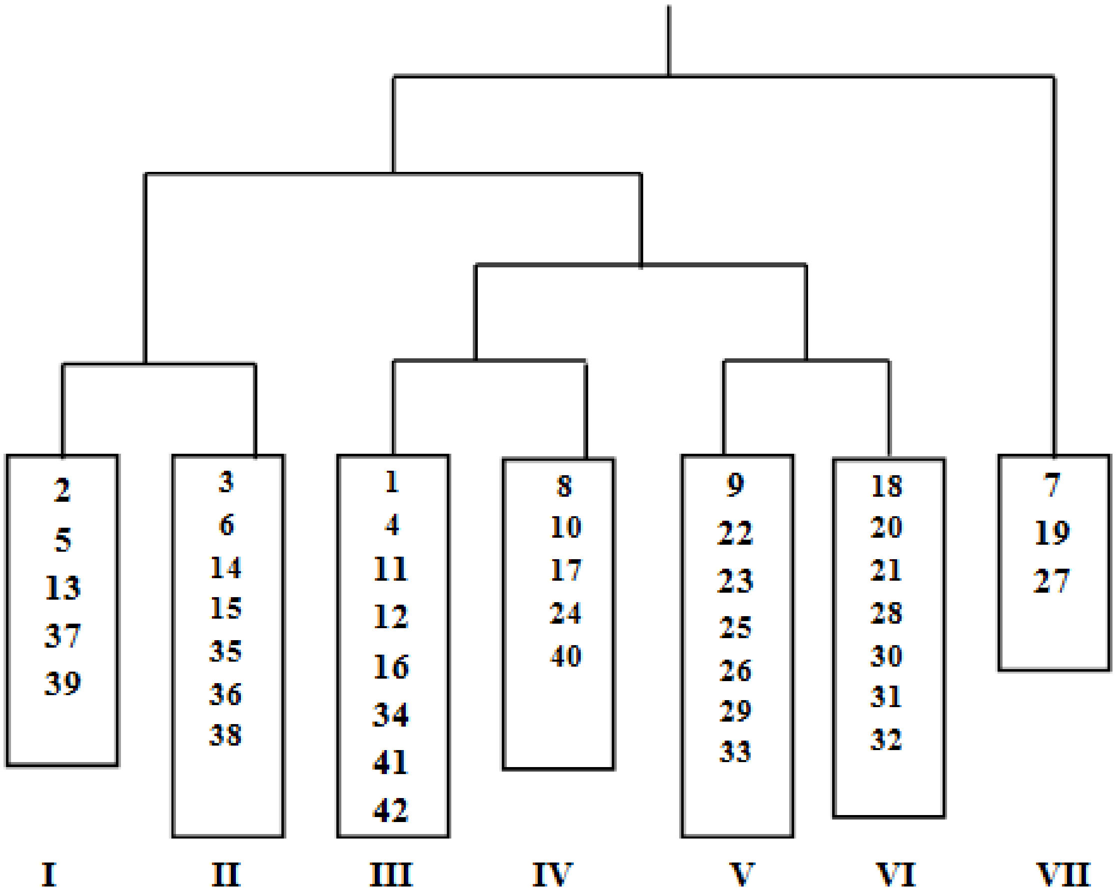

Figure 1. *The dendrogram of TWINSPAN results for the 42 forest quadrats in the Yunmeng Mountain Forest Park in Beijing, China. Roman numerals I - VII refer to the seven forest formations; Arabic numbers in rectangles refer to quadrat number.*

I. Form. *Pinus tabulaeformis.* This is a planted forest formation, distributed in patches from 780 to 1205 m, on hills with sunny slopes of 25 – 38°, and brown forest soil. The soil depth is 20-45 cm, and the litter depth is 3-5 cm. Disturbance intensity is medium. The forest community has a total cover of 85%, a tree layer cover of 70%, a shrub layer cover of 40%, and an herb layer cover of 35%. Common species in the tree layer include *Juglans mandshurica, Popolus cathayana,* and *Quercus mongolica.* Common species in the shrub layer include *Spiraea trilobata*, *Leptopus chinensis, Rhamnus parvifolia,* and *Vitex negundo* var. *heterophylla.* Common species in the herb layer include *Calamagrostis arundinacea*, *Carex rigescens*, *Artemisia lavandulaefolia,* and *Aconitum kusnezoffii*.

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II. Form. *Juglans mandshurica.* This is a secondary natural forest formation, distributed from 790 to 1200 m, on hills with sunny, semi-sunny, and semi-shady slopes of 10 – 39°, and brown forest soil. The soil depth is 25-70 cm, and the litter depth is 3-6 cm. Disturbance intensity is medium. The forest community has a total cover of 90%, a tree layer cover of 75%, a shrub layer cover of 50%, and an herb layer cover of 65%. Common species in the tree layer include *Fraxinus rhynchophylla, Quercus mongolica, Ulmus pumila, Acer truncatum*, and. *Tilia mongolica.* Common species in the shrub layer include *Deutzia parviflora, Philadelphus pekinensis, Corylus mandshurica, Spiraea trilobata*, *Lespedeza bicolor,* and *Leptopus chinensis.* Common species in the herb layer are *Rabdosia japonica, Dioscorea nipponica, Carex rigescens*, *Aconitum nagarum, Circaea quadrisulcata*, *Spodiopogon sibiricus, Ostericum sieboldii*, and *Artemisia lavandulaefolia*.

III. Form. *Fraxinus rhynchophylla + Acer truncatum + Tilia amurensis.* This is a secondary natural mixed forest formation, distributed from 1170 to 1320 m, on hills with semi-sunny, shady, and semi-shady slopes of 10 – 45°, and brown forest soil. The soil depth is 30-60 cm, and the litter depth is 3-7 cm. Disturbance intensity is heavy. The forest community has a total cover of 85%, a tree layer cover of 75%, a shrub layer cover of 40%, and an herb layer cover of 60%. Common species in the tree layer include *Quercus mongolica, Betula dahurica, Syringa pekinensis, Celtis bungeana, Popolus davidiana*, and. *Tilia mongolica.* Common species in the shrub layer include *Myripnois dioica*, *Syringa pubescens, Spiraea trilobata*, *Lespedeza bicolor,* and *Euonymus alatus.* Common species in the herb layer include *Adenophora wawreana, Carex lanceolata, Artemisia lavandulaefolia, Rabdosia japonica, Dioscorea nipponica, Carex rigescens*, *Circaea quadrisulcata*, *Calamagrostis arundinacea, Ostericum sieboldii*, and *Agrimonia pilosa.*

IV. Form. *Quercus mongolica.* This is a secondary natural forest formation, distributed from 1070 to 1360 m, on hills with sunny and semi-sunny slopes of 15 – 46°, and brown forest soil. The soil depth is 42-80 cm, and the litter depth is 4-7.5 cm. Disturbance intensity is medium. The forest community has a total cover of 90%, a tree layer cover of 70%, a shrub layer cover of 45%, and an herb layer cover of 55%. Common species in the tree layer include *Pinus tabulaeformis, Ulmus macrocarpa, Juglans mandshurica, Betula dahurica, Fraxinus rhynchophylla, Acer truncatum, Fraxinus rhynchophylla,* and *Popolus davidiana*. Common species in the shrub layer include *Spiraea trilobata*, *Euonymus alatus, Syringa pubescens, Lespedeza bicolor, Rhododendron micranthum*, and *Leptodermis oblonga.* Common species in the herb layer include *Carex rigescens*, *Adenophora wawreana, Carex lanceolata, Artemisia lavandulaefolia, Saussurea nivea, Aquilegia yabeana*, *Chelidonium majus*, *Clematis brevicaudata,* and *Rabdosia, japonica.*

V. Form. *Betula dahurica.* This is a secondary natural forest formation, distributed from 1190 to 1390 m, on hills with semi-sunny, semi-shady, and shady slopes of 35 – 47°, and brown forest soil. The soil depth is 28-59 cm, and the litter depth is 4-7.9 cm. Disturbance intensity is medium. The forest community has a total cover of 90%, a tree layer cover of 75%, a shrub layer cover of 35%, and an herb layer cover of 45%. Common species in the tree layer include *Betula dahurica, Popolus davidiana, Salix caprea, Betula platyphylla, Acer truncatum,* and *Quercus mongolica*. Common species in the shrub layer include *Abelia biflora, Spiraea trilobata*, *Corylus mandshurica, Euonymus alatus, Syringa pubescens,* and *Rhododendron micranthum.* Common species in the herb layer include *Carex siderosticta, Calamagrostis arundinacea, Aconitum* *kusnezoffii, Carex rigescens*, *Saussurea nivea, Aquilegia yabeana*, *Adenophora wawreana,* and *Clematis brevicaudata.*

VI. Form. *Popolus davidiana + Betula platyphylla.* This is a secondary natural mixed forest formation, distributed from 1200 to 1400 m, on hills with semi-sunny, shady, and semi-shady slopes of 20 – 44°, and brown forest soil. The soil depth is 25-62 cm, and the litter depth is 3-6.6 cm. Disturbance intensity is weak. The forest community has a total cover of 90%, a tree layer cover of 70%, a shrub layer cover of 40%, and an herb layer cover of 45%. Common species in the tree layer include *Betula dahurica, Larix principis-rupprechtii, Salix caprea, Fraxinus rhynchophylla,* and *Acer truncatum.* Common species in the shrub layer include *Corylus heterophylla*, *Deutzia hamata* var. *baroniana*, *Elsholtzia stauntonii, Corylus mandshurica,* and *Euonymus alatus.* Common species in the herb layer include *Agrimonia Pilosa, Calamagrostis arundinacea, Carex rigescens*, *Vicia gigantean, Circaea quadrisulcata*, *Spodiopogon sibiricus*, and *Adenophora wawreana.*

VII. Form. *Larix principis-rupprechtii.* This is a plantation forest formation, distributed from 1200 to 1414 m, on hills with shady and semi-shady slopes of 20 – 40°, and brown forest soil. The soil depth is 27-57 cm, and the litter depth is 4-8.2 cm. Disturbance intensity is weak. The forest community has a total cover of 99%, a tree layer cover of 80%, a shrub layer cover of 35%, and an herb layer cover of 45%. Common species in the tree layer include *Betula dahurica, Popolus davidiana, Betula platyphylla, Quercus mongolica, Tilia mandshurica*, and *Salix caprea*. Common species in the shrub layer include *Spiraea trilobata, Weigela florida, Abelia biflora, Corylus heterophylla*, *Euonymus alatus,* and *Deutzia hamata* var. *baroniana.* Common species in the herb layer include *Calamagrostis arundinacea, Carex rigescens*, *Phlomisum brosa, Saussurea nivea*, *Aquilegia yabeana*, *Convallaria majalis*, *Polygonatum odoratum*, *Phlomisum brosa*, *Adenophora divaricata,* and *Sedum aizoon.*

Forest communities and related environment

In the CCA ordination of 42 quadrats with six environmental variables (*Fig. 2*), the Monte Carlo permutation test indicated that the eigenvalues for the first canonical axis and for all canonical axes were significant ($P<0.01$). The species-environment correlations of the first four CCA axes were 0.919, 0.817, 0.913, and 0.855; and the cumulative percentage variance of species-environment relations for the first four CCA axes were 82.1 %; which showed that CCA performed well in describing relations among species, forest communities, and environmental gradients (Zhang, 2011). The Monte Carlo permutation test also indicated that the species–environment correlations with the CCA axes were significant (ter Braak and Smilauer, 2002).

Ordination results showed that the first CCA axis was significantly correlated with elevation, slope position, aspect, slope and soil depth and that elevation and slope position were the most significant factors related to the first CCA axis (*Fig. 2, Table 1*). Elevation had a positive correlation, and slope position had a negative correlation, with the first CCA axis. The altitudinal and slope position gradients were very clear (from left to right, *Fig. 2*), and along these gradients, elevation increased gradually, and slope position varied from valley bottom to hill ridge. The second CCA axis was significantly correlated with aspect, litter depth, soil depth, and slope position (*Fig. 2, Table 1*). The third CCA axis was correlated with soil depth, slope, litter depth, and aspect, and the fourth axis was correlated with aspect, slope, and litter depth (*Table 1*).

Table 1. *Inter-set correlation coefficients of environmental variables with CCA axes in the Yunmeng Mountain Forest Park in Beijing, China*

	CCA 1	CCA 2	CCA 3	CCA 4
Elevation	0.810^{***}	-0.147	0.243	0.204
Aspect	-0.427^{**}	0.458^{**}	0.304^{*}	0.507^{***}
Slope	0.549^{***}	0.027	-0.530^{***}	0.182
Slope position	-0.698^{***}	0.291^{*}	-0.204	-0.293^{*}
Soil depth	0.469^{***}	-0.337^{*}	0.533^{***}	0.007
Litter depth	0.258	-0.382^{**}	-0.448^{**}	0.303^{*}

* P<0.05, ** P<0.01, *** P<0.001

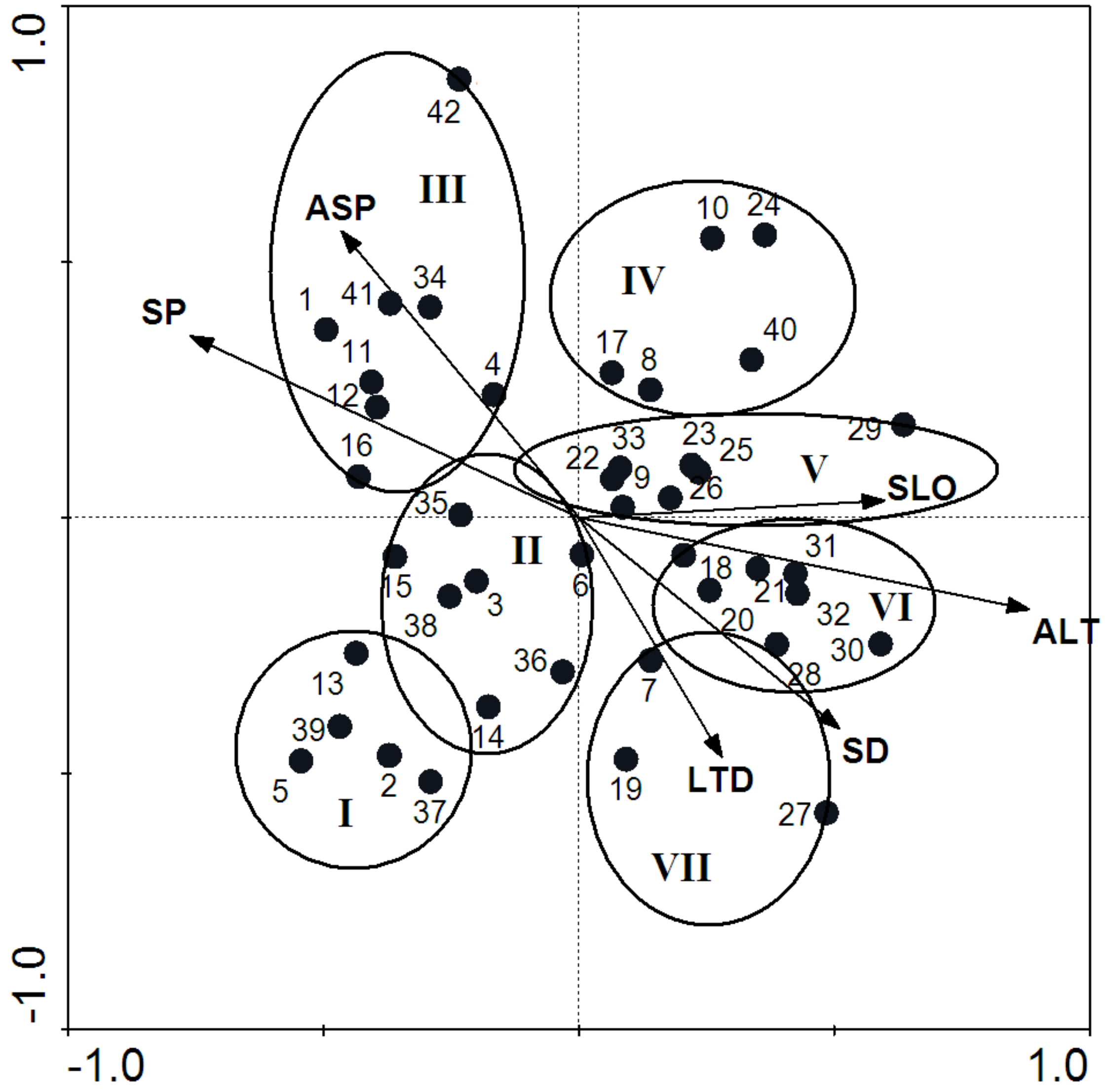

Figure 2. *CCA ordination biplot of the 42 forest quadrats and six environmental variables in the Yunmeng Mountain Forest Park in Beijing, China. ALT, ASP, SLO, SP, SD, and LTD refer to altitude, aspect, slope, slope position, soil depth, and litter depth, respectively. Roman numerals I - VII refer to the seven forest formations; Arabic numbers refer to quadrat number.*

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The distribution of forest communities on the CCA ordination map was related to environmental gradients, e.g., forests on the left were usually distributed in lower hills, with lower slope position and valley bottom, such as Form. *Pinus tabulaeformis,* Form. *Juglans mandshurica* and Form. *Fraxinus rhynchophylla* + *Acer truncatum* + *Tilia amurensis*. Forests on the right were distributed in comparatively higher hills, with upper slope position and hill ridge, such as Form. *Quercus mongolica*, Form. *Betula dahurica,* Form. *Popolus davidiana* + *Betula platyphylla,* and Form. *Larix principis-rupprechtii.*

Some pairs of environmental variables, such as elevation and slope position, elevation and soil depth, slope and aspect, slope and slope position, and soil depth and slope position, were significantly correlated with each other (*Table 2*).

Table 2. *Correlation coefficients between environmental variables in the Yunmeng Mountain Forest Park in Beijing, China*

	Elevation	Aspect	Slope	Slope position	Soil depth	Litter depth
Elevation	1					
Aspect	-0.273	1				
Slope	0.329^{*}	-0.367^{*}	1			
Slope position	-0.940^{***}	0.288^{*}	-0.383^{**}	1		
Soil depth	0.561^{***}	-0.285	0.139	-0.551^{***}	1	
Litter depth	0.263	-0.297^{*}	0.328^{*}	-0.225	0.074	1

* P<0.05, ** P<0.01, *** P<0.001

Species diversity in forests

Species richness, diversity, and evenness showed significant variation within forests of the Yunmeng Mountain Forest Park. Species richness ranged from 11 to 20, the Shannon-Wiener index ranged from 1.129 to 2.114 and evenness ranged from 0.410 to 0.813. We plotted species diversity indices against altitudinal and slope position gradients, which were the two most important factors affecting forest and species distribution, based on the CCA analyses above. Species richness and diversity showed significant correlations with elevation and slope position (*Fig. 3, Table 3*).

Table 3. *Correlation coefficients of species diversity with environmental variables in the Yunmeng Mountain Forest Park in Beijing, China*

	Elevation	Aspect	Slope	Slope position	Soil depth	Litter depth
Shannon-Wiener index	0.401^{**}	-0.134	0.176	-0.424^{**}	0.376^{*}	-0.056
Species evenness	0.075	-0.112	0.122	-0.072	0.194	-0.009
Specie richness	0.529^{**}	0.086	-0.004	-0.453^{**}	0.164	0.060

* P<0.05, ** P<0.01, *** P<0.001

Species richness and diversity (Shannon-Wiener index) increased gradually with increasing elevation, reaching a maximum close to the mountain summit, and decreased gradually as slope position varied from hill ridge to valley bottom. The correlations of evenness with these two gradients were not statistically significant, but the trends were similar to those for species richness and diversity. This finding suggests that elevation and slope position were the most important factors affecting species diversity in the Yunmeng Mountain Forest Park. Species diversity was also correlated with soil depth (*Table 3*).

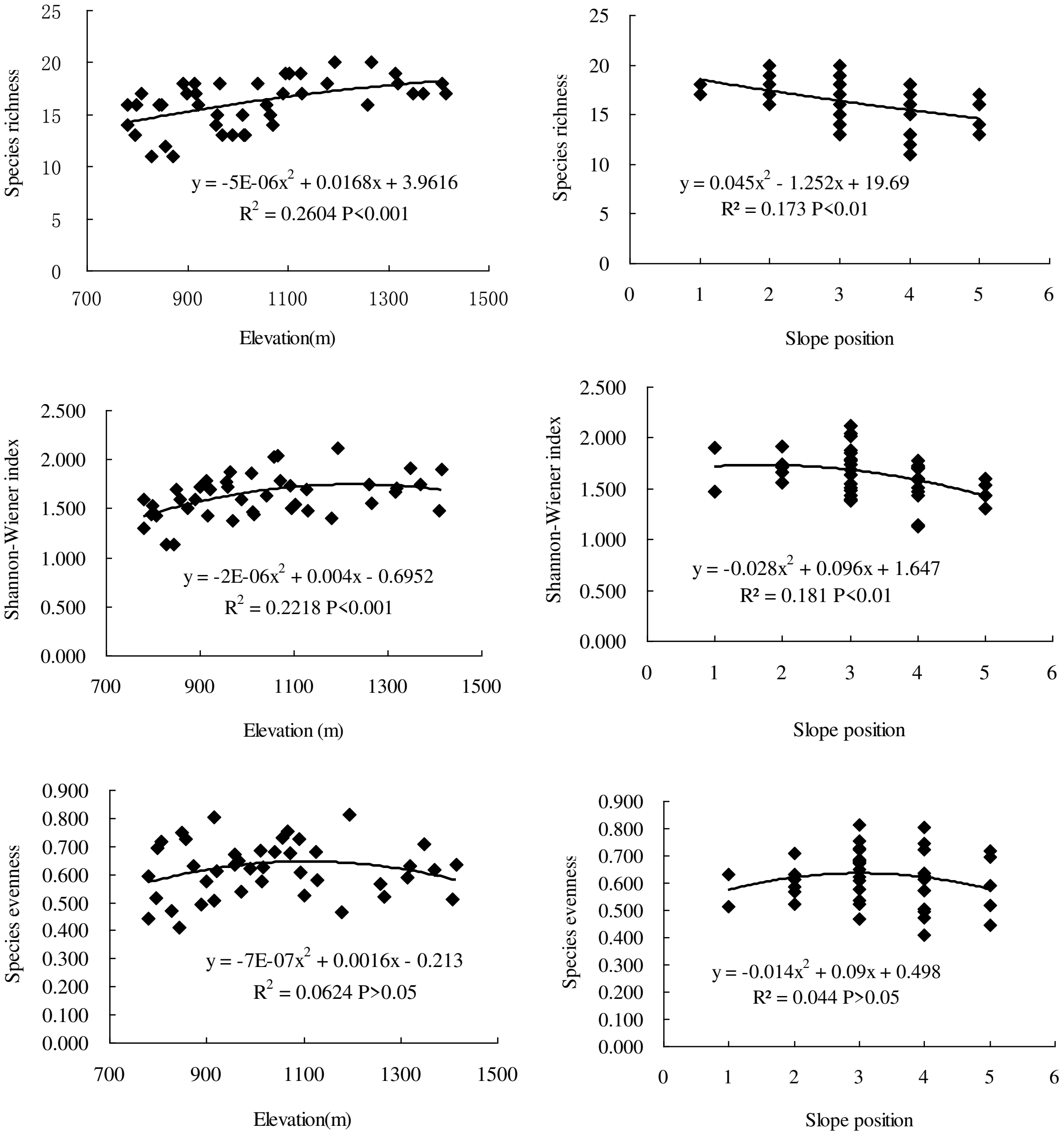

Figure 3. *Variation in species richness, diversity (Shannon-Wiener index) and evenness of forest communities along altitudinal and slope position gradients in the Yunmeng Mountain Forest Park in Beijing, China. Values of 1-5 for slope position represent the hill ridge, upper, middle, lower locations and valley bottom, respectively.*

Discussion

Forest types and distribution pattern

Variation in forest communities (forest diversity) was apparent in the Yunmeng Mountain Forest Park, and TWINSPAN successfully distinguished them as different forest formations. The seven forest formations were representative of the general forest vegetation in this park, and they conform to the Chinese forest classification system (Wu, 1980; Zhang et al., 2013). Most formations were secondary forest vegetation, following destruction of the original warm-temperate broad-leaved deciduous forests and temperate coniferous forests (Dai et al., 1990), with the additions of some plantations (Zheng et al., 2007). The present work is the first systematic and quantitative classification of forest vegetation in the Yunmeng Mountain Forest Park. Dai (1990) described the vegetation in this park as only three formations, i.e., Form. *Vitex negundo* var. *heterophylla,* Form. *Spiraea trilobata*, and Form. *Quercus mongolica* (*liaotungensis*). The former two formations were shrublands in the 1980s, and they have been recovered to forest formations after nearly 30 years conservation (Zhang et al., 2013). The latter formation has been developed and enlarged distribution area in this park. These changes suggest that the conservation of plant species and forest vegetation has had significant effects on forest composition since the park was established (Cui et al., 2008). The seven forest formations recognized in this study belong to three vegetation types: warm-temperate broad-leaved deciduous forest, warm-temperate coniferous forest, and cold-temperate coniferous forest (Wu, 1980). These results correspond to forest descriptions of other mountains in Beijing, such as Baihua Mountain (Zhang et al., 2012) and Songshan Mountain (Suriguga et al., 2010). The Yunmeng, Baihua, Dongling, and Songshan Mountains are typical mountainous regions, and contain over 70% of the natural forests in the Beijing area; they therefore play a significant role in improving the city's ecological environment (Zhang et al., 2013).

The distribution of dominant species determined the forest differentiation and distribution along environmental gradient (Wu, 1980; Lomolino, 2001). The distribution of dominant species such as *Pinus tabulaeformis, Juglans mandshurica*, *Quercus mongolica, Larix principis-ruprechtii, Betula platyphylla,* and *Popolus davidiana,* play important roles in forest patterning in the Yunmeng Mountain Forest Park (Zheng et al., 2007). In addition to different dominant species, each of the seven forest formations has its own specific species composition, structure, function, and environment (Wu, 1980; Zhang et al., 2013). These forest formations are distributed predictably along an altitudinal gradient from lower to upper hills, in a reliable order: Form. *Pinus tabulaeformis,* Form. *Juglans mandshurica*, Form. *Fraxinus rhynchophylla* + *Acer truncatum* + *Tilia amurensis*, Form. *Quercus mongolica*, Form. *Betula dahurica,* Form. *Popolus davidiana* + *Betula platyphylla,* and Form. *Larix principis-rupprechtii.* These distribution patterns are determined by interactions among species, communities, and environment (Liu and Ren, 1992; Zilliox and Gosselin, 2014).

Forest vegetation – environment relation

Forest composition, structure, and distribution pattern are usually related to environmental factors which exhibit heterogeneity over space and time, such as climate, topography, soil and disturbance (Alexander and Millington, 2000; Kikvidze et al., 2006; Korner, 2007). This is true in the Yunmeng Mountain Forest Park. The variation in forest communities was closely related to environmental variables, such as elevation, slope position, slope, aspect, soil depth, and litter depth, with elevation and slope position being the most important factors affecting forest formations in this park. The change of forest formations in CCA space clearly illustrated the relationship of forest communities to environmental variables (Brinkmann et al., 2009; Muenchow et al., 2013). Each forest formation had its own distribution area and was correlated with a specific combination of environmental variables (ter Braak and Smilauer, 2002; Zhang et al., 2012). The first CCA axis was primarily an elevational gradient, i.e., from left to right in the CCA ordination diagram, elevation increased gradually. Elevational change leads to changes in humidity, temperature, soil type, etc., which in turn influence forest formations (Vittoz et al., 2010; Otypkova et al., 2011). The first CCA axis was also a slope position gradient, i.e., from left to right in the CCA diagram, slope position varied gradually from valley bottom to hill ridge. Change in slope position also leads to change in humidity, temperature, soil type etc., which further influences the forest formations (Dai et al., 1990; Meng et al., 2012).

Forest variation was closely related to other environmental variables in the Yunmeng Mountain Forest Park, such as slope, aspect, soil depth, and litter depth. Changes in slope and aspect may lead to a change in hours of sunshine, in humidity or in temperature, which affects forest community development (Virtanen et al., 2010). The soil depth and litter depth are also important for providing nutrients to plants in forests (Austrheim, 2002). All environmental variables were interrelated, and they play a coordinated role in forest development (Zhang, 2011).

Species diversity

Species diversity is an important feature of forest composition and structure, and its change is frequently used as an indicator of forest dynamics (Muhumuza and Byarugaba, 2009). The spatial variation of species diversity corresponds closely to forest vegetation patterns in a region (Rahbek, 2005; Zhang et al., 2012). This spatial change of species richness, diversity and evenness was apparent in the Yunmeng Mountain Forest Park and corresponded to changes in forest structure, composition, function, distribution, etc., related to environmental variables (Littell et al., 2010; Zhang et al., 2011). Species richness and diversity were significantly correlated with elevation and slope position. Elevation and slope position, therefore, are key factors in influencing species diversity in this park. Elevation as a key factor affecting species diversity in mountainous areas has been demonstrated by many researchers (e.g., Fetene et al., 2006, Muhumuza and Byarugaba, 2009; Meng et al., 2012). The most commonly observed pattern is for diversity to be highest at intermediate elevations (Austrheim, 2002; Virtanen et al., 2010). The present study showed a different pattern, with species diversity was increasing gradually with increasing elevation, and reaching a maximum near the mountain summit. This pattern occurs because the altitudinal gradient from 750 m to 1414 m is shorter in the Yunmeng Mountains compared with other mountains, such as the Baihua and Songshan Mountains (Soriguga, 2012; Zhang et al., 2013). This pattern can be recognized as a part of a ‘‘humped’’ distribution curve (Brinkmann et al., 2009; Otypkova et al., 2011).

Implications for management

The forest vegetation in the present study showed different forest formations within the Yunmeng Mountain Forest Park. These formations were secondary natural forests restored from destroyed warm-temperate deciduous forests, and degraded lands (Cui et al., 2008; Zhang et al., 2013). The restoration and conservation of forests have been effective over the past 30 years (Zheng et al., 2007), and as a result, vegetation reflects the effects of elevation, slope position and landform on forest composition, structure and diversity. The identified forest communities, compositions, and diversity are typical for the mountainous areas in Beijing (Suriguga et al., 2010). These forests are very important to the city and its citizens. During the last decade, eco-tourism has developed very quickly along with forest restoration and environmental improvement, and the disturbance to forests has correspondingly increased (Cui et al., 2008). This must be controlled, i.e., tourist numbers should be regulated in summer, according to the tourist capacity of this park (Wang et al., 2004; Zheng, 2007).

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APPENDIX

Electronic Appendix 1: Species Importance Value Data in the Yunmeng Mountain Forest Park in Beijing, China.

Electronic Appendix 2: Environmental data in the Yunmeng Mountain Forest Park in Beijing, China.

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ESTABLISHMENT OF BLUE MUSSEL BEDS TO ENHANCE FISH HABITATS

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Abstract. Human activity has impacted many coastal fjords causing degeneration of the structure and function of the fish habitats. In Nørrefjord, Denmark, local fishermen complained of declining fish catches which could be attributed to eutrophication and extraction of sediments over several decades. This study aimed to establish blue mussel beds (*Mytilus edulis*) to increase structural complexity and increase the abundance of fish and epifauna in Nørrefjord. It was expected that the mussels would improve water transparency and increase the depth range and coverage of eelgrass (*Zostera marina*). New methods for mussel production and -bed construction were investigated in collaboration with local volunteer fishermen. The effect of the artificial mussel beds was most evident on a small scale. Video observations directly at the beds (Impact area) demonstrated increased biodiversity and a three times higher abundance of mesopredator fish compared to the Control area. Water clarity and eelgrass coverage were unchanged. Two methods for establishing mussel beds were tested. A total of 44 tons of blue mussels were produced and established in beds over an area of 121,000 m^2. Production of blue mussels directly on hemp sacs hanging on long-lines was the most effective method. This new method is potentially a useful management tool to improve fish habitats.

Keywords: *habitat complexity, biogenic reef, fish community, benthos, volunteer.*

Introduction

Coastal habitats are under great anthropogenic pressure and 85% of the European coastlines are estimated to be degraded (Bryant et al., 1995; EEA, 1999). Eutrophication, overfishing and destructive dredging fishery have severely affected shellfish and biogenic reefs (Airoldi and Beck, 2007). These pressures may also affect the population structure of fish (Sundblad et al., 2014) as the coastal habitats are important for many commercial fishes for spawning, feeding and as nursery area (Seitz et al., 2014).

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Habitat complexity in coastal habitats is an important component for a number of fish species as more complex bottom structures provide more shelter opportunity from predators and a higher abundance of prey than bare bottom sediments (Heck and Wetstone, 1977; Luckhurst and Luckhurst, 1978; Nelson and Bonsdorff, 1990). The abundance and biodiversity of fauna living within a biogenic reef of bivalves, increase with complexity and structure area, and promotes fish growth and diversity (Carbines et al., 2004; Norling and Kautsky, 2007; 2008). Especially smaller fish species such as common goby (*Pomatoschistus microps*), rock goby (*Gobius paganellus*) and butterfish (*Pholis gunnellus*) but also larger fish like flatfishes use mussel beds as habitat for either direct foraging, breeding or as nursery area (Jones and Clare, 1977). Predatory fish are attracted to the structures by the abundance of prey. The overall effects of increased complexity can thus be relatively substantial for fish. Apart from improving coastal habitats by increasing the complexity (McDermott et al., 2008), mussels are filter feeders and remove suspended inorganics, phytoplankton and detrital particles. The filtration process reduces turbidity and generally improves water quality (Riemann et al., 1988; Nielsen and Maar, 2007). The improved water transparency leads to better conditions for benthic primary producers e.g. sea grasses (Newell and Koch, 2004), allowing them to spread into deeper areas.

Nørrefjord, Denmark, is representative for many coastal areas in northern Europe. It has been subject to substantial nitrogen loadings from agriculture during the last three to four decades (Rask et al., 2000). High nitrogen loadings are known to reduce water transparency and increase the extent and frequency of oxygen depletion events (Krause-Jensen et al., 2011; 2012; Wulff et al., 2014). However, in the last decade, nitrogen loading has dropped markedly in Nørrefjord, whereas phosphorous has remained unchanged but at a low level (data, The Danish Natural Environment Portal, miljoeportal.dk) due to intensive improvements in sewage treatment during the 1980s (Ærtebjerg et al., 2003). Further, the fjord has experienced extraction of sand and gravel from 1950-1990 (N.C. Christensen, local fisherman, pers. com.). Extraction of resources from shallow coastal areas reduces the complexity of the bottom and the habitat quality (Nielsen and Petersen, 2013). In other coastal areas in Denmark, dredging activities with towed fishing gears for fin- and shellfish (Dolmer and Frandsen, 2002; Kaiser et al., 2006) also deteriorate habitat quality. Furthermore, climate change, increased water temperature and acidification may impact coastal habitats (IPCC, 2014; Mackenzie et al., 2014). All these pressures have resulted in deteriorated habitats and a decline in bottom fauna and fish biomass (Pihl et al., 2005; Holm, 2005; Christiansen et al., 2006). Nørrefjord was previously dominated by blue mussel beds (Rask et al., 2000) but hypoxia events is believed to have degraded the benthic habitats with an associated decline in fish populations. This general deterioration of the fjord is of great concern to the local recreational fishermen, who experience declining fish catches. The recreational fishermen therefore initiated this project to improve conditions for fish by promoting fish habitats in Nørrefjord. This project is unique through the close collaboration between local stakeholders, local managers and researchers.

Bivalve restoration is known to have a positive effect on fish communities (reviewed by Peterson et al., 2003). Most studies focus on oyster beds, but the function of structure is more important than the species comprising the structure (Palomo et al., 2007; Norling and Kautsky, 2007). Therefore it was hypothesized that establishment of mussel beds could, in a manner similar to oyster beds, improve fish habitats. When establishing cultured mussels, it is standard procedure to dredge natural bottom mussels for seeds and then transplant the mussels to a different area from a specialized vessel (Dolmer et al., 2012). Neither the destructive dredging nor the expensive machinery was an option in this project in Nørrefjord.

The primary aim of this study was to test if establishment of blue mussel beds would have a positive effect on abundance of fish and epifauna. It was also hypothesized that the established mussel beds would improve water transparency followed by increase in eelgrass (*Zostera marina*) depth range and coverage. The secondary aim was to develop an efficient and effective method for production of suspended blue mussels for the establishment of bottom mussel beds in a Danish fjord using voluntary labour, as this had not been attempted before.

Materials and Methods

Study area

The field study was conducted during 2010 and 2011 in Nørrefjord, Helnæs Bight (10 7.17E 55 9.10N) south-west of the Island of Funen, Denmark (*Fig. 1*). The fjord is a protected bay with two connections to the strait Lille Belt between Funen and the Jutland Peninsula. The mean water depth is 5.5 m and the maximum depth is 12 m (Rask et al., 2000). Two sites resembling each other in terms of depth, sediment and eelgrass cover were chosen 1 km apart, one was the Control and one was the Impact area (*Fig. 1*).

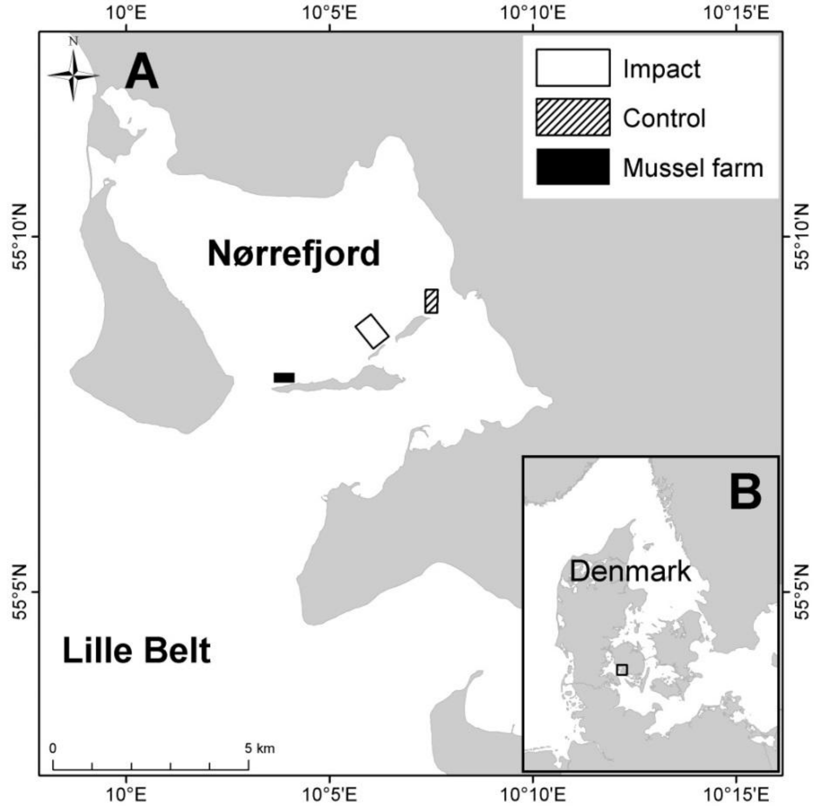

Figure 1. *Study area. A) Location of the mussel farm, the Impact area, and the Control area. B) Location of Nørrefjord in Denmark.*

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Establishment of blue mussel beds

A mussel collection site (mussel farm) was established with help from a local consultant (Nordshell A/S) (*Fig. 1*). The mussel farm consisted of long-line systems (7 x 200 m length) maintained floating by gradually increasing the number of buoys as the weight of the produced mussels increased over the summer.

Blue mussel beds were established on the fjord bottom at 4-6 m depth within the Impact area (*Fig. 2a*). The mussel beds where constructed to increase the complexity of the bottom substrate to improve the beds' value as fish habitat. The overall bed structure was constructed in a patchy distribution to imitate natural mussel beds. The mussel beds were constructed as piles of 1 m diameter and 0.5 m height (= one mussel bed). This was done by piling 28 kg of mussels on top of degradable hemp sacks through a tube (40 cm diameter, 6 m length). Half the piles were placed on top of 3 hemp sacks (60-100 L) containing mussel shells (40 L per sac), thus producing 3-dimensional structures on the seafloor. Another 25% of the piles consisted of mussels placed directly on the fjord bottom without hemp sacks. The remaining 25% consisted of hemp sacks with mussel shells. All mussel beds were placed in grids with 3-10 m distance between single beds resulting in a mussel density of 2.8-9.3 kg mussel m^{-2}.

Effect analysis

Before commencing the effect analysis, diver and video observations were made to estimate the survival rate of the blue mussels and to confirm that the structures still remained on the fjord bottom. No systematic analysis was made based on diver and video observations. However, a rough estimate of the mussel survival rate was found based on the observations.

The effect of the constructed mussel beds was measured in a BACI design, including investigations *before* mussel bed establishment (summer of 2010) and one year *after* mussel bed establishment (summer of 2011). All analysis took place in both *control* and *impact* area. The effect analysis sought to clarify the effect of the mussel beds on fishes, epibenthic invertebrates and important environmental parameters presented in the following sections.

Eelgrass and water quality

Eelgrass coverage in the Impact area and Control area was mapped 1) to locate areas suitable for mussel bed establishment and 2) to analyse the effect of the mussel beds on eelgrass coverage *before* and *after* mussel bed establishment (*Table 1*, *Fig. 2*). Eelgrass coverage was mapped by *in situ* video monitoring of the fjord bottom from a slow drifting boat. GPS position of the drop camera (600 TV lines) and the associated eelgrass coverage was logged every 2 min. Eelgrass coverage was analysed in 5 categories: 0 = no eelgrass, 1 = dead shoots, 2 = single plants, 3 = thin coverage or patches, 4 = dense beds of eelgrass. These categories corresponded to a percentage cover of 0 = 0%, 1 = 0 % (dead shoots), 2 = 1-25%, 3 = 26-75%, 4 = 75-100%. Mussel beds were placed in areas within the Impact area where there was, generally, no eelgrass to avoid damaging eelgrass beds.

The effect of the mussel beds on water transparency was investigated by measuring secchi depth, and measurements were carried out weekly from May to September *before* and *after* mussel bed establishment.

Epibenthic samples

Benthic invertebrates were quantified in the Control area and Impact area *before* and *after* mussel bed establishment using an epibenthic sledge (Modified Ockelman Sledge, KC Denmark, Denmark) (*Table 1*). The sledge was dragged 30 sec at 1 kn over the seafloor at 4-6 randomly selected stations in the two areas, in the depth range of mussel bed establishment (4-6 m), to sample invertebrates and other smaller organisms living on the surface of the bottom substrate. The density of all fauna was estimated based on the area covered by the sledge on each tow (4.6 m^2). All organisms were counted and determined to lowest possible taxonomical group. Fish, blue mussels and snails were not included in this analysis as they could not be quantified properly from sampling with the epibenthic sledge.

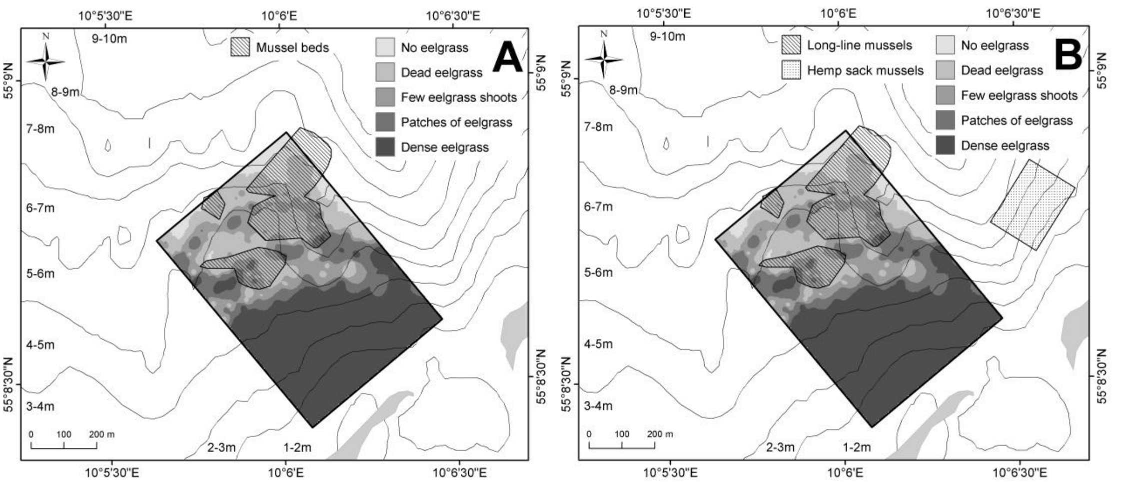

Figure 2. *A) Mussel beds were established in Impact area. B) Two different methods of blue mussel production and establishment of mussel beds were tested in 2010 and 2011, respectively. Eelgrass cover is based on data from 2010.*

Fish community

Fish distribution and abundance was investigated using two different methods 1) gillnets for large scale effect of the mussel beds on the fish community (0-100 m) and 2) video observations for small scale effect (0-2 m). Gillnets were deployed at 9 stations in the Control and Impact area with 3 stations at 0-2 m, 2-4 m and 4-6 m depth (*Table 1*). Each station was sampled once a month from May to October *before* and *after* mussel bed establishment, using multi mesh size gillnets. The mesh sizes in the different panels were 6.5, 8.5, 11, 14.3, 18.6, 24.2, 31.4, 40.9, 53.1, 69, 89.7 and 116.6 mm and applied in random order during sampling. Height of the nets were 1.5 m and length was 3 m (mesh size 6.5-14.3 mm), 6 m (mesh size 18.6-40.9 mm), or 12 m (mesh size 53.1-116.6 mm) (Eigaard et al., 2000). All nets were deployed in the afternoon and hauled the following morning. The catch was identified to species level, and total length was measured to the nearest 0.5 cm below and weighed (± 1 g wet weight). No differentiation was made between sprat/herring, salmon/trout and common/sand goby.

Two cameras (Sport, LH Camera, Denmark) recorded close-up of the mussel structures in the Impact area and sandy bottom in Control area both at 4-5 m depth (*Table 1*). The cameras recorded continuously for 12 h. All video sampling was carried out in late summer *after* mussel bed establishment in five consecutive days. Subsequently, fish appearance was counted for every second minute for the first 10 minutes of each hour. The remaining video sequences were viewed but not analysed. To avoid bias from the deployment operation, the first 30 min after deployment of the camera was omitted.

Development of new method

The local community of recreational fishermen (Danish Organization for Amateur Fishermen in Faaborg) contributed to the project on a volunteer basis and did most of the practical work (i.e. crowdsourcing).

Mussel beds were established in 2010 and 2011 with two different methods. In 2010 blue mussels were produced in a mussel farm on suspended long-lines and harvested from a specialized vessel in November 2010. The harvested mussels were thereafter used to establish mussel beds in the Impact area as described above (*Fig. 2b*). In 2011 blue mussels were produced directly on hemp sacks (100 L) filled with 40 L of shells hung from the long-line system. The mussel beds were then constructed in September 2011 outside the Impact area by transporting the long-line with the hemp bags between two boats to the Impact area. The mussel bags were then detached from the line and allowed to sink to their placement with approximately the same distance between the bags as in 2010. The effect of the second mussel bed establishment 2011 was not investigated.

Table 1. *Samples carried out in 2010 and 2011. For secchi depth, the first number represents the number of samples statistically analysed and the number in parenthesis is the actual number of samples.*

Sampling	**2010**			**2011**		
		Impact			**Impact**	
	Control	**Off structure**	**On structure**	**Control**	**Off structure**	**On structure**
Epibenthic sledge	4	6		5	5	
Gillnet	54	54		54	54	
Video obs.				11 h		23 h
Eelgrass cover	701 obs	1201 obs		501 obs	591 obs	
Secchi depth	0 (31)	0 (30)		14 (99)	14 (106)	11 (22)

Data analysis and statistics

Secchi depth: The difference between average secchi depths was analysed in the Control area, Impact area and directly on the mussel bed area in 2011. Only averages on days where secchi depth was measured in all 3 areas or minimum in Control and Impact area were included in the analyses to ensure that the differences were caused by area and not by time. Data was tested using a GLM (model: area + day) where area was either Control or Impact and day was a random effect.

Eelgrass: The difference in eelgrass coverage was tested at 4-6 m depth (mussel bed establishment depth) with logistic regression. The independent variable in the model was eelgrass coverage with category values between 0 to 4 while the dependent variables were areas (Control, Impact) and years (2010 (*before*), and 2011 (*after*)).

Epibenthic Samples: Using the BACI design (Underwood 1992) ensured that any detected changes found were a result of the establishment of blue mussel beds and not temporal or spatial variability. Standardized cross effect of Control-Impact and *before-after* was estimated by LSmean function in proc-GLM (model: Year*Area). Abundance data were log-transformed. The following variables were used: year (2010 and 2011), area (Control and Impact) and species.

Fish Community: Standardized cross effect of BACI was estimated by LSmean function in proc-GLM (Abundance model: Year*Area). Abundance data were log-transformed. The following variables were used: year (2010 and 2011), area (Control and Impact), species (the most dominant species was analysed separately while the remaining species were grouped into "Other species"), depth (0-2 m, 2-4 m and 4-6 m), low/high impact area (0-4 m and 4-6 m) and season (May+Jun, Jul+Aug and Sep+Oct).

The abundance of fish pr. video sequence followed a negative binomial distribution and data was analysed for any effects of area and time of day by LSmean function in proc-GLM (abundance = area month hour). Data were log-transformed and observations were divided into morning (8:00-11:00), noon (12:00-15:00) and evening (16:00-19:00).

The threshold for rejection of the null hypothesis was defined as P=0.05. Data was statistically analysed in SAS 9.4.

Results

Establishment of blue mussel beds

The naturally occurring blue mussel beds in Nørrefjord were generally in poor condition consisting primarily of empty, crushed shells and very few live mussels. The natural beds were small (<5-7 m in diameter) and occurred mainly from 4-6 m. Therefore the produced blue mussels were established in beds to imitate the size and placement of natural mussel beds in other areas of Nørrefjord: 1-2 m diameter spaced 3-10 m apart at 4-6 m depth corresponding to 121.000 m^2 of mussel bed in total (*Fig. 2A*).

A rough estimate based on diver and video observations showed that approximately 5% of the mussels had survived until spring of 2011. The structures of the beds were intact as the empty mussel shells still remained on the fjord bottom.

Fish communities

A total of 19 different fish species were caught in gillnets in 2010 and 15 in 2011. In both years the catches were dominated by three species: cod (*Gadus morhua*) black goby (*Gobius niger*) and three-spined stickleback (*Gasterosteus aculeatus*), which combined accounted for 81% of the total catches. The statistical tests focused on these three species, as all other species occurred in low numbers (*Table 2*) and were combined in the category "Other species" for statistical testing.

On the video recordings 112 primarily smaller fish were observed in both areas in 2011. Seven taxonomical groups were recorded (*Table 2*) and goby was the most common group represented by black, sand and undetermined goby comprising approximately 66% of the observations in the Impact area. Undetermined species comprised 29% of the catches but was most likely from the G*obiidae* family. Only one fish species was observed in the Control area and four species were observed in the Impact area, disregarding the goby sp. and unidentified fish species.

A significant cross effect on fish abundance of year and area, i.e. a direct effect of mussel bed establishment was only found for black goby at 0-4 m depth (P=0.004 – se all P-values in *Table 3*). The mean abundance of black goby decreased from 4.7 to 0.2 ind. day^{-1} in the Control area and from 5.2 to 0.02 ind. day^{-1} in the Impact area from 2010 to 2011.

Yearly variation in abundance was significant for cod, black goby and three-spined stickleback in both 0-4 m depth and 4-6 m depth (all P<0.05), with an increase in cod and three-spined stickleback and decrease in black goby from 2010 to 2011. A significant effect of area was only observed for black goby (P=0.009). Significant changes in abundance caused by season were observed for cod in the 4-6 m depth and black goby in 0-4 and 4-6 m depth (*Fig. 3*). For all other fish species no significant changes were observed for any of the analysed variables.

Table 2. *Abundance of fish species caught in gillnets and observed in video observations in the Control and Impact area.*

Scientific name	Gillnet catch pr. day n	Gillnet catch pr. day n	Video obs pr. day n	Video obs pr. day
	Control n=108	Impact n=108	Control 11 h	Impact 23 h
Agonus cataphractus	0.3			
Ammodytes tobian	1.7	1.8		
Belone belone	2.0	0.5		
Ctenolabrus rupestris				5.2
Eutrigla gurnardus		0.2		
Gadus morhua	48.2	50.7		
Gasterosteus aculeatus	109.3	149.2		
Gobius niger	69.7	51.7		25
Merlangius merlangus	0.2			
Myoxocephalus scorpius	13.0	26.7		
Pholis gunellus	0.2			
Platichthys flesus	3.3	2.5		
Pleuronectes platessa	0.3	0.5		
Pomatoschistus minutus/microps	6.3	4.8		7.3
Salmo salar/trutta	2.5	0.7		
Scomber scombrus	3.5	4.5		
Spinachia spinachia	9.0	4.0		
Sprattus sprattus/Clupea harengus	12.5	4.7		
Syngnathus typhle		0.2	17.5	
Zoarces viviparus	6.7	16.7		1
*goby				51.1
Undetermined			24	36.5
Total	**288.7**	**319.2**	**41.5**	**126.1**

There was a highly significant difference between the abundance of fish observed on video (P<0.0001) in the Impact area compared to the Control area, with three times as many fish observed directly on the mussel beds (*Table 2*). In addition to this, a diel variation occurred, as significantly more fish were observed in the morning compared to noon and evening (P<0.0001). There was no significant difference between noon and evening (P=0.19).

Outside the processed 5x2 minute observations, cod, trout and flatfish were observed on several occasions but only in the Impact area.

Epibenthic samples

In total, 14 taxa were recorded in epibenthic samples of which 9 were identified to species level (*Table 4*). Two of the taxa were fish (*Sygnathus typhle* and *Pomatochistus microps*) and one was blue mussel (*Mytilus edulis*). All three were omitted from the analysis leaving 11 taxa of benthic invertebrates in the analysis. Crustaceans dominated the community both in terms of numbers and taxa with 8 of the 11 invertebrate taxa.

Table 3. *P-values for mean abundance of fish caught in gillnets. Significance levels are set at: * 0.05, ** 0.01, *** 0.001. The ÷ for Gobius niger in Year*Area column at 0-4 m depth indicate that the cross effect of year and area was significantly negative for this species.*

Abundance	**Impact**	**Year**	**Area**	**Year*Area**	**Season**	**Depth**
Gadus morhua	High (4-6 m)	***	P=0.2	P=0.1	P=0.8	-
	Low (0-4 m)	***	P=0.7	P=0.5	**	P=0.07
Gobius niger	High (4-6 m)	***	P=0.1	P=0.1	***	-
	Low (0-4 m)	***	**	÷ **	***	P=0.8
Gasterosteus aculeatus	High (4-6 m)	*	P=0.4	P=0.5	P=0.3	-
	Low (0-4 m)	**	P=0.8	P=0.7	P=0.09	*
Other	High (4-6 m)	P=0.9	P=0.4	P=0.6	P=0.07	-
	Low (0-4 m)	P=0.8	P=0.1	P=0.6	P=0.4	P=0.06

Only one species in each of the taxonomical phylums Echinodermata, Annelida and Urochordata was found.

In general, year and area had an effect on abundance of most species of epibenthos. A positive significant effect of mussel beds on abundance was found for Polynoidae (P=0.04), *Praunus flexuosus* (P=0.04) and *Idotea baltica* (P=0.006) (cross effect of year and area) (*Table 4*). The abundance of *Idotea* increased only in the Control area, so the effect of mussel beds in the Impact area seems to be negative for this species. No significant effect of mussel beds was observed for all species suited as fish prey (all species except tunicate (*Ascidiae*) and starfish (*Asterias rubens*)) or all species combined.

Starfish increased 15 to 32 fold in the Impact and Control area, respectively, from 2010 to 2011. All starfish were relatively small ranging from 4 mm to 9 cm with 70% measuring < 15 mm.

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Table 4. _Abundance of epibenthic invertebrates m^{-2} in the Control and Impact area before (late October 2010) and after (early November 2011) mussel bed establishment. "-" signifies too few observations for statistical analysis. Significance levels are set at: * 0.05, ** 0.01, *** 0.001._

Benthic invertebrates	**2010 No m^{-2}**		**2011 No m^{-2}**		**Fish prey**	**Significant changes**		
	Control n=4	**Impact n=6**	**Control n=5**	**Impact n=5**		**Year**	**Area**	**Year*Area**
Crustacea								
Corophiidae	1.2	2.3	0.3	4.7	Yes		**	
Crangon crangon	0.9	5.7	1.6	3	Yes			
Gammaridae	2	1.4	0.3	20.1	Yes			
Idotea baltica	0.1	2.8	17.4	3.3	Yes	***		***
Ostracoda	0	0	0	0	Yes	-	-	-
Palaemon adspersus	2.3	2	0.3	0	Yes	**		
Phtisica marina	0	0	0.3	9.6	Yes	***		
Praunus flexuosus	0.5	0.1	6.7	18.6	Yes	***		*
Echinodermata								
Asterias rubens	0.7	2.2	26.9	28.3	No	***		
Annelida								
Polynoidae	0.1	0	0.9	3.1	Yes	**		*
Urochordata								
Ascidacea	0	0.1	1	0.4	No	***		
n total	**8**	**16.5**	**55.9**	**91.3**		***		
n fish prey	**7.3**	**14.2**	**28**	**62.5**		**		

Eelgrass and water quality

All secchi measurements varied between 3.4 and 6.0 m with slightly deeper measurements during early summer compared to late summer as could be expected due to seasonal variation in planktonic blooms (data not shown). No significant difference was found between the Control and Impact area (P=0.36).

The restored mussel beds did not affect eelgrass coverage significantly when comparing eelgrass coverage in the Control area and Impact area in 2010 or 2011 (P>0.05, logistic regression). Neither were there significant differences when comparing areas with and without restored beds in the Impact area at 4-6 m depth (P>0.05, Chi-Square test). Depth was the only factor that had a significant effect on eelgrass coverage (P<0.0001, Chi-Square test). Video observations of eelgrass cover showed dense mats until 4-5 m depth and a maximum depth of 7.4 m in both the Control and Impact area.

Development of new method

In 2010 the mussels were produced on suspended long-line systems. Based on mussel coverage, weight and long-line length in the mussel farm, it was estimated that a total of 28 tons of blue mussel were produced in 2010. The harvest and subsequently construction of mussel beds was labour-intensive and 14 men and 5 boats worked for 8 days.

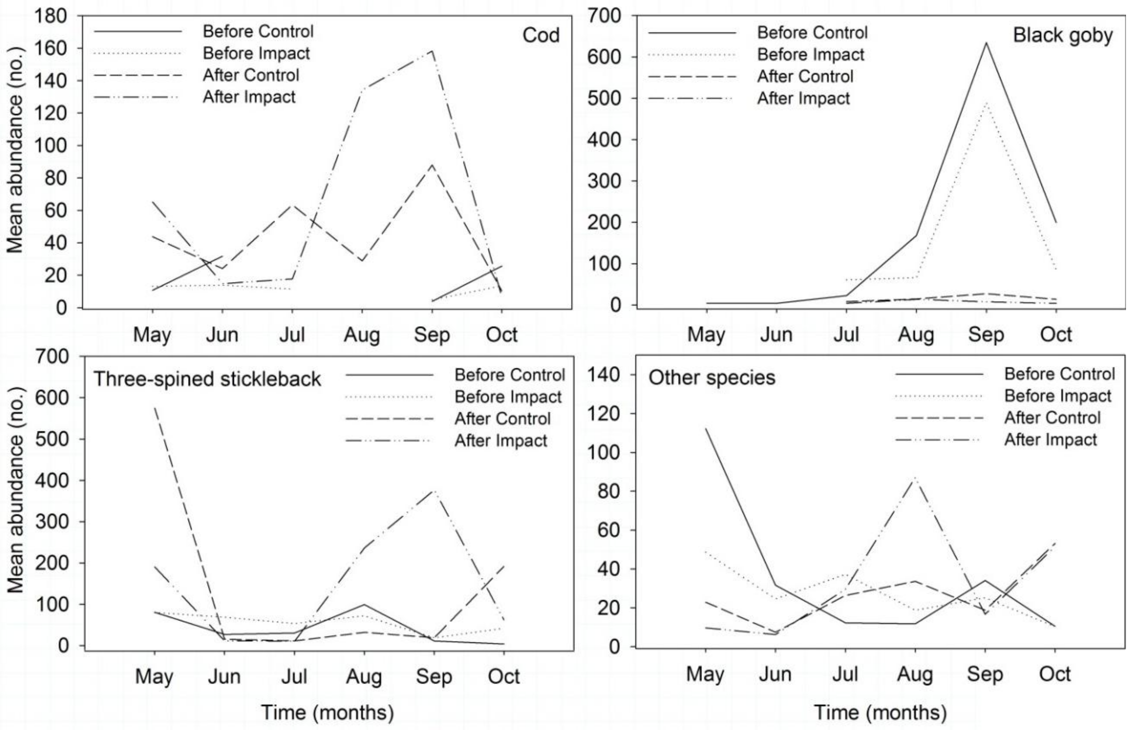

Figure 3. *Monthly (May-Oct) fish catches for cod, black goby, stickleback and other species caught in gillnets in the Control and Impact area before and after mussel bed establishment. Notice the difference in abundance between species.*

To reduce the work load in the harvest process, blue mussels were in 2011 produced on hemp sacks on the long-lines. It was estimated that 16 tons of blue mussels were produced using this method. The harvest and construction of mussel beds required 12 men and 5 boats in 1 day.

Discussion

Effect of mussel bed establishment

The mussel bed structures improved fish habitat on a local scale resulting in a higher abundance and biodiversity of fish directly on the introduced mussel structures. In particular, small gobies were observed circling around the structures for extended periods. Similar observations were made around stone reefs and wind turbine foundations in the Baltic Sea where gobies were observed to occur in significantly higher numbers within a few meters from the structures (Wilhelmsson et al., 2006; Andersson and Öhman, 2010; Hansen, 2012). Also larger fish (e.g. cod) are known to be surprisingly stationary (Lindholm et al., 2007; Karlsen, 2011). The very local effect of structures could explain why the effect of the established mussel beds in the present study was greatest in the video observations rather than the larger scale gillnets and epibenthic sledge.

Very few mussels survived the starfish predation, but the structure remained intact as empty shells. The increase in starfish abundance in spring 2011 could not be related to the establishment of the mussel beds as the increase in starfish abundance occurred both

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in the Control and Impact area. High abundance of starfish was also reported from adjacent waters (Lille Belt) (pers. com. Allan Buch). The structures comprised by the empty mussel shells are reported to be just as important as live mussels as they still function as shelter for associated fauna (Palomo et al., 2007; Norling and Kautsky, 2007). In this study, the persistence of the local effect on fish abundance and biodiversity, despite the high predation rate by starfish on the blue mussels, supports the finding that the fish habitat function of the mussel bed remains intact with its structure, despite the loss of live blue mussels.

Gobies are mesopredators and attract larger piscivorous species, such as cod and trout (Fjøsne and Gjøsæter, 1996; Wennhage and Pihl, 2002; Almqvist et al., 2010). The observation of large piscivorous species (trout and cod) in the Impact area suggests that the same attraction mechanism was present around the established mussel bed structures. There was a tendency towards increased cod abundance after mussel bed establishment (*Fig. 3).* This increase in predation pressure could explain the decreased black goby abundance. We did not see the same decrease for three-spined stickleback. This may be due to the relatively large spines of the stickleback that make it a less attractive prey compared to the goby (Wennhage and Pihl, 2002).

The goldsinny wrasse (*Ctenolabrus rupestris*) is a fish species occurring in higher densities near rocky substrates and exhibits high affinity to these types of complex habitats (Gjøsaeter, 2002). The presence of goldsinny wrasse on the established mussel structures suggest that the mussel structure provided a complex habitat similar to rocky reefs that could attract this reef-associated species.

Eelgrass and water quality

The establishment of the mussel bed in the present study did not affect the eelgrass coverage or depth range. Eelgrass cover was generally in good condition in Nørrefjord with patches as deep as 7-8 m in depth. The reason for the good condition in Nørrefjord is probably the reduced nitrogen loading compared to the 1980s (data, The Danish Natural Environment Portal, miljoeportal.dk, Rask et al., 2000). The decrease in nitrogen loading has gradually increased the secchi depth (data, The Danish Natural Environment Portal, miljoeportal.dk) and improved the light conditions for eelgrass.

An effect on secchi depth after the establishment of the new mussel beds could not be expected due to the magnitude of the established mussel beds. A conservative estimate of potential filtration rate with 5% survival of the 28 tons mussels established in beds in autumn 2010 would be 5600 m^3 d^{-1} (based on filtration rate for 25 mm blue mussels found by Winter, 1973). The total body of water in Nørrefjord is 213 x 10^6 m^3 and according to maximum tidal amplitude, the exchanged body of water is estimated to 15.6 x 10^6 m^3 twice a day, not taking into account any wind effect. Thus, even if all the mussels had survived, the filtration rate would have been 0.1 x 10^6 m^3 and still not enough to filter the water body exchanged by the tide alone. However, other studies have demonstrated a depletion of phytoplankton around blue mussel long-line systems with up to 80% and up to 1.5 m increase in secchi depth (Petersen et al., 2013). It has been estimated that an increase in secchi depth of 12 cm in Skive Fjord (another Danish fjord resembling Nørrefjord in area and mean depth) could be achieved by 18.8 ha of mussels on suspended long-lines (Petersen et al., 2013). That is 13 times larger than the mussel farm used in Nørrefjord. As bottom living mussels experience depletion of food items due to less exchange of water near the bottom compared to suspended mussels (Petersen et al., 2013), Nørrefjord would need even more mussels and thus a larger

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proportion of the fjord bottom, to see the same change as for the suspended mussels studied in Skive Fjord. However, since an increase in secchi depth would in time increase macro algae and eelgrass depth range (Nielsen et al., 2002) the establishment of mussel beds in these areas may be one way to improve local environmental conditions in semi-enclosed fjords.

Development of a new method

The method with mussel production on hemp sacks on the long-lines followed by direct establishment of the mussel beds was the most effective method both in time and labour compared to the traditional long-line system. The hemp sack method can be applied to many other geographic locations. The heavy involvement of local volunteers can be recommended in future ecological improvement- and restoration projects. Crowdsourcing allowed us to conduct the experiment cost-effectively. As long as the projects evolve in collaboration with researchers and local managers, this development of bottom-up initiated projects may be beneficial to society and increase environmental awareness of the local community (Grese et al., 2000). The increased awareness was reflected in the wide interest in the project from local and regional newspapers, radio stations as well as the attendance at stakeholder meetings (Assens Municipality, Developing Fyn Municipal Ltd (Lag Fyn), The Danish Nature Agency of Odense, the Danish Ministry of the Environment and local interest organizations such as the sailing club and the Danish Organization for Amateur Fishermen). It was even suggested by the stakeholders that this collaboration with the local community should be best practice in all future habitat restoration projects.

Conclusions

In conclusion, this study showed that it was possible to improve fish habitats on a local scale. The blue mussel structures established in Nørrefjord improved shelter and food especially for small mesopredator fish. The quantity of blue mussels established in Nørrefjord was insufficient to observe any effect on secchi depth and eelgrass cover and range. A new method was introduced, as we succeeded to establish mussel beds in a cost-effective way using crowdsourcing (local volunteer fishermen). The hemp sacks attached to the long-lines proved to be the most effective method of the two methods tested.

This study shows that with the help of volunteers, this habitat improvement strategy is a potential useful management tool to increase fish abundance and improve fish communities in Danish fjords in the future. Therefore, we recommend more local involvement in future improvement and restoration projects.

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HABITAT USE BY BIRDS IN AIRPORTS: A CASE STUDY AND ITS IMPLICATIONS FOR BIRD MANAGEMENT IN SOUTH AMERICAN AIRPORTS

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Abstract. The concentration of birds around airports has significantly increased the occurrence of aeronautical accidents worldwide. One of the main techniques to modify the avian habitat in airports, and therefore exert controls over bird population density, is to regulate the height of surrounding grasslands. The objective of this work was to record the seasonal use of habitat by birds in an airfield in Argentina, and to evaluate the effect of grassland height in the abundance of different components of the avian community. A total of 44 sampling events were carried out between October 2005 and July 2011, comprising all seasons. Short grassland areas (≤ 30 cm), registered 53 % of the total bird abundance, and were consistently for all seasons the most utilized habitat by those species that registered the highest abundances (e.g. *Milvago chimango* and *Vanellus chilensis*) and that pose a highest risk to aerial operations. In general, the species *Patagioenas picazuro*, *Zenaida auriculata*, *Myiopsitta monachus* and *Sturnus vulgaris* also made preferential use of short grassland areas rather than tall grasslands. Maintaining grassland areas at heights > 30 cm, might be a successful habitat manipulation strategy and represent a tool to control birds in South American airports. In turn, this would be a relatively simple and economic management strategy.

Keywords: *bird strikes, wildlife hazard, human-wildlife conflicts, grass management, habitat management*

Introduction

Since the beginnings of aviation the occurrence of birds in and around airports and airfields has contributed to an increase in aeronautic accidents worldwide (Sodhi, 2002). Airports are attractive areas for some bird species because they are built around open spaces

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with grasslands and tree groves of various heights, and are adjacent to areas of high urban density, providing resources such as food, shelter, and resting and nesting grounds (Froneman, 2000; Sodhi, 2002; Transport Canada, 2002; Cleary and Dolbeer, 2005).

In military aviation about 54 % of aircraft collisions with birds take place on the airstrip or its surroundings, and this figure goes up to 95 % in civil aviation (Burger, 1983; Smith, 1986; Neubauer, 1990; Cleary et al., 1999; ICAO, 2009; ATSB, 2010; Galvão Novaes and Del Valle Alvarez, 2010). In order to reduce the occurrence of accidents, the International Civil Aviation Organization has exhorted all its affiliate countries to evaluate bird-related risks in airports, and to develop appropriate management and control programs (ICAO, 2001).

Among the variety of bird control methods in airports (Hygnstrom et al., 1994; Transport Canada, 2002; Cleary and Dolbeer, 2005), avian habitat management seems to be the most effective in the long run. However, habitat management does not necessarily guarantee complete control over birds, and it is generally required that this strategy is combined with active controls (Barras and Seamans, 2002; Sodhi, 2002; Transport Canada, 2002; Cleary and Dolbeer, 2005). Adequately managing grassland height by keeping it below 30 cm, constitutes one of the main habitat management techniques in airports, reducing bird abundances and therefore the chance of accidents (Mead and Carter, 1973; Brough and Bridgman, 1980; Dekker and van der Zee, 1996; Deacon and Rochard, 2000; Civil Aviation Authority, 2002; ATSB 2010).

However, the management of grassland height has somehow yielded opposing results; while it was effective in the UK (Deacon and Rochard, 2000), in the USA it has produced contradictory or ambiguous results, probably due to the different bird and grass species at play (Seamans et al., 1999; Barras and Seamans, 2002; Sodhi, 2002; Washburn and Seamans, 2004; Seamans et al., 2007; Carragher et al., 2012). Studies on habitat use by birds in South American airports are fairly recent and few, and the grassland management recommendations that arose from them are very broad, short-term, and not do not incorporate seasonality and inter-annual variations (e.g. Guedes et al., 2010; Marateo et al., 2011).

The relationships between avifauna and vegetation in South American airports, might differ from the patterns described for other parts of the world, given that the ecological communities are not the same. Therefore, it is of utmost importance to carry out mid- and long-term studies, in order to provide effective management strategies for this region. The aim of this study was to record seasonal habitat use patterns by bird species that pose a higher risk for aircraft, in an airfield in Argentina, and to evaluate the effect of grassland height on their abundances.

Materials ant methods

Study area

This study was carried out at Campo de Mayo airfield (CMA) (surface area = 130 ha), located in NE Buenos Aires Province, Argentina (34º 32’ 04.80” S – 58º 40’ 17.63” W) (*Fig. 1*). The area is a mosaic of open and forested areas, and densely-populated urban centers. The airfield has a 1,800 m-long paved runway, and is surrounded by grassland patches and tree groves, taxiways, heliports, areas of pavement, hangars and other buildings.

Since 2005, an uninterrupted bird control program has been implemented at CMA, mainly aimed at controlling Chimango Caracaras (*Milvago chimango*). As a result of the first field studies, two groups of this species were identified in relation to their use of space, residence time and degree of aggregation (Marateo et al., 2012). Accordingly, the two groups were: visiting Chimango Caracaras (with maximum abundances > 6,000 individuals), which are controlled by pyrotechnics (Marateo et al., 2012); and resident Chimango Caracaras, which are controlled by a combination of measures including pyrotechnics, elimination of shrubs and nest removal.

A total of four accidents involving birds have been reported for the airfield since 2005, namely: two involving Chimango Caracaras, one involving the Southern Lapwing (*Vanellus chilensis*), and one involving the Campo Flicker (*Colaptes campestris*). However, there are a number of unreported incidents with airplanes and helicopters, which involved mainly the first two species mentioned (CMA authorities, pers.com.).

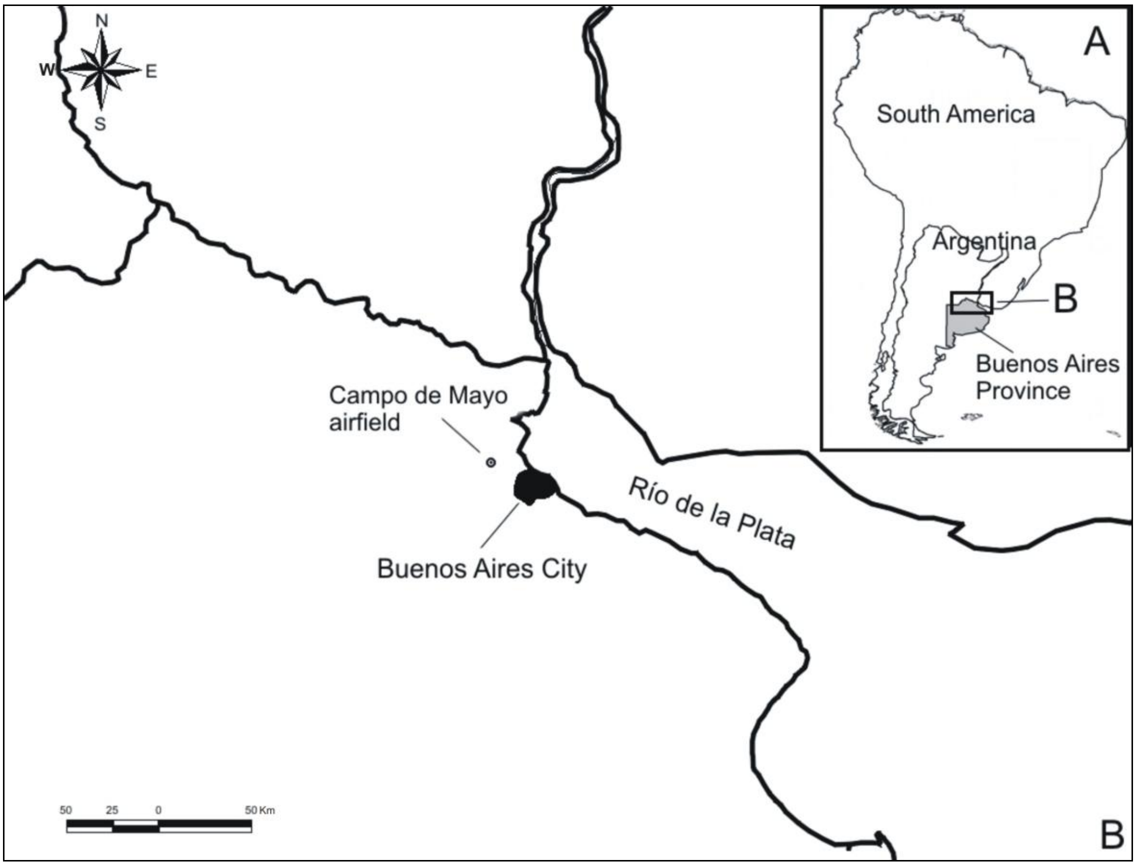

Figure 1. *Study area. Campo de Mayo airfield located in NE Buenos Aires Province, Argentina (34º 32' 04.80" S – 58º 40' 17.63" W).*

Sampling

A total of 44 bird sampling events were carried out between October 2005 and July 2011 at CMA, comprising all seasons. Three observers were placed at different sectors of the airfield in order to sample these simultaneously by covering the total area with trajectories lasting ~30 min. Observers rotated between sectors in order to even out possible differences in data recording between them. Sampling took place at different times throughout the day in order to minimize possible differences in the daily activity of birds.

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In each sampling event the total number of (observed and heard) birds were counted at different sectors of the airfield, recording the species, number of individuals and their use of habitat. Avian habitats were classified as:

1. Short grassland: areas with a dominance of herbaceous species and grasses < 30 cm in height,
2. Tall grassland: areas with a dominance of herbaceous species and grasses > 30 cm in height,
3. Trees: wooded patches of varying extent or isolated trees,
4. Runway,
5. Taxiways,
6. Buildings.

Finally, the category “in flight” was considered in those cases in which individuals only used the aerial space as a transit zone and did not make use of any other habitats during sampling.

The extent of the tall- and short grassland areas varied throughout the study, hence two grassland heights were considered (≤ 30 cm, and ≥ 30 cm), independently of their location within the airfield and the surface area covered by either one. Landscape management of grassland areas was not performed consistently at CMA, and therefore we assumed no significant effect of the cover area and location of the tall- and short grasslands.

Data analysis

Differences in species abundance between habitats were analyzed for each season by means of Friedman’s non-parametric two-way ANOVA, or repeated measure ANOVA. *A posteriori* multiple comparisons were based on the mean values for each treatment rank, and the variances of the ranks (Conover, 1999).

Results

Averaged total abundance was 236 birds (SD = ± 117; max = 645; min = 48; N = 44). The most abundant species were the Chimango Caracara (36 % total abundance); the Southern Lapwing (34 % total abundance); the Picazuro Pigeon (*Patagioenas picazuro*) and the Eared Dove (*Zenaida auriculata*; 11 % total abundance); the Monk Parakeet (*Myiopsitta monachus*; 9 % total abundance); and the Common Starling (*Sturnus vulgaris*; 6 % total abundance). Short grassland areas registered 53 % of total bird abundance, followed by the runway with 14 %, the aerial space with 13 %, tree groves with 8 %, tall grassland areas with 5 %, and taxiways and buildings with 3 % in each.

Significant differences were recorded between habitats in the abundances of Chimango Caracaras, Southern Lapwing and pigeons and doves, for all seasons. Chimango Caracaras and Southern Lapwing were consistently more abundant in short grassland areas. Pigeons and doves made a more extensive use of the aerial space of CMA, and were also more abundant in short- than in long grassland areas, during fall, winter and spring. Monk Parakeet showed significant differences in their abundances between habitats only in winter, and were generally more abundant in short grassland areas (*Table 1, Fig. 2*).

There were no significant differences between habitats in the abundance of the Common Starling, although they were more abundant in the short grassland in winter and spring (*Table 1, Fig. 2*).

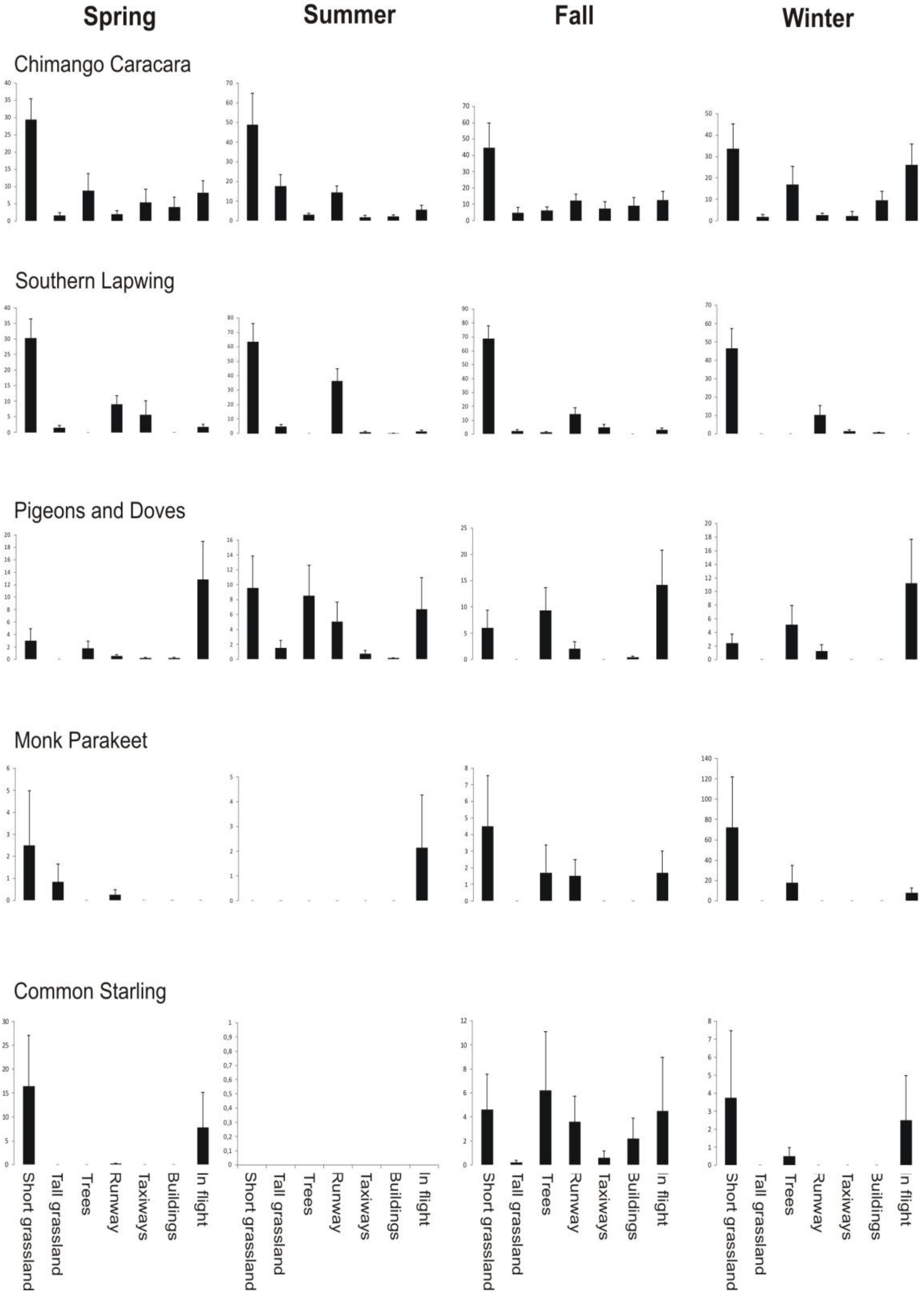

Figure 2. *Bird species abundance by habitat and season for CMA for six consecutive year.*

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Even when the use of trees was not important on average, higher abundances in this habitat were recorded for pigeons and doves in most seasons, for the Monk Parakeet in fall and winter, and for the Common Starling in the fall. Relatively important abundances of the Southern Lapwing were recorded in the runway, mainly during summer (*Fig. 2*).

Table 1. *Results from Friedman's ANOVAs, and a posteriori tests on the number of individuals for the most abundant bird species at Campo de Mayo airfield (CMA), listed by season for six consecutive years. Only a posteriori tests are shown for comparisons between tall- and short grasslands.*
χ_r^2 *= Friedman's statistic.*
SD = standard error
Max = maximum abundance
** statistically-significant differences (a posteriori tests) between bird abundances in the short- vs. tall grassland (p ≤ 0.05).*

Species	Season	N	X_r^2	p	Short grassland (≤30cm)			Tall grassland (≥30cm)		
					Mean (SD)	Max.	Mean rank	Mean (SD)	Max.	Mean rank
Chimango Caracara	Spring	12	5,84	0,0001	29,3 (6,21)	70	6,33	1,6 (0,88)	9	3,21*
	Summer	14	7,67	<0,0001	48,9 (15,95)	165	5,89	17,6 (6,14)	69	4,54*
	Fall	10	2,83	0,0181	44,6 (15,23)	167	5,95	5 (3,42)	35	3,2*
	Winter	8	15,18	<0,0001	33,8 (11,54)	103	6,5	1,8 (1,28)	10	2,06*
Southern Lapwing	Spring	12	20,38	<0,0001	30,3 (6,22)	73	6,83	1,6 (0,73	8	3,67*
	Summer	14	47,73	<0,0001	63,6 (12,58)	152	6,57	4,9 (1,47)	14	4,36*
	Fall	10	16,49	<0,0001	69,1 (9,15)	118	7	1,9 (1,37)	13	3,1*
	Winter	8	21,34	<0,0001	46,5 (10,82)	111	7	0	0	2,94*
Pigeons and Doves	Spring	12	3,56	0,0041	3 (1,92)	23	4,54	0	0	3,17*
	Summer	14	3,84	0,0021	9,6 (4,3)	61	5,25	1,5 (1,08)	15	3,32*
	Fall	10	3,4	0,0064	6 (3,46)	35	4,5	0	0	3*
	Winter	8	5,95	0,0001	2,4 (1,39)	10	4,31	0	0	3,13
Monk Parakeet	Spring	12	1,3	NS	2,5 (1,69)	15	-	0,8 (0,83)	10	-
	Summer	14	1	NS	0	0	-	0	0	-
	Fall	10	1,39	NS	4,5 (3,07)	30	-	0	0	-
	Winter	8	7,87	<0,0001	72,4 (49,74)	397	6,06	0	0	3,38*
Common Starling	Spring	12	2,04	NS	16,5 (10,68)	120	-	0	0	-
	Summer	14	-	-	0	0	-	0	0	-
	Fall	10	0,98	NS	4,6 (2,99)	30	-	0,2 (0,2)	2	-
	Winter	8	0,73	NS	3,8 (3,75)	30	-	0	0	-

Discussion

Short grassland areas were the most frequently-used habitat by the most abundant species (i.e. Chimango Caracaras and Southern Lapwing), regardless of the season considered, and also by Monk Parakeet in winter. Pigeons and doves used short grasslands more frequently than tall grasslands, and were also the most abundant group in the aerial space. In turn, these species represented a higher risk for aircraft, not only due to their abundances, but also due to their size and degree of aggregation.

Even if it did not present significant differences in its abundance between habitats, the Common Starling was mainly recorded in short grassland areas.

The Southern Lapwing nests on the ground and feeds on invertebrates, while the Common Starling feeds on soil invertebrates and seeds (Feare, 1984; Piersma and Wiersma, 1996). Therefore, short grasslands (< 30 cm in height) constitute a very favorable avian habitat for these species, as it has been demonstrated for starlings (Whitehead et al., 1995). The Picazuro Pigeon and the Eared Dove feed on seeds, mostly on the ground (Baptista et al., 1997), while the Monk Parakeet is a generalist that feeds on the grasslands when there is an abundant supply of seeds and grains (Collar, 1997).

The Chimango Caracara is a generalist species that feeds largely on soil insects (Willis, 1994; Biondi et al., 2005), and at CMA in particular this was observed

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coinciding with population outbreaks of the beetle *Abderus anomalus* (pers.obs.). Chimango Caracara have also been pointed as important predators on Southern Lapwing's nests (White et al., 1994), which happened to be the second-most abundant bird species at the study site, and reproduce in short grassland areas.

Additionally, a visiting group of Chimango Caracaras makes overnight use of short- and tall grasslands at CMA, indistinctly. Control over these has been successful by means of repulsion and banishment techniques (i.e. pyrotechnics; Marateo et al., 2012), given that grassland height has no effect over them.

Tall grasslands of a uniform density can prevent the displacement and social contact among birds, posing an obstacle to anti-predatory vigilance behavior and the search for food. This has been noted for starlings and the Northern Lapwing (*Vanellus vanellus*) in Canada and Europe (e.g. Solman, 1966; Brough and Bridgman, 1980; Deacon and Rochard, 2000; Devereux et al., 2004; Butler and Gillings, 2004). Maintaining tall grasslands has yielded successful results in UK airports, especially for the control of the Northern Lapwing (Brough and Bridgman, 1980; Deacon and Rochard, 2000), a related and morphologically- and ecologically-similar species to the Southern Lapwing of South America. This strategy has also been effective in the control of starlings and pigeons (Brough and Bridgman, 1980). On the other hand, maintaining grassland height > 30 cm would also contribute to the numerical reduction of nests of Southern Lapwings and other species, which might in turn represent important food sources for opportunistic rapace birds such as the Chimango Caracara.

Given their wide distributional range, environmental plasticity and ability to expand their geonemia, the bird species considered here, might pose similar risks in other South American airports, as it has been mentioned for the Southern Lapwing in NE and S Brazil, and Uruguay (Bastos, 2000; Galvão Novaes and Del Valle Alvarez, 2010; Guedes et al., 2010; Crocce, 2011). Therefore, it seems reasonable to assume that maintaining grasslands at > 30 cm heights, might be a successful habitat management technique in the control of avifauna at CMA, and it might also prove effective for other South American airports.

One of the most debated aspects of maintaining grassland height > 20-25 cm, is the potential increase in rodents and insects, which might in turn attract rapaces and other predatory bird species (Barras and Seamans, 2002; Cleary and Dolbeer, 2005). Therefore, a sound management strategy should also incorporate the monitoring of rodent and rapace bird abundances and prevent further population increments. On the other hand, maintaining tall grasslands might prove difficult and costly in arid zones (Cleary and Dolbeer, 2005). For South America specifically, this might not be a big constraint, given that large warm and temperate areas have humid to very humid climate, for which this would be a relatively simple and economic management strategy.

This study represents a starting point for a series of future analyses that should be carried out, considering the management of grassland height and also incorporating its structure, composition and cover extent as potentially important variables. We believe that the incorporation of these variables will shed light into the effectiveness of this habitat management strategy for the control of birds in South American airports.

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MULTIVARIATE EVALUATION OF VARIABILITY OF BROMUS GENOTYPES FROM NORTH-EASTERN POLAND

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Abstract. In the years 2008–2010 phenotypic observations of the populations of *Bromus secalinus* L. at 14 sites in North-Eastern Poland were carried out. The populations were characterised by measurements of the following parameters: tuft height, number of culms in a tuft, height of generative shoots, length of panicle, number of spikelets in panicle, number of kernels in spikelet, number of kernels in panicle and seed production per plants. Cluster analysis by the Ward method distinguished 4 groups of genotypes of multivariate similar populations of rye brome. The greatest distances between the values of characters analysed were found between groups 1 and 4. On the basis of spatial autocorrelation analysis a significant autocorrelations for height of generative shoots, length of panicle and number of spikelets in panicle was found, which means that these traits were similar for spatially close populations.

Keywords: *autocorrelations, Bromus, genotypes, multivariate, spatial, variability*

Introduction

Over recent years a decrease in genetic diversity in segetal communities has been observed. This phenomenon leads to the disappearance of species characteristic of a given field agrocenosis and spreading of nitrophilous taxa, including those from Poaceae family (Peterson, 2006). The changes are interpreted as consequences of changes in the conditions of plat vegetation related to the new technologies of field production including the use of herbicides. As a result the appearance of the impoverished communities is observed, in which one or two species dominate (Pysek et al., 2005; Baessler and Klotz, 2006; Pinke and Pál, 2008; Holubec, 2010). In Poland about 100 species of field weeds have the status of threatened with extinction (Warcholińska, 1994). According to Siciński (2001), over the last 50 years not only the number of weed species but also the bank of their diaspores in soil have decreased by 20–40%. In view of the above, measures should be taken to restrict this phenomena by setting up agro-reserves as it has been done in West European countries. It seems a good idea to leave small enclaves in which segetal species should have their ecological niche.

The existing populations of threatened segetal species should be monitored and favourable conditions should be made for their development. Prior to taking up these

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measures, the genetic diversity of the threatened species should be evaluated on the sites of their occurrence to establish the degree of inbred and assess the narrowing of the genetic pool.

It is known that genetically homogeneous populations are to a greater degree threatened with extinction as individuals of the same genetic composition have the same resistance to adverse environmental factors such as diseases or pests, which can lead to their fast elimination. Within the large project aimed at protecting biodiversity in Poland, we wanted to emphasise the situation of segetal community species, especially those threatened with extinction.

The aim of the study undertaken was to evaluate the phenotypic variation of rye brome populations in North-Eastern Poland. In particular we wanted to establish the degree of genetic diversity of populations of this species and to check if similar genotypes occur in spatially close localities.

Material and methods

Area of study

In the years 2008–2010 phenotypic analysis was made of Bromus secalinus L. populations occurring at 14 localities (Bryzgiel, Leszczewo, Magdalenowo, Horodnianka, Rzepiska, Gołaszyn, Rudnik, Popławy, Niwiski, Bojmie, Jagodne, Groszki Stare, Barcząca and Tyborów) in North-Eastern Poland. The area studied covered about 250,000 km^2 (*Fig. 1*).

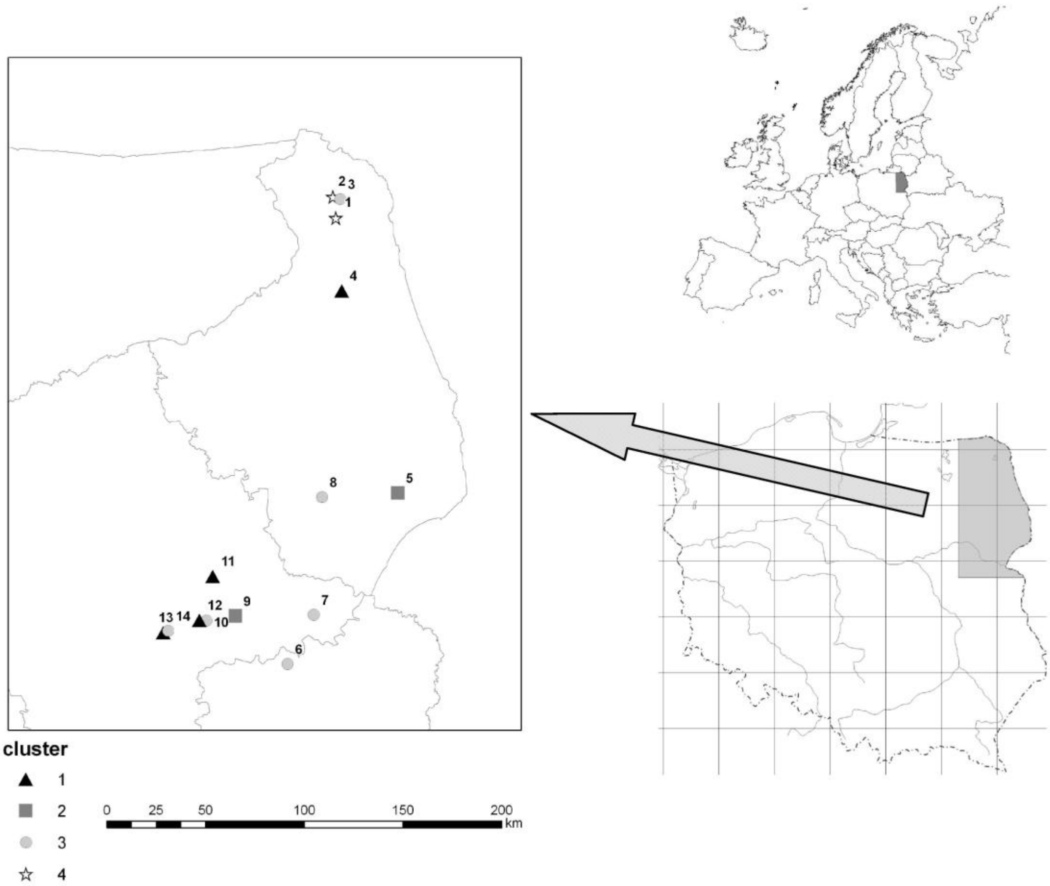

Figure 1. *Location of study area and distinguished groups of genotypes in the sites.*

In the phase of wax maturity 30 plants of B. secalinus were collected from each site and for them the following traits were measured: tuft height, number of culms in a tuft, height of generative shoots, length of panicle, number of spikelets in panicle, number of kernels in spikelet, number of kernels in panicle and seed production per plants. Results are presented as mean values and the range of variation, the coefficient of variation (CV) was calculated.

Statistical analysis

The collected data were used for univariate and multivariate statistical analysis. For each location basic statistical parameters were calculated i.e. means, ranges of values and coefficients of variations (standard deviations/mean).

For further multivariate statistical analyses means for each location were used (Johnson and Wichern, 2002). Cluster analysis was used for distinguishing similar genotypes based on eight traits. Square Euclidean distance was used as a measure of dissimilarity between the genotypes and Ward's method was used for agglomeration of the object into groups. The analyses were performed on standardized variables.

Means for the same traits were used for principal component analysis (PCA) to describe relations between the examined traits and to present multivariate variability of the genotypes.

Spatial statistics were used for estimation of spatial autocorrelation. For each trait Moran's I autocorrelation coefficient was calculated (Moran 1950; Mitchell, 2005).

For the analyses Statistica 8.0 (StatSoft, 2008) statistical package and ArcGIS 9.3 (ESRI) geografic information system software were used. For all analyses significance level was set at 0.05.

Results

Between the examined traits the highest relative variability expressed by coefficient of variation (CV) was observed for seed reproduction (*Table 1*). Coefficient of variability for all locations was equal to 127.8% and was from about 50% (sites: 2, 3, 6 and 7) to more than 200% (site number 5). High variability was observed for number culms in tuft. Coefficient of variation for this trait for all data set was equal to 91.6% and for examined sites was from 32 to 140%. The trait with the lowest variability was tuft height; CV for all data set was equal 17.1% and for examined sites was between 7 and 22%. Quite low relative variability was observed for number of kernels in spikelet and height of generative shoots. CV for number of kernels in spikelet was equal to 23.2% for all data set and 22.4% for height of generative shoots. For both traits range of CV for examined sites was between 10 and 30%.

On the basis of Ward's method of cluster analysis performed using mean values of eight traits 4 groups of genotypes were distinguished. The four groups are presented in the dendrogram (*Fig. 2*). To the first group belong four genotypes from following sites: 4, 11, 12 and 13. The group is characterized by the lowest means of all examined traits i.e. traits described height of plants and shoots as well traits connected with yielding of plants had much lower means in comparison with other groups of genotypes (*Table 2*).

***Table 1**. Mean values, variability range and coefficient variability of studied features in Bromus*

Location	Tuft height			Number culm in tuft			Height of generative shoots			Seed reproduction		
	Min - max	Mean	CV	Min - max	Mean	CV	Min - max	Mean	CV	Min - max	Mean	CV
1-Bryzgiel	119-153	138 ±10.6	7.7	2-12	4.5 ±2.5	55.1	105-143	124.7±10.3	8.3	230-1450	601.8±341.7	56.8
2-Leszczewo	126-153	141±9.6	6.8	2-12	5.1±2.9	56.3	116.2-143	127.3±9.0	7.1	230-1450	739.9±425.4	57.5
3-Magdalenowo	119-149	134±9.2	6.9	1-6	3.7±1.2	32.2	99-142	118.7±11.4	9.6	154-1005	441.8±228.1	51.6
4-Horodnianka	70-142	110±19.7	17.9	1-11	2.5±2.6	106.3	70-128.5	104.7±17.5	16.7	20-1702.2	280.3±401.2	143.1
5-Rzepiska	97-149	120±14.2	11.9	1-19	3.9±4.3	112	55.3-141	96.2±24.6	25.5	45-3615	463±942.9	203.6
6-Gołaszyn	85-136	115±14.3	12.4	1-4	2.3±1.2	50.8	62.7-136	102.7±22.2	21.7	40-420	220.2±97.9	44.4
7-Rudnik	92-145	122±13.6	11.2	1-7	2.1±1.7	79.5	10.6-135	101.1±29.6	29.3	60-529	201.9±107.3	53.2
8-Popławy	102-146	126±15.1	11.9	1-10	2.9±2.8	95.1	60.7-145	100.6±23.5	23.4	56-898	290.5±237.8	81.8
9-Niwiski	89-155	129±17.1	13.3	1-24	4.4±6.2	139.6	38.7-147	105.4±28.3	26.9	20-3185	511.7±898.0	175.5
10-Bojmie	90-153	127±17.5	13.7	1-9	2.7±1.8	66.5	46.5-155	106±27.3	25.8	51-1568	273.6±286.2	104.6
11-Jagodne	59-120	85±18.3	21.6	1-6	2.9±1.5	52.3	44.2-113	77±19.3	25	15-720	196.7±176.1	89.5
12-Groszki Stare	80-135	109±13.8	12.6	1-4	1.9±1.0	50.5	73-135	99.4±15.0	15.1	30-420	137.3±82.4	60.0
13-Barcząca	60-141	105±17.8	16.9	1-13	3.5±3.4	98.1	60-121.6	97.5±16.0	16.4	12-1928	345.7±448.8	129.8
14-Tyborów	78-142	117±15.4	13.1	1-10	3.2±2.6	80.7	68.3-137	105.7±17.2	16.2	21-1206	354.6±286.1	80.7
total	59-155	120±20.5	17.1	1-24	3.3±3.0	91.6	10.6-155	104.8±23.5	22.4	12-3615	361.4±461.9	127.8

Location	Length of panicle			Number of spikelets in panicle			Number of kernels in panicle			Number of kernels in spikelet		
	Min - max	Mean	CV	Min - max	Mean	CV	Min - max	Mean	CV	Min - max	Mean	CV
1-Bryzgiel	11-22.2	16.1±2.5	15.7	10-39	20.7±6.8	33	74-235	136.7±40.9	29.9	5.3-7.8	6.7±0.7	10.8
2-Leszczewo	14-22.2	17.2±2.2	12.6	12-39	21.3±7.2	33.8	76-235	142.2±40.2	28.3	5.3-7.8	6.8±0.8	12.5
3-Magdalenowo	10-20.2	15.1±3.0	19.5	9-31	19.4±6.5	33.3	57-210	118.9±42.6	35.8	4.2-7.1	6±0.9	15.1
4-Horodnianka	5-18	12.8±3.3	25.9	4-29.7	15.5±7.6	49.2	20-168	81±41.5	51.3	4.0-6.0	5.1±0.6	11.0
5-Rzepiska	7.3-16	11.4±2.6	22.5	4.6-30	13.8±7.3	52.5	2-255	87.6±64.3	73.5	2.8-11.0	6.6±1.5	23.1
6-Gołaszyn	8-25	14.4±4.8	32.9	8.3-37	19.6±7.0	35.8	40.8-240	113.1±56.6	50	4.0-8.0	5.5±1.0	18.9
7-Rudnik	6.3-18	12.5±2.9	23.5	10.6-31	20±6.5	32.5	40-232	121±52.8	43.7	3.6-8.0	5.8±1.2	19.8
8-Popławy	6-17	10.8±3.4	31.3	11-34	18.2±8.0	44	56-374	145.1±91.5	63.1	4.0-11.1	6.2±1.9	31
9-Niwiski	6-18	12.4±3.0	24.1	4-31	14.8±6.9	46.5	15-266	101.4±64.7	63.8	2.7-12.0	6.5±2.0	31.1
10-Bojmie	1.5-22	13±4.8	37.1	3.7-39	15.2±7.7	50.8	9-234	105.2±60.8	57.8	1.7-9.5	6.4±1.9	29.7
11-Jagodne	4.4-15.5	10.1±3.1	31.2	3-26	11.2±6.2	55.4	9-176	68.3±45.9	67.2	2.9-9.0	5.8±1.2	20.7
12-Groszki Stare	8.5-17	12.3±2.3	18.8	7-23	14.1±4.8	34.1	30-142	75.1±32.2	43	4.0-6.6	5.4±0.7	12.2
13-Barcząca	4-27	12.3±3.9	31.4	3-47	14.9±8.8	58.7	12-313	85.9±58.4	68	4.0-7.4	5.5±0.9	15.7
14-Tyborów	6.5-23	14.7±3.7	25.3	2.6-36	19.6±7.7	39	13-309.6	119.3±71.4	59.9	4.2-13.6	6.4±1.9	28.9
total	1.5-27	13.2±3.8	29.0	2.6-47	17±7.6	44.9	2-374	107.2±60.7	56.7	1.7-13.6	6.1±1.4	23.2

The second group contains genotypes from sites number 5 and 9. These genotypes had quite low means of most traits in exception of number culms in tuft, number of kernels in spikelet and seed reproduction. In the third group are genotypes from six sites; these genotypes had quite low number culms in tuft and seed reproduction and quite high means for length of panicle, number of kernels in panicle and number of kernels in spikelet. Group 4 contain genotypes from two sites; the genotypes characterize the highest means for all traits. Especially very high mean was observed for seed reproduction, which was much higher in comparison with other groups; very high mean was observed for number culms in tuft.

Variability of examined population based on principal component analysis (PCA) is presented in *Figure 3*. Principal component analysis (PCA) proved strong relationships between all traits. All traits were negatively correlated with the first principal

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component (*Fig. 4, Table 3*), which explained almost 70% of the total variability of all traits. The second principal component explained much less variability (16.7%), it means that diversity of genotypes presented in plot of the first and the second principal components is mainly along X axis (PC1), in smaller degree along Y axis (PC2).

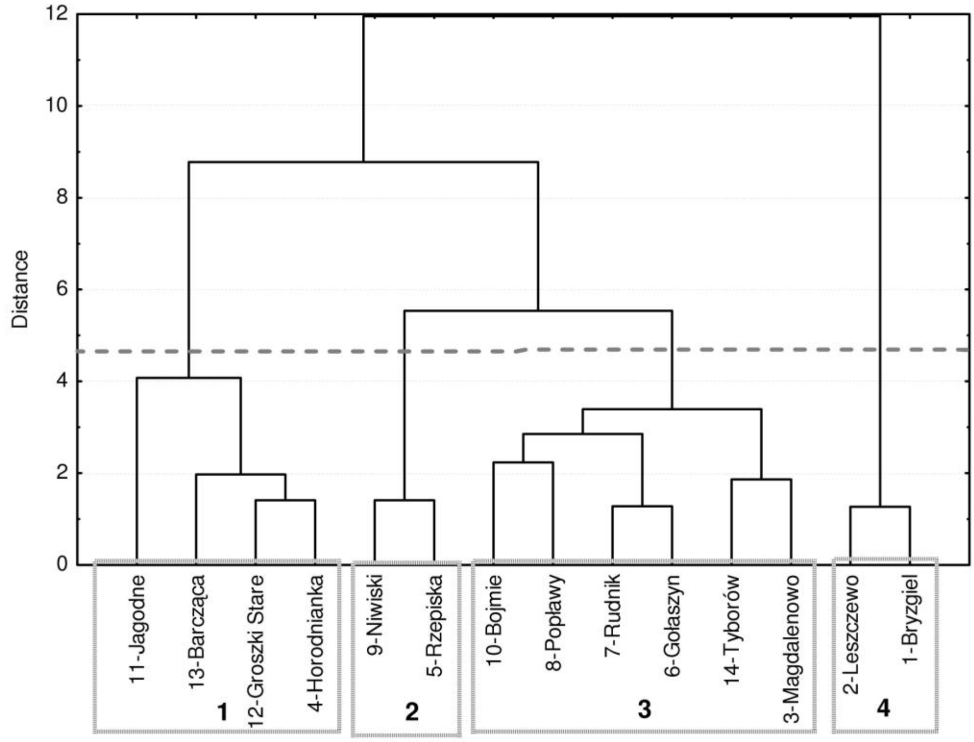

***Figure 2**. Dendrogram on the basis of cluster analysis with distinguished 4 groups of genotypes from the different sites*

***Table 2.** Mean values and standard deviations for distinguished clusters in cluster analysis*

	Group			
Features	**1**	**2**	**3**	**4**
Tuft height	102.3±11.9	124.3±6.3	123.6±6.9	139.1±2.2
Number culms in tuft	2.68±0.66	4.15±0.4	2.84±0.59	4.78±0.45
Height of generative shoots	94.6±12.1	100.8±6.6	105.8±6.7	126±1.9
Length of panicle	11.9±1.2	11.9±0.7	13.4±1.6	16.7±0.8
Number of spikelets in panicle	13.9±1.9	14.3±0.7	18.7±1.8	21±0.5
Number of kernels in spikelet	5.43±0.29	6.54±0.06	6.07±0.35	6.76±0.08
Number of kernels in panicle	77.6±7.6	94.5±9.8	120.4±13.4	139.5±3.9
Seed reproduction	240±92	487±34	297±89	671±98

The biggest difference was observed between groups 1 and 4 which are located in opposite parts of the *Figure 3*. Positive correlation between the second principal component (PC2) and tuft height, height of generative shoots, length of panicle i.e. traits which describe plants habit was observed. Negative correlation was observed between PC2 and number culms in tuft, number of kernels in spikelet, seed reproduction i.e.

traits connected with plant productivity. The genotypes which are located in the top of the figure characterize by higher productivity and lower height, while at the bottom are genotypes which have higher plants but less productive.

On the basis of Moran's I spatial autocorrelation it is possible to detect for which traits spatial dependence exists i.e. similar mean values of the traits in sites located in lower distance.

Significant autocorrelation was observed for height of generative shoots, length of panicle and number of spikelets in panicle (*Table 4*). These traits had similar values for neighbouring sites and the difference between means of these traits was higher for distant locations.

Table 3. *Correlation of coefficients between the traits and the first and the second principal components*

Trait	PC1	PC2
Tuft height	-0.91	0.09
Number culm in tuft	-0.75	-0.62
Height of generative shoots	-0.92	0.20
Length of panicle	-0.83	0.27
number of spikelets in panicle	-0.79	0.56
Number of kernels in spikelet	-0.74	-0.47
Number of kernels in panicle	-0.83	0.32
Seed reproduction	-0.86	-0.45

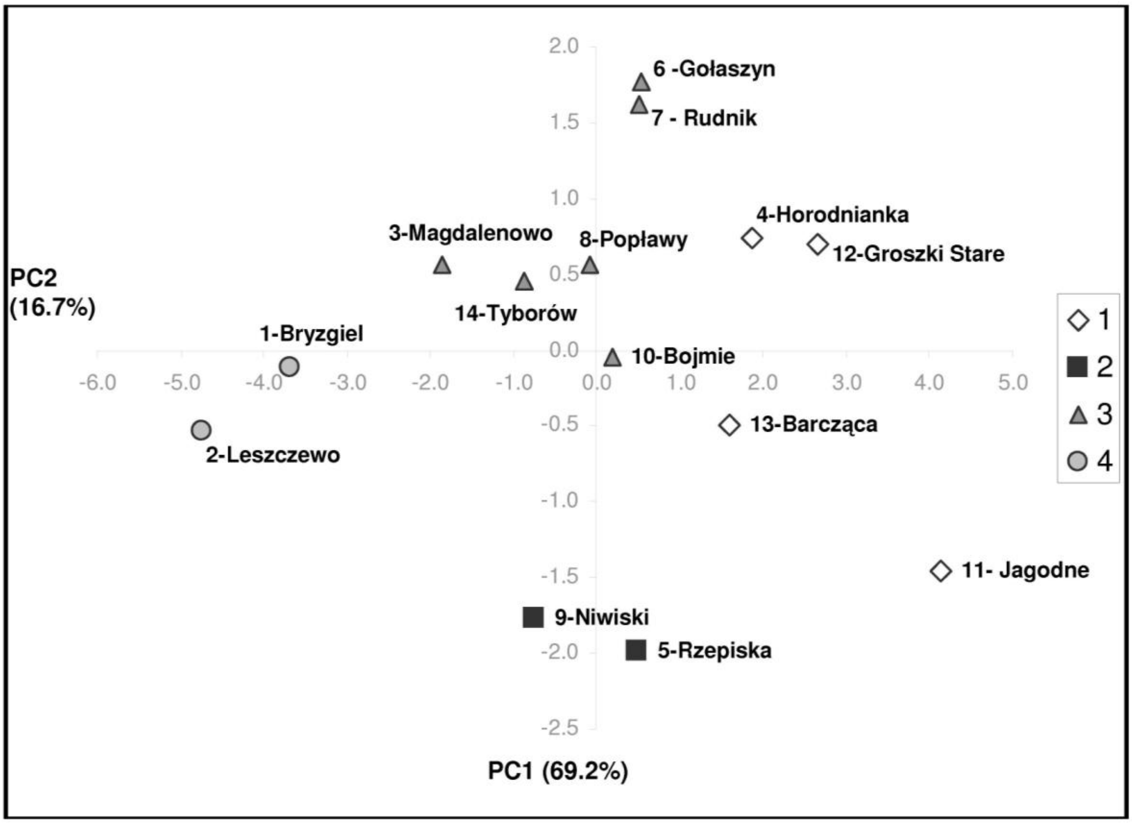

Figure 3*. Plot of PC1 and PC2 for genotypes from examined locations*

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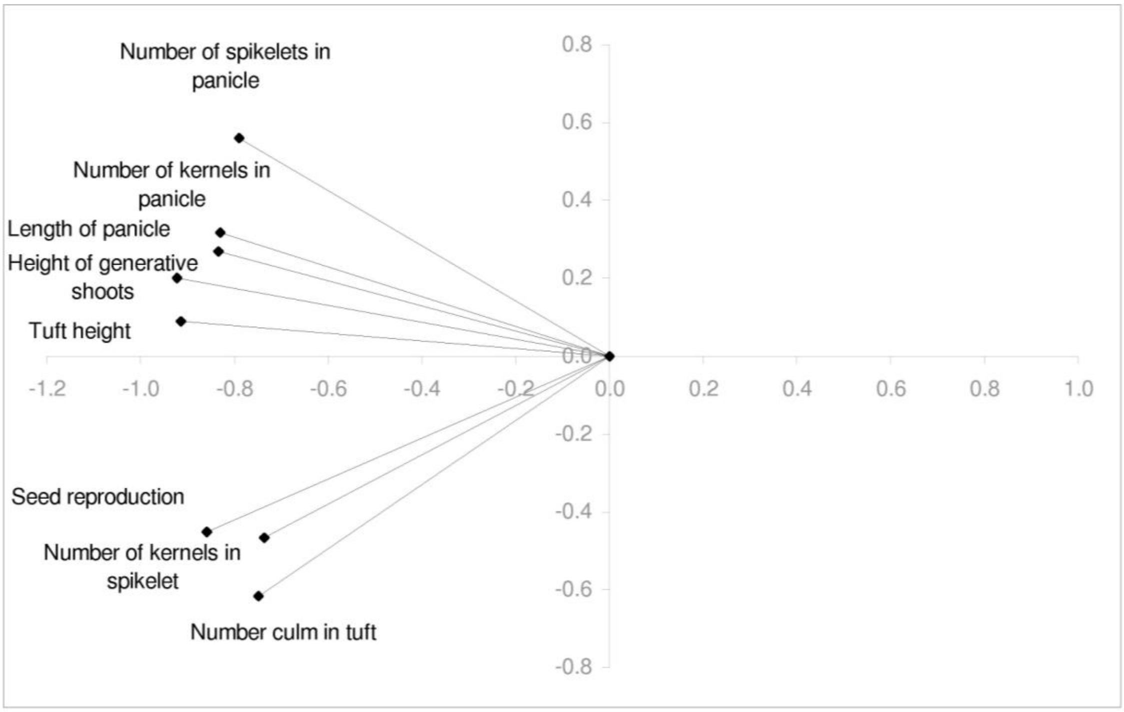

Figure 4. *Investigated features in plot of two principal components (PC1 and PC2)*

Table 4. *Moran's I spatial autocorrelation coefficients*

	Trait	**Moran's I**	**p-value**
X1	Tuft height	0.115	0.212
X2	Number culm in tuft	-0.020	0.727
X3	Height of generative shoots	0.372	0.003
X4	Length of panicle	0.303	0.019
X5	Number of spikelets in panicle	0.279	0.031
X6	Number of kernels in spikelet	-0.231	0.350
X7	Number of kernels in panicle	0.131	0.209
X8	Seed reproduction	0.141	0.170

P<0.05 means significant spatial autocorrelation

Discussion

Bromus secalinus L. is an anthropogenic archeophyte, which means that its development has been stimulated by man by selection from parent populations (Zając et al., 2009). It is an annual tetraploidal species growing in fields with winter crop, especially in rye and wheat in Poland and its presence is related to extensive type of cultivation. It prefers moderately abundant, trophic habitats of pH weakly acidic or neutral (Zarzycki et al., 2002). Both in Poland and in other European countries rye brome has been also met on poor soils related to the community of Scleranthion anuuai (Šilc and Čarni, 2007). It is capable of adapting to non-segetal habitats, but there it occurs in less abundant populations. According to Warcholińska (1994) and Nowak et al. (2003), rye brome is threatened with extinction. However, in recent years an increase in the number of sites of its occurrence and in the number of individuals in populations of this species has been noted in the world (Stone et al., 2001; Moray, 2005) as well as in Poland, especially in eastern parts of Poland (Skrajna et al., 2005; Korniak and Dynowski, 2009; Skrajna et al., 2012).

Diversity of agrophytocenoses depends on many factors such as the climatic and soil conditions, culture of farming, technologies of cultivation and kind of crops (Tichonowa, 2007).

The rye brome populations studied differed significantly in the above traits as evidenced by high coefficients of variation. The lowest coefficients of variation were found to characterise the tuft height and generative shoot height, at three localities of rye brome (1, 2 and 3). Taking into regard these traits, the three populations of rye brome showed a significant genetic homogeneity. So low coefficients of variation were noted in homozygous inbred lines (Kubicka and Dec, 2001) and some variations and local forms of rye (Kubicka et al., 2006). For rye, relatively high coefficients of variation were observed for hybrids, but the CV values were not higher than 50% (Kubicka et al., 2001).

The highest coefficients of variation were obtained for the number of set seeds, i.e. plant fertility, ranging from 44.4% to 203.6%. Similar CV values have been reported for this feature in Polygonum species (Matusiewicz et al., 2010).

Although the analysed populations of rye brome are phenotypically different, only four groups of homogeneous genotypes were distinguished on the basis of the cluster analysis. Some populations of *Bromus secalinus* L. growing at close by localities revealed a considerable genetic differences. The most pronounced differences in all traits considered were noted between groups 1 and 4. On the basis of polymorphism of storage proteins in caryopses of these populations of rye brome only three groups of homogeneous genotypes were distinguished (Skrajna et al., 2012).

Multidimensional statistical analyses permitted characterisation of variation of rye brome population from the localities studied and grouping of objects showing multivariate similarity from the traits analysed. Preservation of phenotypic variation of local forms of plant species is of great importance as it can be used for development of varieties characterised by greater heterozygous state and thus a greater plasticity of harvesting (Kubicka et al., 2006; Sanni et al., 2008). As far as segetal species are concerned, their higher genetic variation permits a better adaptation to a habitat and leads to greater production of seeds per plant, which ensures higher abundance of plants of a given species.

Maintenance of rye brome populations showing high genetic variation needs careful monitoring and control of the historical and present sites of its occurrence.

Conclusions

Phenotypic variation of the population of *Bromus secalinus* L. growing at 14 sites in part of North-Eastern Poland, taking into account the following traits: tuft height, number of culms in a tuft, height of generative shoots, length of panicle, number of spikelets in panicle, number of kernels in spikelet, number of kernels in panicle and seed production per plants.

The lowest coefficients of variation were observed for the height of tuft and height of generative shoots for the populations at three sites (1, 2 and 3), while the highest coefficient of variation of 203.6% was found for the plant fertility at site 5. High coefficients of variation attest to a relatively high variation between the rye brome populations studied.

Cluster analysis according to Ward's method permitted distinction of 4 groups of genotypes of multivariate similar populations. The most pronounced differences in the values describing all traits analysed were found between groups 1 and 4.

On the basis of analysis of spatial autocorrelation with the use of I Moran coefficient, a significant autocorrelation was found for the three following traits: height of generative shoots, length of panicle and number of spikelets in panicle. This result means that the mean values describing the traits were similar for *Bromus secalinus* populations at close lying sites and the differences increased with increasing distance between the sites compared.

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CHARACTERISTICS OF THE SHAPE ASYMMETRY OF LEAF TIP AND BASE IN *POPULUS NIGRA* L. UNDER INDUSTRIAL DUMP CONDITIONS

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Abstract. The scope of this study is to reveale the nature and variability of shape asymmetry of leaf tip and base in *Populus nigra* L. under conditions of two types of industrial waste dumps: coal mine and overburden rock dumps. The coefficient of shape asymmetry of leaf tip ranges from 0.000 to 0.253 in the samplings of *P. nigra* (in ascending order): City Park → coal mine waste dumps → overburden rock dumps. The coefficient of tip shape asymmetry less than 0.050 is characteristic of most leaves of all analyzed samples. The coefficient of shape asymmetry of leaf base varies from 0.000 to 0.152. This value was statistically significantly higher in the leaves sampled in the overburden rock dumps. The asymmetry coefficient of base shape less than 0.015 is characteristic of most leaves of all analyzed samples. The shape asymmetry of leaf tip and base in *P. nigra* is fluctuating. Among three indices analyzed (overall shape pseudosymmetry index, and coefficients of shape asymmetry of leaf tip and base), the shape asymmetry coefficient of leaf base is the most informative indicator. It is most useful for differentiation of leaf blades sampled in less degraded ecosystems and those sampled in stressful ecosystems.

Keywords: *woody plants, morphological variation, fluctuating asymmetry*

Introduction

Plants are ideal organisms for studying genotypic and environmental influences on developmental stability. Because they may have numerous leaves, flowers, and stems, one can study variation in developmental stability in a single individual (Cowart and Graham, 1999).

Plants in man-disturbed environments are constantly affected by adverse environmental factors. Leaves are plant organs highly sensitive to environmental conditions, thus morphological changes are observed in leaves under the influence of various factors (Givnish, 1987; Stakovetskaya et al., 2012).

Many authors suggest that leaf morphology changes within the species are associated with the changed growth conditions (Givnish, 1978; Isakov et al., 1984; Zakharov, 2000; Bukharin et al., 2007; Niinemets et al., 2007; Bessonova, 2009; Migalina et al., 2009; Vogel, 2009; Khuzina, 2010; Zaitseva, 2012 etc.). Environmental factors influencing leaf development may have a significant effect on the features of its final structure and shape (Givnish, 1984; Migalina et al., 2009).

It is extremely difficult to study the influence of plant growth conditions on leaf shape directly and experimentally, but this influence can be traced in indirect way by plant material sampling in the sites with varying conditions (Isakov et al., 1984).

Asymmetry is one of the fundamental concepts of modern biology. There are three types of asymmetry: directional, fluctuating and antisymmetry (Palmer and Strobeck, 1986). Fluctuating asymmetry (FA) implies some minor and random (non-directional)

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deviations from a strict bilateral symmetry of biological objects. It is suggested to use this type of asymmetry to measure the developmental stability of an organism at the macroscopic level (Zakharov, 1987, 2001; Zakharov et al., 2000). The level of fluctuating asymmetry is minimal only under optimal environmental conditions and non-specifically increases in any stress conditions (Gelashvili et al., 2004). Some authors (Palmer and Strobeck, 1986, 2003; Palmer, 1994; Kryazheva et al., 1996; Møller and Swaddle, 1997; Cowart and Graham, 1999; Baranov and Gavrikov, 2008) suggest determining of fluctuating asymmetry as one of morphological methods to evaluate condition and dynamics of biological systems. The index of fluctuating asymmetry in this case is regarded to be an organism stability index. Directional asymmetry differs from fluctuating by the fact that the mean value of a feature for one side is higher than for the other. Antisymmetry takes place in the case when manifestation of the feature asymmetry is the standard, no matter what is the direction of the differences between sides. Statistically, it is reflected in the fact that frequencies deviate from normal distribution towards the negative kurtosis or bimodality (Palmer and Strobeck, 1986, 2003; Zakharov, 1987). The phenomenon of antisymmetry, as well as its direction, is to a great degree determined genotypically (Gelashvili et al., 2004), thereby rendering these forms of asymmetry useless for studies of developmental instability) (Graham et al., 1993). Fluctuating asymmetry allows defining developmental disorders based on the single genotype (Thoday, 1958).

There are different methods and approaches to the studies of fluctuating asymmetry. Some of them are described by Graham et al. (2003).

There is a large amount of studies on fluctuating asymmetry in different plant species, yet this problem is still under-explored. The factors underlying fluctuating asymmetry, especially in plants, remain poorly understood (Wilsey et al., 1998; Wilsey and Saloniemi, 1999). Levels of fluctuating asymmetry may be partly determined by compensational growth, and the efficiency of such compensation may be affected by stress (Møller and van Dongen, 2003).

Applicability of fluctuating asymmetry manifestations to studies of plant developmental stability and biological indication of environment is debatable and further investigations are necessary.

For example, Wilsey and Saloniemi (1999) noted that there was no relationship between leaf FA and shoot growth in *Betula pubescens* Ehrh. ssp. *tortuosa.* The investigation of Mal et al. (2002) suggests that FA in *Lythrum salicaria* L. may be used as an ecological indicator to identify environmental stress caused by certain heavy metal pollutants.

Wuytacka et al. (2011) noted no direct relationship between FA in the leaves of *Salix alba* L. and air pollution that makes this biological indicator useless for air pollution monitoring.

Velickovica and Perisica (2006) obtained the first quantitative data on *Plantago major* L. indicating that plants living in the stressful sites are more symmetrical and leaf FA for plant species with wide ecological distribution such as *P. major* should be considered as an ‘index of habitat quality‘.

Leaf asymmetry appears to be a poor indicator of nutrient stress in young plants of *Acer platanoides* L. and *Betula pendula* Roth (Black-Samuelsson and Andersson, 2003).

There are indications of the relationship between leaf FA and its position within a plant. For example, Cowart and Graham (1999) indicate that leaf size and asymmetry in the common *Ficus carica* L. vary within a plant, depending on height (lower, middle, tip) and position (inside, outside). Leaves from the outside top are the largest, and those from the inside bottom are the smallest. Outer leaves, which may experience greater stress from cold, heat, sunlight, and desiccation, are more asymmetrical than inner leaves. Outer leaves, which may experience greater stress from cold, heat, sunlight, and desiccation, are more asymmetrical than inner leaves.

In the study of Premchamd et al. (1998) on the effect of salinity on leaf FA in *Glycine max* (L.) Merr., FA turned out a poor indicator of plant condition, whereas other measures of developmental instability were more informative.

One of the most general types of stress experienced by plants is water-limitation, which becomes particularly pronounced during periods of drought. Fair and Breshearsa (2005) noted that FA in *Quercus undulata* K. Koch are related to precipitation.

According to Hódarf (2002), the more symmetrical plants of *Quercus ilex* L. respond less to yearly variations in drought stress.

At the same time, it is evident from the study of Møller (1995) that plants of *Ulmus glabra* Huds. infected with mining insect *Rhynchaenus rufus* (Schrank, 1781) were characterized by a greater level of FA.

The study of Cornelissen and Stiling (2005) indicated that leaf miners might use leaf FA as a cue to plant quality. According to these data, asymmetric leaves of *Quercus laevis* Walter and *Quercus geminata* Small contained significantly lower concentrations of tannins and higher concentrations of nitrogen than symmetric leaves for both plant species.

Møller (1999) noted that the plants of *Ulmus glabra* Huds. with a higher level of FA were more susceptible to Dutch elm disease, than plants with more symmetrical leaves.

The parts of leaf are variable to a different degree. For instance, our studies on *Betula pendula* Roth growing in ecosystems transformed to a different degree have shown maximal variation of leaf basal part near the petiole, compared to other leaf parts.

Leaf length increases mainly due to intercalary growth. Leaf tip is the first to stop growing, and the base is the last. Accordingly, leaf tip is shaped first that is indicative of the general order of development of this organ, ‘from top to bottom’ (Nizhegorodtsev, 2010).

The scope of this study is to determine character and variability of shape asymmetry of leaf tip and base in *Populus nigra* L. growing in industrial waste dumps of two types, namely coal mine and overburden rock dumps.

P. nigra in the south-east of Ukraine is found in different types of habitats that enabled us to investigate its leaf morphological variation under different environmental conditions and applicability of this species as a biological indicator of environmental condition.

Materials and methods

Leaves were sampled during 2010 to 2012 summers from short shoots of the crown lower part of mature generative trees. Due to high hybridization with closely related species, we selected specimens with distinct species-specific morphological features. Leaves were collected in coal mine dumps and overburden rock dumps dating back to various times and located in industrial sites of the south-east of Ukraine. Ecotopes of coal mine waste dumps are characterized by acidic substrates; alkaline ones characterize those of overburden rock dumps. The textures, fertility, edaphotope salinity of these dumps are to a high degree similar.

For control we used parameters of *P. nigra* leaves sampled in the Donetsk City Park. There the soils are less saline with pH ~7. On the whole, parkland is less exposed to pollutants than industrial sites.

The leaves were scanned and measured using ImageJ 1.43u program. The sample size was 380 leaves in coal mine waste dumps, 307 ones in overburden rock dumps, 264 ones in the City Park.

To assess variability of the shape asymmetry of leaf tip and base we used the numeric index, calculated using Gendels and Budantsev (1991) technique. The radius-vectors obliqued from the center (according to the mentioned method) at the angle of 20°. The numeric index was calculated for the left and right side of the leaf. Asymmetry coefficient of tip (or base) shape of the leaf blade was calculated using the formula:

$$K = \frac{/ I_L - I_R /}{(I_L + I_R)} \tag{Eq.1}$$

where K is a asymmetry coefficient of tip (or base) shape of the leaf blade, I_L and I_R are numeric values of this index for tip (or base), respectively, for the left and right side of the leaf blade.

To determine the nature of the shape asymmetry of leaf tip and base by the analyzed parameters we performed a significance test for the kurtosis and compared the index values calculated for the left and right sides by Wilcoxon criterion.

To assess informative value of analyzed measures for biological indication, we assessed general symmetry of the leaf shape within the mentioned samples and then conducted discriminant analysis. To estimate general leaf symmetry we used a program for pseudosymmetry level calculations in relation to mirroring for flat bilaterally symmetrical objects (BioPs) – biological pseudosymmetry.

Estimation of pseudosymmetry in the case is based on the expression of integral convolution:

$$\eta = \frac{\sum_{i,j} A_{i,j} \times B_{i,j}}{\sum_{i,j} A_{i,j}^2} \tag{Eq.2}$$

where η is a symmetry level, A is a matrix of original image intensities, B is a matrix of intensities, obtained as a result of reflection of A matrix against a drawn plane. As we deal with a sum of positive values defining intensity pixels, the range of symmetry change level is within the limits from zero (for absolutely non-symmetrical object) to one (for absolutely symmetrical one). In the procedure of calculation of pseudosymmetry index pixel intensities were not taken into account and nonhomogeneity of the object was ignored. The intensity of background pixels is 0, and the intensity of object pixels is 255. In other words, an object in this program is a dark spot against the white background with a symmetry plane drawn across it (Nizhegorodtsev, 2010). Thus, invariance degree of congruency (shape) was taken into account and color intensity of the leaf was ignored. The reflection plane was chosen by several points of main vein.

Morphological diversity of samples was analyzed basing on representation of leaves of different gradations, distributed according to asymmetry indices. Morphological diversity was estimated using Shannon index. This index was calculated according to a formula:

$$H = -\sum_{i=1}^{N} p_i \ln p_i \qquad \text{(Eq.3)}$$

where H is Shannon's index, p_i is the percentage of leaves from the sample, which belong to i-gradation of a parameter, N – total number of gradations of the analyzed parameter.

Significance test for the differences in Shannon's index is conducted using Hatcheson method according to calculation formulas indicated by Magurran (1988).

Statistical data processing was carried out using the STATISTICA 6.0 package.

Results

The coefficient of shape asymmetry of leaf tip ranged from 0.000 to 0.253 for all of the analyzed samples. The coefficient of shape asymmetry of leaf base ranged from 0.000 to 0.152. Leaves with the maximum values of asymmetry coefficient of tip shape and with the maximum values of coefficient of base shape asymmetry are shown in *Figure 1.*

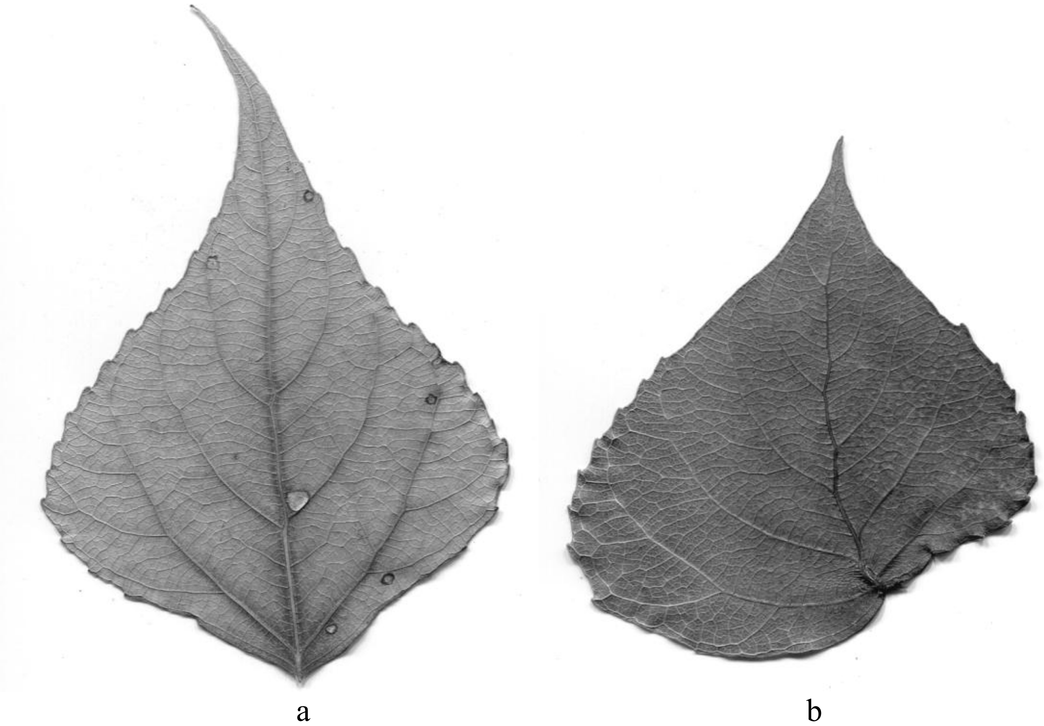
a b

Figure 1. *Leaf blades of Populus nigra L.: a – with the maximum values of coefficient of tip shape asymmetry (0.253); b – with the maximum values of coefficient of base shape asymmetry (0.152)*

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Pseudosymmetry index (invariance level) of general leaf shape for *P. nigra* samples ranged from 0.5485 to 0.9796. Leaves with the minimal and maximal values of coefficient are shown in *Figure 2*.

a b

Figure 2. *The leaves of Populus nigra L. with minimal and maximal values of pseudosymmetry of the general shape: a) minimal (0.5485); b) maximal (0.9796)*

Waste dumps of coal mines

The coefficient of shape asymmetry of *P. nigra* leaf tip varied from 0.000 to 0.154, the coefficient of variation was 91.7 % and the mean value was 0.043 ± 0.0037 (from now on the confidence interval is specified for P = 0.05). In the analyzed sample, leaves with a asymmetry coefficient of tip shape less than 0.050 constituted 67.1 % (including 16.4 % of those with the value of 0.000), those with a coefficient of 0.050 to 0.099 constituted 21.0 %, those with a coefficient of 0.100 to 0.149 constituted 11.2 %, those with a coefficient of 0.150 and more constituted 0.7 % of the sample (*Fig. 3*).

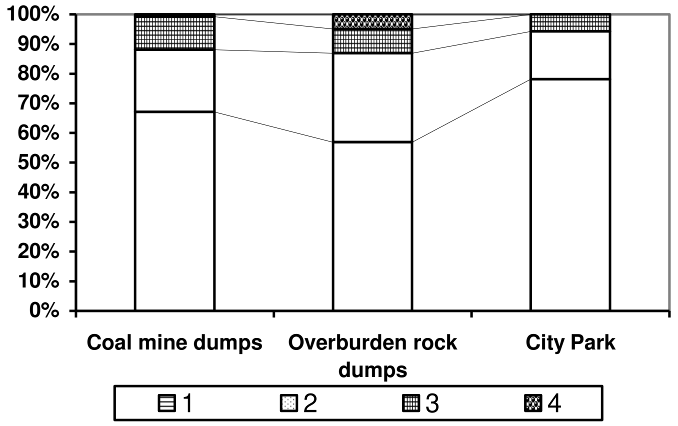

Figure 3. *Distribution of Populus nigra L. leaves sampled in different ecosystems according to the values of the asymmetry coefficient of tip shape: 1) less than 0.050; 2) 0.050–0.099; 3) 0.100–0.149; 4) 0.150 and more*

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The coefficient of shape asymmetry of leaf base varied from 0.000 to 0.064, the coefficient of variation was 81.9 %, and the mean value was 0.015 ± 0.0014. Leaves with a coefficient of base shape asymmetry less than 0.015 constituted 58.6 % (including 21.1 % of those with the value of 0.000), those with a coefficient of 0.015 to 0.029 constituted 31.6 %, those with a coefficient of 0.030 to 0.044 constituted 7.2 %, those with a coefficient of 0.045 to 0.059 constituted 1.9 %, those with a coefficient of 0.060 and more constituted 0.7 % of the sample (*Fig. 4*).

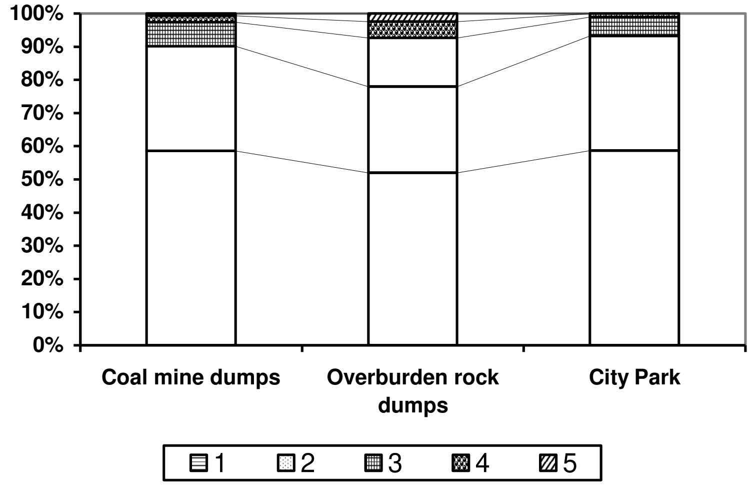

Figure 4. *Distribution of Populus nigra L. leaves sampled in different ecosystems according to the values of shape asymmetry coefficient of leaf blade base: 1) less than 0.015; 2) 0.015–0.029; 3) 0.030–0.044; 4) 0.045–0.059; 5) 0.060 and more*

Pseudosymmetry index of leaf general shape of *P. nigra* ranged from 0.7119 to 0.9796, coefficient of variation is 4.55 %, and mean value is 0.9267 ± 0.00421. Leaves with pseudosymmetry index of general shape 0.7000–0.7999 constituted 2.1 % of overall sample, those with index of 0.8000–0.8999 – 13.2 %, 0.9000 and more – 84.7 % (*Fig. 5*).

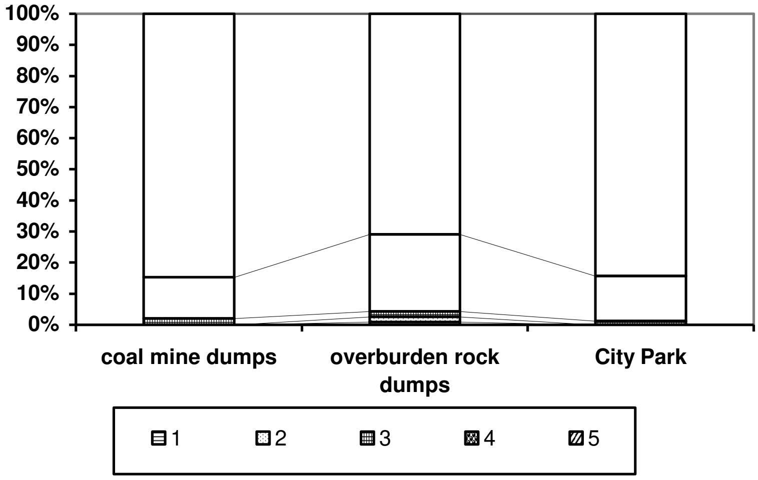

Figure 5. *Distribution of Populus nigra L. leaves sampled in different ecosystems according to the values of asymmetry coefficient of leaf general shape: 1) less than 0.6000; 2) 0.6000–0.6999; 3) 0.7000–0.7999; 4) 0.8000–0.8999; 5) 0.9000 and more*

Overburden dumps

The value of shape asymmetry coefficient of the *P. nigra* leaf blade tip varied from 0.000 to 0.253, the coefficient of variation is 85.1 %, and the average value is 0.055 ± 0.0050. Leaf blades with a coefficient of tip shape asymmetry less than 0.050 constitute 56.9 % of the sample (including 5.7 % of in the leaves with asymmetry coefficient of 0.000), those with a coefficient of 0.050 to 0.099 constitute 30.1 %, those with a coefficient of 0.100 to 0.149 constitute 8.1 %, those with a coefficient more than 0.150 constitute 4.9 % of the sample (*Fig. 3*).

The value of the shape asymmetry coefficient of leaf blade base ranges from 0.000 to 0.152, the coefficient of variation is 107.5 %, and the mean value is 0.020 ± 0.0017. Leaf blades with a coefficient of base shape asymmetry less than 0.015 constitute up to 52.0 % of the analyzed samples (including 13.8 % of those with the coefficient value of 0.000), those with a coefficient of 0.015 to 0.029 constitute 26.0 %, those with a coefficient of 0.030 to 0.044 constitute 14.7 %, those with a coefficient of 0.045 to 0.059 constitute 4.9 %, those with a coefficient of 0.060 and more constitute 2.4 % of the sample (*Fig. 4*).

The value of pseudosymmetry index of leaf general shape varied from 0.5485 to 0.9681, the coefficient of variation is 7.0 %, and the average value is 0.9097 ± 0.00714. Leaves with pseudosymmetry index of general shape less than 0.6000 constitute 0.9 % of the sample, those with a coefficient of 0.6000 to 0.6999 constitute 1.7 %, those with a coefficient of 0.7000 to 0.7999 – 1.7 % of the sample, those with a coefficient of 0.8000 to 0.8999 constitute 24.8 %, and those with a coefficient more than 0.9000 – 70.9 % (*Fig. 5*).

City Park

The value of shape asymmetry coefficient of the *P. nigra* leaf blade tip varies from 0.000 to 0.111, the coefficient of variation is 97.2 %, and the average value is 0.029 ± 0.0025. Leaf blades with the value of a coefficient of leaf blade tip asymmetry less than 0.050 constitute 78.2 % (including 20.7 % of those with the value of 0.000), those with a coefficient of 0.050 to 0.099 constitute 16.1 %, and those with a coefficient value of 0.100 to 0.149 constitute 5.7 % of the sample (*Fig. 3*).

The value of shape asymmetry coefficient of leaf blade base ranges from 0.000 to 0.045, the coefficient of variation is 71.0 %, and the average value is 0.015 ± 0.0013. Leaf blades with the asymmetry coefficient of base shape less than 0.015 constitute 58.7 % (including 18.4 % of those with the value of 0.000), those with a coefficient of 0.015 to 0.029 constitute 34.5 %, those with a coefficient of 0.030 to 0.044 constitute 5.7 %, and those with a coefficient of 0.045 to 0.059 constitute 1.1 % of the sample (*Fig. 4*).

The value of pseudosymmetry index of leaf general shape varied from 0.7792 to 0.9673, the coefficient of variation is 3.71 %, and the average value is 0.9268 ± 0.00417. Leaves with pseudosymmetry index of general shape with a coefficient of 0.7000 to 0.7999 constitute 1.2 % of the sample, those with a coefficient of 0.8000 to 0.8999 constitute 14.5 %, and those with a coefficient more than 0.9000 – 84.3 % (*Fig. 5*).

Shannon's index values that represent morphological diversity of sampled in different ecosystems *P. nigra* leaf blades by asymmetry indexes are showed in *Table 1.*

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***Table 1.** Shannon's index values that represent morphological diversity of sampled in different ecosystems Populus nigra L. leaf blades by asymmetry indexes*

Leaf blade asymmetry index	**Ecosystem**		
	Coal mine dumps	**Overburden rock dumps**	**City Park**
	Shannon's index values, nats		
Shape asymmetry coefficient of leaf blade tip	0.874	1.034	0.651
Shape asymmetry coefficient of leaf blade base	0.945	1.209	0.896
Pseudosymmetry index of leaf general shape	0.488	0.769	0.477

Discussions

Most leaf blades of all analyzed samples are characterized by the tip shape asymmetry coefficient less than 0.050. According to significance estimates of differences of the analyzed parameters using Student t-test ($P < 0.05$), the values of shape asymmetry coefficient of leaf tip were statistically significantly different when comparing pairs of analyzed samples, and they increased in the ecosystems as follows: City Park → waste dumps of coal mines → overburden dumps.

Most leaf blades of all analyzed samples are characterized by the base shape asymmetry coefficient less than 0.015. The values of asymmetry coefficient of base shape are statistically significantly different when comparing the following pairs of samples: coal mines dumps with overburden dumps, overburden dumps with the City Park. This parameter is higher for the leaf blades of the plants growing in overburden dumps.

Most leaf blades of all analyzed samples are characterized by the pseudosymmetry index of leaf general shape more than 0.9000. The values of pseudosymmetry index of leaf general shape are statistically significantly different comparing samples of overburden rock dumps with two other analyzed samples. As the result of comparing of shape pseudosymmetry coefficient of the *P. nigra* leaf general shape values between coal mine dumps and City Park no significant statistical difference was established.

The maximum values of the analyzed asymmetry parameters are registered in leaves sampled in overburden dumps that is obviously related to alkaline reaction of the substrate.

The maximum values of morphological diversity of leaf blades by asymmetry parameters counted using Shannon's index are also registered in leaves sampled in overburden dumps.

There were no cases when empirical values of the kurtosis of numeric index for the tip and base of leaf left and right sides surpassed the critical one, thus antisymmetry is not found (*Table 2*). Using Wilcoxon test we have not found any statistically significant differences in the value of numeric index of tip and base for left and right sides of the leaf, thus the statistically significant directional asymmetry is not found (*Table 3*).

***Table 2**. Empirical values of numeric indexes kurtosis for left and right sides of tip and base of Populus nigra L. leaf*

Analyzed part of leaf blade	**Ecosystem**		
	Coal mine dumps	**Overburden rock dumps**	**City Park**
Tip	-0.524	-0.226	0.259
Base	-0.798	0.723	-0.297

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Table 3. *Wilcoxon test values received by comparing of tip and base numeric indexes of left and right sides of Populus nigra L. leaf blade*

Analyzed part of leaf blade	**Ecosystem**		
	Coal mine dumps	**Overburden rock dumps**	**City Park**
Tip	14990.5	12075.0	10740.5
Base	12904.5	12306.0	10050.0

On this basis it is possible to make a conclusion about fluctuating character of shape asymmetry of *P. nigra* leaf blade tip and base.

Previously reported gives the possibility of using *P. nigra* shape asymmetry coefficient of leaf blade tip and shape asymmetry coefficient of leaf blade base to determine resistance level of this species, compliance of plantations to various technogenic loads, and makes them perspective for indicating of environment state in general.

Conducted discriminant analysis showed following results. From the analysis of discriminant functions coefficients it should be noted that for the first discriminant function shape asymmetry coefficient of leaf blade base is most significant. The impact of other two variables is considerably lower (*Table 4*). According to the values of mean canonical variables the first discriminant function separates City Park sample from two other samples (*Table 5*).

The second discriminant function is marked mostly by shape asymmetry coefficient of leaf blade tip, impact of pseudosymmetry index of leaf general shape is lower, but at the same time it is also valuable comparing with the smallest impact of shape asymmetry coefficient of leaf blade base (*Table 4*). This discriminant function separates mostly sample of coal mine dumps and also other samples, but less qualitative (*Table 5*).

Table 4. *Standardized coefficients for canonical variables, received as a result of discriminant analysis*

Variables (asymmetry indexes of *Populus nigra* L. leaf blade)	**Root 1**	**Root 2**
Pseudosymmetry index of leaf general shape	-0.04903	-0.454729
Shape asymmetry coefficient of leaf blade tip	-0.02986	0.792767
Shape asymmetry coefficient of leaf blade base	-1.00363	0.078718

Table 5. *Means of canonical variables, received as a result of discriminant analysis*

Ecosystem	**Root 1**	**Root 2**
Coal mine dumps	1.33742	-0.175946
Overburden rock dumps	0.20904	0.308783
City Park	-2.63625	-0.122323

This interpretation is confirmed by a scattering diagram (*Fig. 6*).

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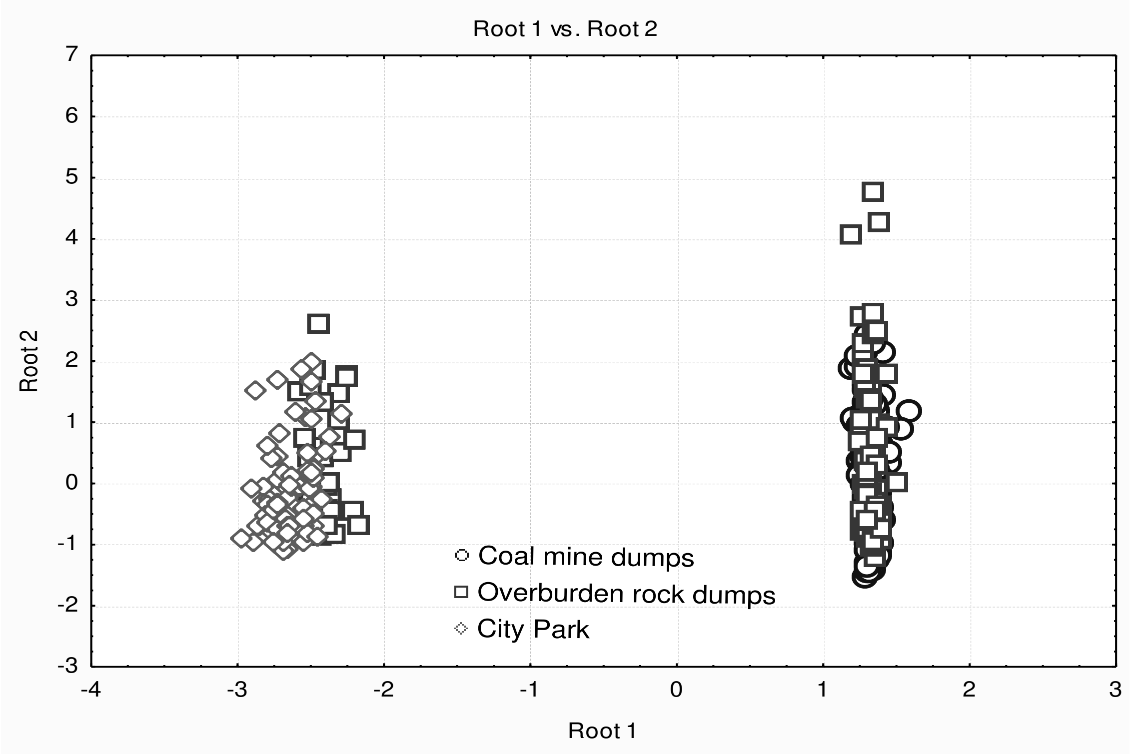

Figure 6. *Populus nigra* L. leaf blades from different ecosystems allocation in canonical space

So, from three analyzed indices of *P. nigra* leaf blade asymmetry (pseudosymmetry index of leaf general shape, shape asymmetry coefficient of leaf blade tip, and shape asymmetry coefficient of leaf blade base) the most informative indicator of environment state is shape asymmetry coefficient of leaf blade base. This parameter separates in the best way samples of leaf blades from less transformed ecosystems and ecosystems that are under significant anthropogenic pressure.

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POLLEN MORPHOLOGY OF THE GENUS ALNUS MILL. IN HYRCANIAN FORESTS, NORTH OF IRAN

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Abstract. This study evaluated and compared the morphological characteristics of the pollen grains in the different taxa of the genus Alnus in Hyrcanian forests, north of Iran**.** The pollen characteristics of five new taxa of the genus Alnus were studied and compared in these forests for the first time. The male catkins from 84 alder individuals of 25 populations were collected throughout the Hyrcanian forests and then recognized and grouped in the eight taxa. After extraction and washing with the standard method of the acetolysis procedure, pollen grains were photographed using light and electron microscopy imaging. Their morphological characteristics were measured afterwards. Alder pollen shape was oblate, with 3 to 6 pores: 1.2% triporate, 42.2% tetraporate, pantoporate 53%, 6/3% hexaporate. The aperture type also was annulate or annulus. About 10.8% of the grains were classified in small size (less than 25 µm) and the rest were in average size (25-50 µm). The large diameter of the investigated pollen varied from 20.7 to 39.3 micron (mean= 29.5 µm) and the small diameter from 19.8 to 36.7 micron (mean= 28.5 µm). About 36% of the pollen grains were concave, 37.2% mixed and 26.8% also convex based on wall shape. Cluster analysis based on the quantitative traits of pollen grains grouped altogether taxa in single cluster and none of taxa were categorized into separate cluster. Discriminante analysis based on six quantitative pollen traits showed high similarity and overlap among different alder taxa. Totally, it was found that eight alder taxa have shown little divergence in terms of pollen morphological characteristics, and consequently determining the limitation of these taxa could not easily practicable.

Keywords: *alder tree, pollen grain, exine ornamentation, oblate, SEM*

Introduction

The genus Alnus Mill. is one of six genera of Betulaceae and the only member of this family that has the ability to fix nitrogen through symbiosis relationship with soil filamentous bacteria called *Frankia* (Benson and Silvester, 1993; Chen et al., 1999; Chen and Li, 2004). Alder species widely distributed in temperate regions of northern hemisphere, with the exception of *Alnus glutinosa* (L.) which disperses also throughout the Andes, and small parts of North Africa (Benson and Silvester, 1993; Chen et al., 1999). Since most of alders live in river sides and marsh environments, they fall later than the other tree species. They are generally monoecious plants with the wind pollinated flowers. The pollens positioned into cylindrical male catkins in reddish colored with the length of 10.5 cm and will change to yellow, after they ripened in the fall (Chen et al., 1999).

Alder species has high potential to generating subspecies and hybrids because of the sympatric distribution of this genus (Ren et al., 2010; King and Ferris, 2000). That

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means that the populations of the species are mixed together as a result of relatively similar biological and ecological needs (King and Ferris, 2000); Thus, the formation of species, subspecies or hybrids is found in the distribution area of the genus (Ren et al., 2010; King and Ferris, 2000) creating uncertainties in the classification of the genus Alnus (Ren et al., 2010).

Based on phylogenetic studies using molecular and morphological data, number of Alnus species have already mentioned about 29-35 in the universe that Asia had the largest contribution (23-18 species) (Murai, 1964; Furlow, 1979; Ashburner, 1986; Chen and Li, 2004; Chen et al., 1999). However, according to the latest data provided by the plant list database, a total of 62 taxa is presented and confirmed of which 44 are considered as species (The Plant list, 2010).

In flora Iranica (Browičs, 1972), a subspecies of the black alder called *Alnus glutinosa* ssp. *barbata* C. A. Mey. with two varieties of Caucasian alder namely *A. subcordata* var. *subcordata* C. A. Mey. and *A. subcordata* var. *villosa* (Regel) H.J.P.Winkl have been formerly recorded.

But based on new morphological research the number of 8 taxa have been noted for the genus Alnus of Iran (Zare and Amini, 2012). The presence of the two other subspecies of black alder namely *A. glutinosa* ssp. *glutinosa* (L.) Gaertn and *A. glutinosa* ssp. *antitaurica* Yalt. had previously been reported in flora of Turkey mentioned also in Hyrcanian forests of Iran (Zare and Amini, 2012). According to the recent study, another species of alder which is native to the Mediterranean regions called *A. orientalis* Decne. has been also reported in Iran by Zare and Amini (2012). Moreover, two new species of Alnus was recorded as *A. dolichocarpa* Zare, Amini and Assadi, and *A. djavanshirii* Zare (Zare and Amini, 2012).

Pollen morphological characteristics have been widely used to facilitate the classification of some plants and investigation of the complications existing among them. For example, the evaluation of quantitative and qualitative characteristics of pollen grains was very useful for the classification of the genus Picea (Lindbladh et al., 2002), Artemisia (Lin et al., 2005), Lamiaceae (Hong, 2007), Rosaceae (Chung et al., 2010), Petrorhagia (Aktaş et al., 2010), Cactaceae (Aguilar-García et al., 2012), Annonaceae (Doyle and Thomas, 2012), Agavoideae (Lopes et al., 2013), etc..

Due to much confusion about the taxonomy of the genus Alnus and recent studies as well, the micromorphological features of the mentioned species is still unknown in Iran. Therefore, the purpose of the current study is investigation of micromorphological characteristics of pollen grains of Alnus taxa in Iran using light and electron microscopy. This study tries to assess the efficiency of micromorphological characteristics of pollen in the genus Alnus taxa with applying multivariate statistical tests.

Material and methods

Pollen samples were collected from 84 alder individuals of 25 separate populations throughout Hyrcanian Forests in fall 2011. Individuals were sampled from West to East, including Anzali, Siyahkal, Deilaman, Lahijan, Roudsar, Ramsar, Noushahr Sangdeh, Veysar, Galandroud, Peimoud, Nour, Veresk, Touskacheshmeh, Zarringol valley, Neharkhoran, Ziarat valley, Touskestan, Ramiyan valley, Minoodasht, Sasng, Loveh and Golestan national park. Furthermore, the pollens collected from the herbarium samples of Noushahr, Kelarabad, Fooman (Abrod village) and Sangdeh were used.

Furthermore, perfect herbarium samples collected from each individual (containing leaves and female cones) were identified in the Botanical Garden of Noushahr by botanists and experts. Collected Male catkins were prepared using standard method of acetolysis (Erdtman method) and studied by light microscope (Erdtman 1960; Bennett and Willis, 2001; Aguilar-García et al., 2012). In this procedure, the pollen bags were removed by first rubbing down the male catkins mechanically and then high speed centrifuging with acetic acid. The acetolysis solution (1 to 9 ratio of sulfuric acid and anhydride acetic acid) was added to them and then put in a hot water bath were centrifuged again twice. Finally, they were centrifuged and washed with ethanol and distilled water in two phases. In this method, the outer layer of pollen (Exine) will be cleared of external pollutants and quite pellucid and bright due to the effects of the acids (Erdtman 1960; Aguilar-García et al., 2012). Pollen grains then mixed with glycerol gels and were photographed under a light microscope. Micromorphological characteristics of pollen grains, measured based on validated descriptive methods (Furlow, 1979; Kapp et al., 2000; Blackmore et al., 2003; May and Lacourse, 2012).

Six quantitative morphological traits were measured for each pollen grain. These characteristics were: pollen large and small diameter, annulus width, annulus height, arci width and exine thickness (Furlow, 1979; Kapp et al., 2000; Blackmore et al., 2003; May and Lacourse, 2012).

In order to ensure accurate measurements, several time measurements was applied for each traits and then mean value was recorded (May and Lacourse, 2012). To assess the pollen large and small diameter, all diameters of grains were determined and the smallest and the largest values were selected. *Figure 1* shows the pattern by which the quantitative traits of a triporate pollen is measured.

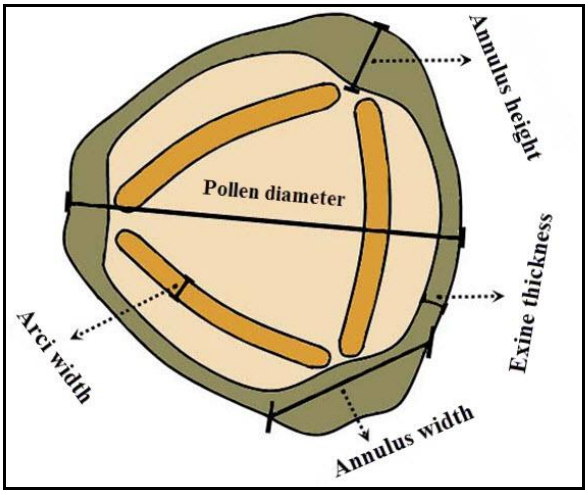

Figure 1. *Measuring quantitative morphological traits in a triporate pollen*

Moreover, two qualitative morphological traits evaluated: pollen shape and annulus protrusion. Besides, the number of pollen pores was counted for each grain separately. Pollen wall shape was classified to three convex, concave and mixed types (May and Lacourse, 2012). The measurements were conducted based on 20 pollen grains of each sample. However, 80 pollen grains of each sample were surveyed in order to estimate the number and percentage of quantitative traits. A total of 4,500 pollen grains were reviewed in this study.

For imaging electron microscopy, washed pollen (based on acetolysis method), were attached using double-sided adhesive strip from the wall of falcon (pollen container), on the surface of aluminum stubs, and then placed into the sputter coater (BAL-TEC Swiss company model SCDOOS). The samples were then coated with a thin layer of gold and put up the chamber of SEM (Philips Netherland-Model XL30) and photographed (2500 to 5100 x, 20 KV).

The samples of all the individuals were grouped in the eight-taxon after identification. First, the total number of clusters determined based on all attributes using the two-step cluster analysis and similarity of taxa were considered. The discriminant analysis was used to evaluate the accuracy of the allocation of tree individuals into composed clusters by stepwise method. The importance of the six quantitative characteristics of alder's pollen, in order to distinguish 8 taxa was determined by current test.

Pollen morphological characteristics measured using Zeiss AxioVision 4.8 software and ImageTools 3.0 with the accuracy of 0.02 µm. Adobe Photoshop 7.0 and Snagit Editor 11.0 software were used to edit and improve the quality of the images.

All measurements were under the Isopolar view of (seeing from polar axis) the pollens.

Results

The results showed that the classification of the genus Alnus based on the pollen shape is consider as oblate (The polar axis pollen is shorter than its equatorial one) from equatorial view. They were triporate to hexaporate. Aperture type of grains was annulate (annulus). Alder pollen is psilate it means there is no exine ornamentation under a standard light microscope. However, the electron microscopic images showed that the pollen surfaces were partly coarse and therefore classified as granulate-regulate in terms of exine ornamentation (Hesse et al., 2009; Blackmore et al., 2003).

Pollen classification was conducted based on dimensions by methods of Erdtman (1986) and Hesse et al (2009). About 10.8 % of pollen grains had small size (less than 25 µm) and the other had medium size (25-50 µm). The large diameter of the alder pollen varied from 20.7 to 39.3 micron (mean=29.5 µm) and the small diameter from 19.8 to 36.7 micron (mean=28.5 µm). Pollen annulus width ranged from 6.5 to 12.8 micron (mean=9.7 µm) and the annulus height from 1.66 to 5.33 micron (mean=3.38 µm).

Arci width had changes from 1.54 to 4.83 micron (mean=3.17 µm) and the exine thickness from 1.28 to 2.67 micron (mean=1.86 µm). About 36% of pollens were concave, 37.2% mixed and 26.8% convex in terms of pollen wall shape. Approximately 1.2 percent of the genus Alnus pollens were triporate, 42.2% tetraporate, 53% pantoporate and 3.6% hexaporate.

Table 1 shows the mean values of quantitative and qualitative morphological characteristics in eight separate taxa of Alnus.

Figure 2 shows box plots related to the six quantitative measured traits of pollens of the Hyrcanian alders. Box *plots* show inter- and intraspesific variation and also overlaps among eight taxa. The solid dark lines bisecting each box plot represent the trait median. Bottom and top border lines of boxes represent the first and third quartiles respectively.

Table 1. *Mean (± standard error) values of quantitative and qualitative characteristics of each 8 taxa of Alnus*

Pollen morphological characteristics	***A. subcordata var. subcordata***	***A. subcordata var. villosa***	***A. djavanshirii***	***A. dolichocarpa***	***A. orientalis***	***A. glutinosa ssp. barbata***	***A. glutinosa ssp. glutinosa***	***A. glutinosa ssp. antiaurica***
Large diameter (µm)	29.4±0.52	28.9±0.70	32.5±0.37	33.2±0.55	27.7±0.10	26.1±1.02	30.5±1.23	28.3±0.59
Small diameter (µm)	28.5±0.53	27.8±0.70	31.8±0.52	32.2±0.69	27.2±0.28	25±1.02	29.5±1.25	27.2±0.68
Annulus width (µm)	9.8±0.21	9.4±0.26	10.1±0.05	11.3±0.06	9.2±0.32	8.7±0.22	9.7±0.65	9±0.28
Annulus height (µm)	3.5±0.07	3.4±0.11	3.9±0.05	4±0.05	3.3±0.11	3.2±0.12	3.2±0.56	3.3±0.07
Arci width (µm)	3±0.09	3.2±0.12	3.8±0.02	3.6±0.05	3.1±0.35	2.6±0.18	2.9±0.05	2.7±0.18
Exine thicknes (µm)	1.9±0.05	1.8±0.04	2.1±0.01	2.3±0.03	2±0.14	1.7±0.06	1.9±0.10	1.8±0.09
Number of pores (%)								
Triporate	3.7	0.6	0	2.3	0	0.8	0	0.4
Tetraporate	48.4	44.6	18.8	44.2	44	27.7	66.5	19.3
Pantoporate	44.7	51.7	74.3	53.5	54.1	68.7	32	71.3
Hexaporate	3.2	3.1	6.9	0	1.9	2.8	1.5	9
Pollen wall shape (%)								
Concave	34.7	38.8	22.3	6.8	64.3	28.0	34.5	14.8
Mixed	37.4	37.4	45.7	59.1	29.8	51.1	41.1	43.3
Convex	27.9	23.8	34.1	34.1	5.9	20.9	20.5	42.1

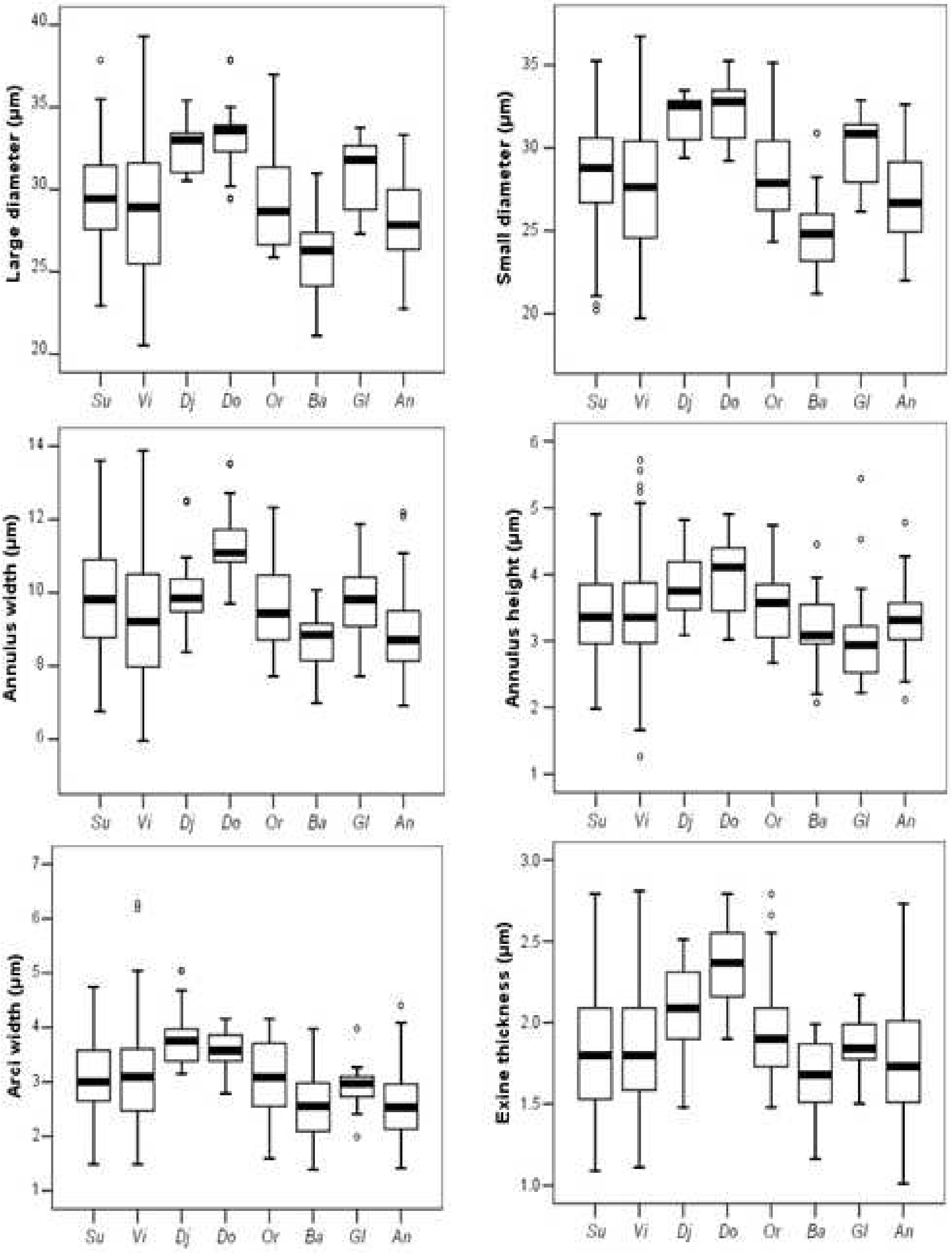

Figure 2. *Box plots of six quantitative characteristics related to the eight Alnus taxa. Su=A. subcordata var. subcordata, Vi = A. subcordata var. villosa, Dj =A. Djavanshirii, Do =A. dolichocarpa, Or =A. orientalis, Ba = A. glutinosa ssp. barbata, Gl =A. glutinosa ssp. glutinosa and An =A. glutinosa ssp. antitaurica.*

Figure 3 shows the light microscope photographs related to pollens of Hyrcanian alder taxa.

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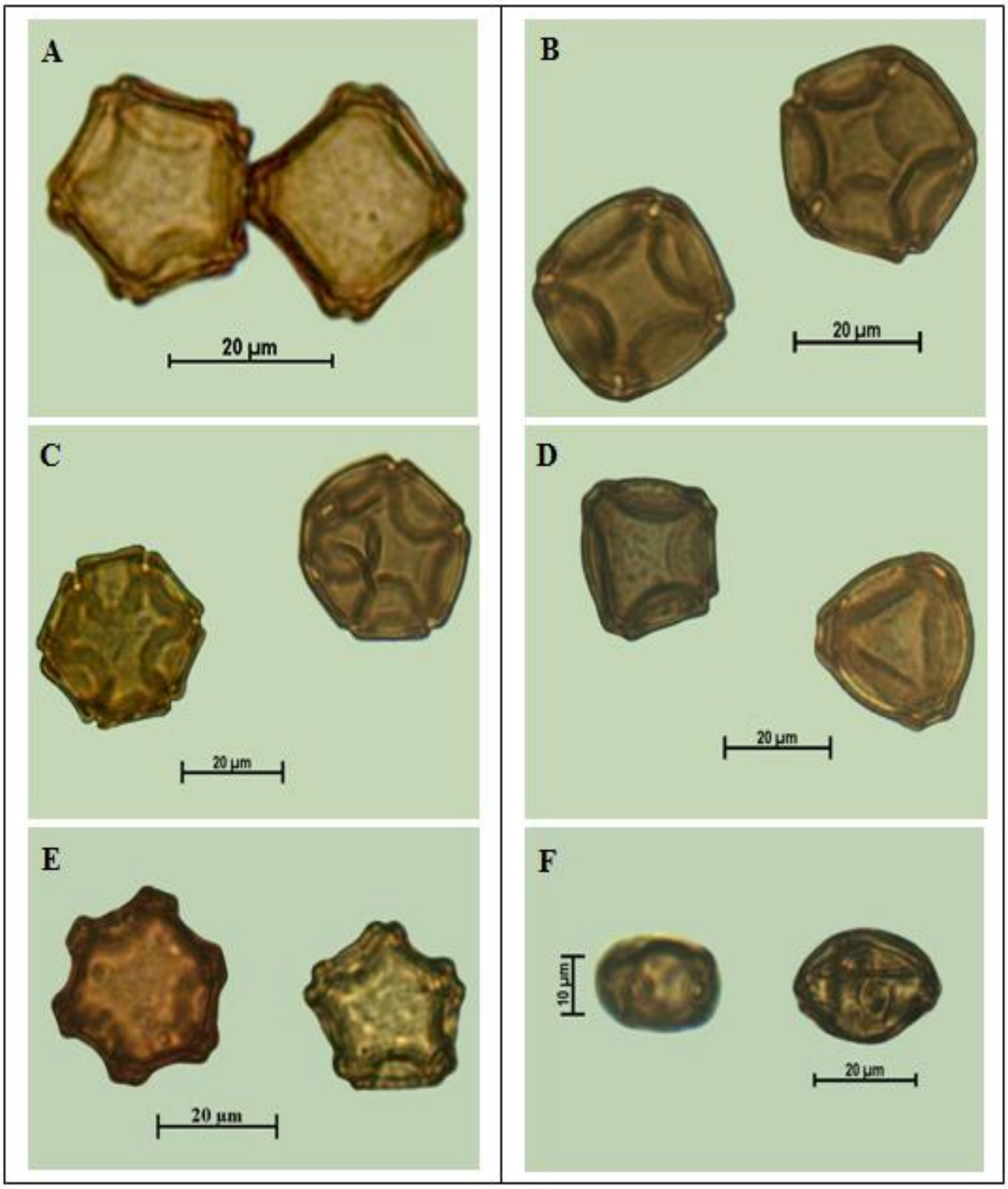

Figure 3. *Images from alder pollens photographed using light microscope with a magnification of 2525x. Left to right respectively (isopolar view): A) Concave tetra- and pantoporate pollen of A. subcordata var. subcordata, B) Convex tetra- and pantoporate pollens related to A. glutinosa ssp. glutinosa, C) Convex and mixed hexaporate pollen of A. djavanshirii, D) Mixed tetraporate and convex triporate pollen of A. subcordata var. villosa, E) Concave panto- and hexaporate pollen of A. subcordata var. villosa, F) equatorial view of pantoporate pollen related to A. glutinosa ssp. antitaurica (left) and tetraporate pollen of A. glutinosa ssp. barbata (right).*

Figure 4 and *5* also shows the SEM microscopy images of alder pollen grains from isopolar and equatorial view with the magnification of 2635x to 5153x.

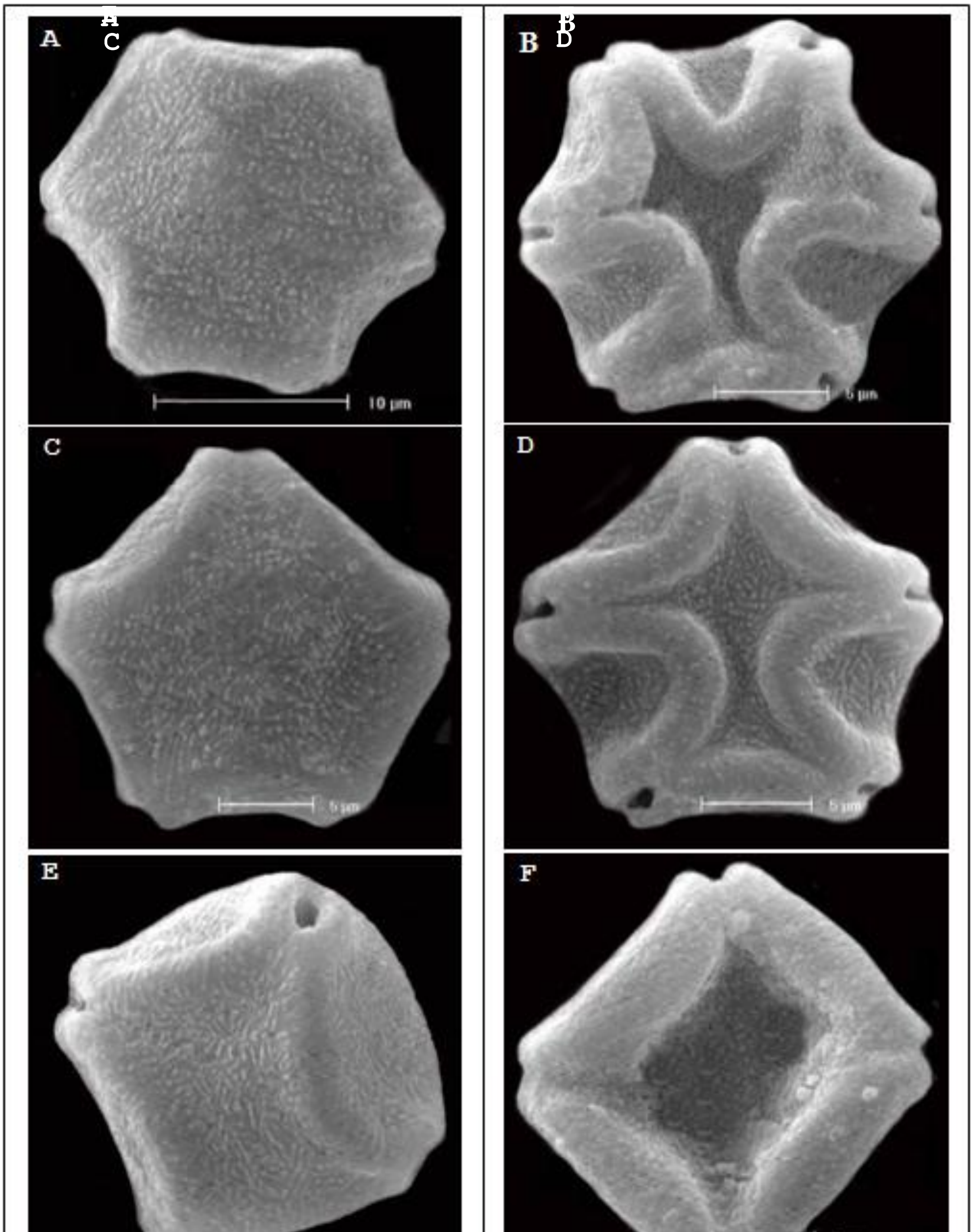

Figure 4. *SEM microscopy images of alder pollens from isopolar view. A) Concave and hydrated hexaporate pollen of A. djavanshirii (2635x), B) Concave and collapsed hexaporate pollen of A. glutinosa ssp. barbata (3523x), C) Concave and hydrated pantoporate pollen of A. glutinosa ssp. antitaurica (3133x), D) Concave and collapsed pantoporate pollen of A. glutinosa ssp. glutinosa (3364x), E) Mixed and hydrated tetraporate pollen of A. orientalis (2751x), F) Mixed and collapsed tetraporate pollen of A. dolichocarpa (3074x).*

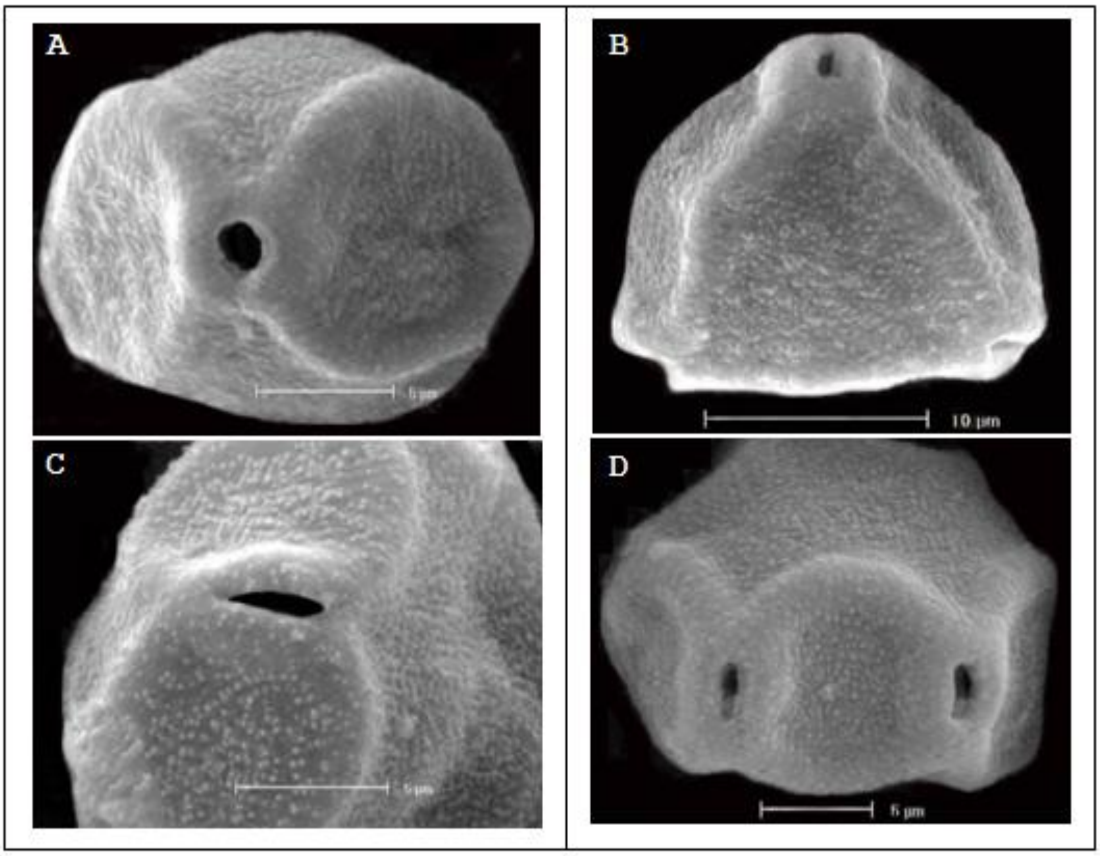

Figure 5. *SEM microscopy images of alder pollen. B) Convex triporate pollen of A. glutinosa (2756x) from oblique polar view, A) Concave tetraporate pollen of A. glutinosa ssp. glutinosa (4794x) in equatorial view, C) Concave pantoporate pollen of A. orientalis ssp. glutinosa (5153x) from equatorial view, D) Concave pantoporate pollen of A. subcordata var. villosa (3263x) in equatorial view.*

Two step cluster analysis was conducted to classify Alnus taxa based on six quantitative measured traits of pollen. *Figure 6* shows the results of two step cluster analysis.

As is known in *Figure 6*, all taxa were clustered together in a common group based on quantitative measured traits of pollen grains and no taxon has been classified in independent and distinct group.

The discriminant analysis was run based on the pollen quantitative characteristics. *Table 2* listed the values canonical correlation, percentage of variance and eigenvalue parameters related to five functions determined by discriminant analysis.

Table 2. *The eigenvalue, percentage of variance and canonical correlation values related to five functions determined by discriminant analysis.*

Functions	Canonical correlation	% of variance	Eigenvalue
1	0.490	39.4	0.315
2	0.414	25.9	0.207
3	0.323	14.5	0.116
4	0.276	10.3	0.083
5	0.213	5.9	0.048

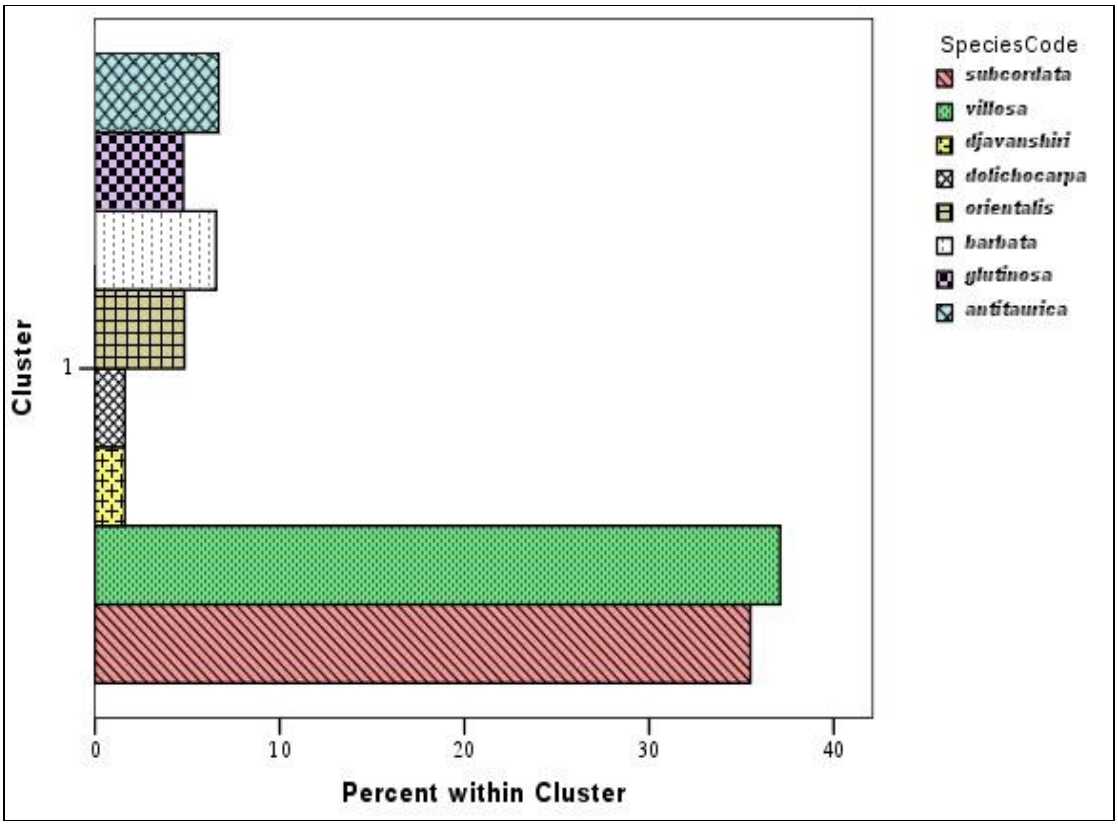

Figure 6. *The result of two step cluster analysis.*

As the results shown in *Table 2*, none of the measured characteristics were suitable to distinguish the Alnus taxa. Low eigenvalues of each of these functions is clearly assign the function is inappropriate to separate the taxa. On the other hand, only 65% of the variance is explained by the first two functions. Therefore, the six measured morphological traits associated with alder pollen can not possibly reflect the distinction between the studied taxa.

Figure 7 shows the distribution of sampled trees associated with correlation of pollen characteristics with the first two functions.

Figure 7 clearly indicates that eight taxa have shown little difference in terms of pollen morphological characteristics and consequently determining the limitation of these taxa using measured traits was not simply possible. The results of cluster analysis in consistent with discriminant analysis, indicated much similarity of Alnus taxa in the north of Iran based on pollen charactristics.

The results of Pearson correlations showed that there was a statistically significant correlation (99% confidence level) between pollen diameter and annulus width (Sig.= 0.000, r_p= 0.743), annulus height (Sig.= 0.000, r_p= 0.346), arci width (Sig.= 0.000, r_p= 0.555), and pollen exine thickness (Sig.= 0.000, r_p= 0.355) whereas there was no significant correlation between pollen diameter and the number of pollen pores (Sig.= 0.191, r_p= 0.056).

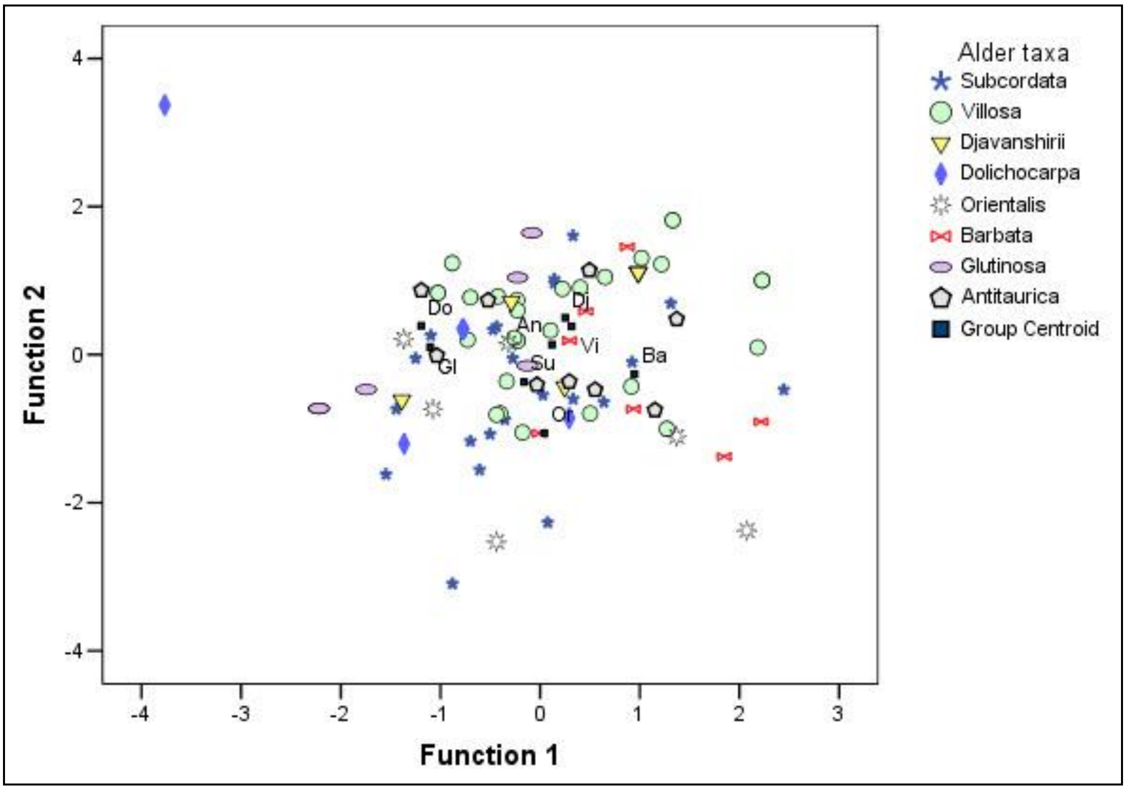

Figure 7. *Distribution of sampled alder around the first and second function based on morphological analysis of the pollen*

Discussion

What clearly indicated by the pollen morphological analysis of the genus Alnus was that none of the morphological traits was useful to distinguish the taxa at the species level.

The highest mean value of each pollen morphological characteristics, often observed in *A. dolichocarpa* and *A. djavanshirii* (*Table 1* and *Figure 2*) and the least commonly was associated with *A. glutinosa* ssp. *barbata* and occasionally the two other subspecies namely *A. glutinosa* ssp. *glutinosa* and *A. glutinosa* ssp. *antitaurica*. In other cases, we couldn't find certain trend among taxa. Eigenvalues, percentage of explained variances and correlation between pollen traits with two functions of discriminant analysis, were very low which identification of taxa was not possible by these traits (*Table 2*).

The pollen type and shape, exine ornamentation and aperture type of pollens, did not reveal significant difference among eight taxa and in this respect there is no support reason in order to identify the structural differences due to evolutionary evidence in classification of the taxa.

In all taxa, tetra- and pantoporate pollens were observed. However, the latter prevailing in six taxa was the most dominant (53% of total pollen grains), followed by tetraporate pollens (42% of total pollens) having the highest frequency. The hexaporate pollen was observed in all taxa except *A. dolichocarpa*. However, the triporate pollens had the lowest frequencies among the taxa; So that the triporate taxa pollen was found in three taxa. The study conducted by Reinink-Smith (2010) on the pollen fossils of the genus Alnus in Alaska, showed that pollen of this genus are often tetra- and pantoporate and tri and hexaporate grains have lower frequency. He also stated that 7-, 8- and even

9-porate pollens are rarely observed in the genus Alnus. For example, in *Alnus firma* 7-porate pollens are prevalent. But he also acknowledged separating and distinguishing the species based on pollen pore number, is not feasible; because presence of the specified number of pore may be similar or very close among the species. Moreover, when the number of pore in a same species is concerned, it can be expected that the greater number of pores are visible in larger grains (Reinink-Smith, 2010).

It is mentioned that the number of pores is influenced and controlled by both genetic and environmental factors (Flenley, 2003). Today, it is demonstrated that the formation of pollen grains are sensitive to specific environmental factors such as temperature and humidity, and response of plants against changes in this environmental factors are relatively fast (Dzyuba, 2006). Furthermore, the number of pores in pollens may be related to climatic factors during the growing season (Flenley, 2003). Even the position of the pollen sacs and anther on the flower and eventually flower on the tree can cause large differences in the number of pores and size of the pollen (Kurtz and Liverman, 1958; Flenley, 2003). These factors not only affect the pollen fertility, but it also affects the distribution of grains (Flenley, 2003).

Mikkelsen in 1949 demonstrated the increasing about 14°C temperature during the growing season can reduce the size of the pollen more than 6 percent in some species of Brassicaceae. Besides the temperature, he mentioned the importance of nutrients on the plant pollen production.

Based on the findings of this paper, larger pollen grains in the genus Alnus had greater exine thickness, arci width and annulus width. However the relationship between pollen diameter and the number of pores were not significantly differed. The study of Lee (1978) showed those pollen grains with greater number of pores, are relatively large, and they need thicker exine to keep the grain structure. Mulcahy (1974) also acknowledged larger pollen need to have more number of pores in order to absorb enough water from the stigma with the rate of smaller grains. Rapid absorption of water is a selective advantage because it allows the development of pollen tubes with higher rate and eventually faster fertility. Therefore, larger pollen grains with more number of pores can better compensate the need of water absorption (Lee, 1978).

Proportion of pollen shape (wall type) which was divided into three types of concave, convex and mixed pollen was almost similar in all Alnus taxa and cannot be stated that each of these types are unique to specific taxon; it was also similar about the number of pores per pollen, as mentioned before.

SEM microscopy results also did not show difference in the type of exine ornamentation in all taxa (*Figure 4* and *5*).

Results of classification by two-step cluster analysis and discriminant analysis clearly indicated that the morphological characteristics of pollen in the genus Alnus cannot even distinguish one taxon from other taxa; Since the population of individuals in each taxon are mixed completely within other taxa, and certain limits can not be identified to separate them (*Figure 7*).

Totally, based on the whole results of the study using light and electron microscopy in this paper, should be noted that pollen related characteristics in the genus Alnus can not resolve the taxonomical and systematical problems of the genus; The main reason for this can be slight variation of pollen characteristics in terms of structural properties of pollen and quantitative traits. However, in many cases, the pollen types and its attributes has been largely used in the classification of plants (Wen and Nowicke, 1999; Lin et al., 2005; Aktaş et al., 2010; Liu et al., 2011; Doyle and Thomas, 2012;). The pollen analysis of the genus Picea was very useful for distinguishing species in North America (Lindbladh et al., 2002). In some genera of plants, the results of pollen analysis was in agreement with molecular studies (Wen and Nowicke, 1999).

However, the results of another study conducted on three species of alder in the U.S. showed similar results with our study. May and Lacourse (2012) stated that pollen characteristics are not suitable for distinguishing three taxa of *A. rubra*, *A. incana* subsp. *tenuifolia* and *A. viridis* subsp. *sinuate* and despite significant differences in the characteristics of these taxa decision can not be made for separating at the species level, due to high degree of overlap in the characteristics of the taxa.

Conclusion

According to the results of this paper, pollen characteristics were not found suitable to separate the Alnus taxa. Also the five new taxa recently added to the floristic list of Hyrcanian Alders of Iran (Zare and Amini, 2012) did not show significant differences with other taxa based on pollen characteristics. The classification of these taxa, two of which have been introduced as new species to the world list of Alnus for the first time (*A. dolichocarpa* and *A. djavanshirii*), still needs to be further tested using DNA barcoding techniques and other genetic methods. However, the non-discrimination (unseparability) of Alnus taxa, based on pollen analysis, is related to the very little variations in the type and other traits of pollen in this genus.

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CLIMATIC CORRELATES OF HUMAN SUBSISTENCE: A GLOBAL ANALYSIS

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Abstract. Subsistence links the behavioural and biological dimensions of human adaptability. We perform a cross-cultural investigation over an unpublished database containing approximately 2,700 ethnographic populations. The distribution of those *Basic Economies* is analysed by *Latitude, Temperature* and *Rainfall.* Results show unequal geographic distributions, although the climatic variables are not good discriminators in all cases. Two strategies (*Food production with fishing/foraging,* and *Foraging/fishing*) are distinct. The generalist form composed by extensive agriculture, coupled with foraging and fishing, is the most adaptable and widespread form of food acquisition. The exceptions are cold deserts, which foragers/fishers provide the best fit to the marginality concept.

Keywords: *subsistence strategies, hunter-gatherers, marginality, cross-cultural analysis*

Introduction

Subsistence and reproduction are the most apparent links between the behavioural and the biological dimensions of life. They are key to the understanding of human adaptation and adaptability to environmental conditions. In terms of subsistence, humans have developed a range of strategies based on different combinations of two basic activities: to produce their own food (by controlling the reproduction of either or both animals and plants) or to forage (by collecting food from the natural ecosystem). However, the climatic correlates of those choices remain poorly explored.

Review of Literature

The Ethnographic Atlas (Murdock, 1967) was a milestone in cross-cultural analyses of ecological constraints, social relations, biological diversity, and culture. Among its 229 hunter-gatherer populations, animal foods are relatively constant (26–35% subsistence), regardless of latitude (Cordain et al., 2000). In contrast, plant foods decrease with increasing latitude, reaching a threshold at 40° N or S. No human groups were found to rely mostly or wholly on gathering plants.

Kelly (1999) investigates the relationship between the plant component of human diet and climate further. He uses “Effective Temperature” (ET= 18W–10C/(W- C) + 8, where W= mean temperature of the warmest month, and C= mean temperature of the coldest month) and “Primary Production” (PP, a measure of plant biomass in the

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environment) to predict gathering dependence of 123 North American foraging groups. Both variables are related to the percentage of plants in the diet (r=0.75, p<0.01), but not of meat. The resulting relationship between the dependence on plant foods and climatic and environmental patterns reveals the presence of dietary complements in the form of aquatic resources and traded foodstuffs. Arctic populations are an example. Living in an environment of high aquatic productivity, they seem to replace plants for aquatic animals. Other studies that have addressed the issue of what factors shape the geographic distribution of human subsistence strategies have used somewhat different climatic and/or environmental predictors. So Binford (2001) uses Mean Annual Temperature as environmental covariant in his analyses, and both him and Marlowe (2005) use Primary Biomass (kg/m^2) as a measure of environmental productivity. Similar to the results obtained by Kelly (1999), these studies provide further evidence for the increased importance of fishing and hunting near the Poles as primary biomass decreases.

Therefore, the most useful correlations between subsistence and the environment are revealed by meteorological phenomena. These variables are the least affected by human activity, and are systematically and continuously recorded (Ellen, 1982), and as the studies above show, they play a critical role in constraining the possible geographic extent of particular modes of subsistence, specially at the extreme ends of the environmental gradient.

While it may be expected that foragers are more vulnerable to climatic and environmental factors, agriculturalists also deal with environmental limitations. Decreasing ecosystem diversity and the reliance on a small number of species make farmers susceptible to environmental fluctuations. This raises the issue of how such potentially unstable systems could spread so widely around the World.

It is generally accepted that all contemporary foragers are aware of the techniques of food production in that they understand the process (means, timing, etc.) of the reproduction of the plants and animals they consume in a regular basis which would allow them to control productivity. Nonetheless, the transition from foraging to farming requires radical shifts in behaviour, which have high adaptive costs. The causes, conditions and circumstances surrounding the origins of agriculture have been and continue to be the subject of intense study. Although numerous and different local factors seem to have shaped the timing and nature of the transition in different parts of the world, it has become clear that the spread of agriculture is related to population densities. Hunter-gatherers live at a density of *ca.* 0.1/km^2, while rice agriculturists in Java, for example, live at 1,000/km^2, a ten-thousand-fold difference (Kennett and Winterhalder, 2006). Considering there are more than seven billion people alive (USCB), agriculture seems the inevitable means of food procurement. In spite of that, contemporary hunting and gathering is a reality under specific conditions, in particular, in ecologically marginal areas where farming is unsustainable. Thus, it is assumed that hunter-gatherers occupy these marginal areas as the result of being out-competed by agriculturalists for the more productive lands (Kormondy and Brown, 1998; Milton, 2000; Marlowe, 2005).

This paper uses the principles disclosed by these earlier studies to address specific questions about the relationships between climate and mode of subsistence at a global scale. In particular, it aims to test whether different subsistence strategies occupy specific climatic ranges by analysing an unpublished cross-sectional database containing ethnographic information on populations from around the world. This design also allows for inferences on the distribution of contemporary foragers and the spread of agriculture. The general objective is to clarify the environmental limits imposed on human adaptive options.

Material and Methods

The database used was constructed as part of the "King's College Human Diversity Project" (Foley and Mirazón Lahr, unpublished database). It contains anthropological and socio-economic information on *ca.* 2,700 ethnographic populations in Africa, East, Middle and West Asia, South, Central and North America and Oceania. The data were compiled from books, articles, compilations, and reports referring to early ethnographic observations (thus reflecting a recent past), when "intrinsic" patterns were more evident. The information used in the subsequent analyses includes location (latitude and longitude), subsistence strategies (basic economy), and language (family and primary branch).

Derived from the usually descriptive data in the source, a qualitative variable called *Basic economy* was created with four categories as defined in *Table 1*.

Table 1. *Categories of the variable Basic economy*

"Basic economy"*	
Category	**Description**
1.Food-production	Subsistence based on agriculture and/or herding of animals.
2.Food Production plus fishing/foraging	Subsistence based on food production (agriculture or herding) allied to hunting, gathering and/or fishing.
3. Foraging/fishing	Subsistence based on hunting and/or gathering plus fishing.
4. Foraging	Subsistence based on either hunting, gathering or on both.

*Throughout the text, categorical and continuous variables are in *italic* for better identification.

Language generally defines the sample unit (namely, the population), but sources are often unclear about whether they deal with a whole linguistic population, a tribe, a group, a subgroup, etc., and which units are related to each other. This is known as the Galton's Problem (Naroll, 1965), and it is minimized here by the large sample size of groups included, coupled with the search for general, broad patterns. Furthermore, many languages still show classification problems (Lewis et al., 2013), and as linguistic identity forms the main form of discriminating amongst peoples, this remains an issue. However, all the data were cross-checked against Murdock's Ethnographic Atlas (1967), the Encyclopaedia of World Cultures (Levinson, 1996), and the first volume of the "Guide of World's Languages" (Ruhlen, 1991).

The climatic data were derived from the "Global Ecosystems Database" (Kineman, 1992). This database provides information on temperature and rainfall at two degrees resolution throughout the world, and allows for matching to the coordinates of a central point within the distribution of each of the human groups in the database. *Mean Annual Temperature* is the average taken from monthly measurements, while *Total Annual Rainfall* expresses annual precipitation. Real latitudes were used instead of "absolute latitude" because the northern and southern hemispheres are not mirror images either in the biophysical or in the anthropologic sense (Kummu and Varis, 2010).

The statistical analyses were performed using SSPS (9.0 for Windows). First, we describe the amplitude of *Latitude, Temperature* and *Rainfall* of each *Basic economy*. Second, the homogeneity of these distributions is tested using the Kruskal Wallis analysis of variance. Lastly, outliers are excluded and discriminant analyses used to test the extent to which climatic variables can discriminate between, and thus account for, different subsistence strategies in the recent (ethnographic) past throughout the world.

Results

General patterns

The global distribution of subsistence strategies is revealed when the coordinates of the 2,725 human groups are plotted according to their category of *Basic economy*. No *Basic economy* is restricted to a single region of the Globe, either by continent or by latitude. Yet, certain categories are clearly unevenly distributed along the latitudinal axis (*Figure 1*).

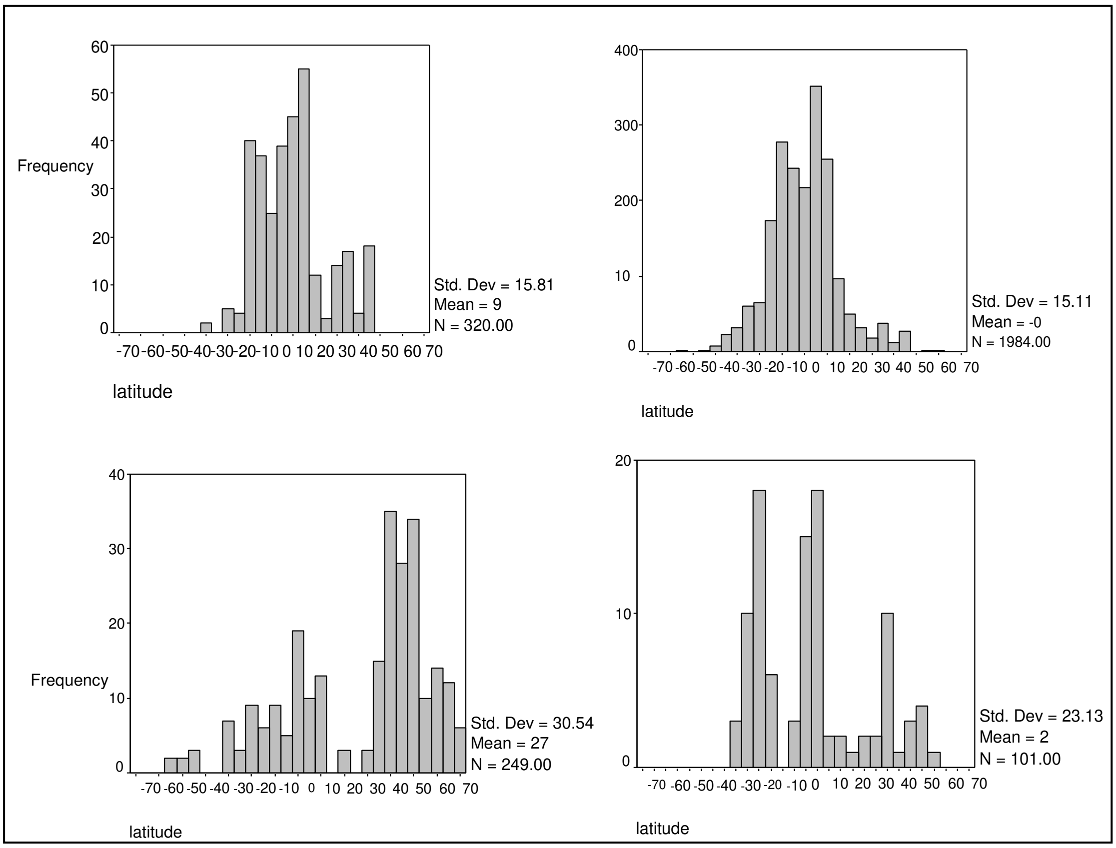

Figure 1. *Numbers and distribution of Basic economies by Latitude. Top left: Food production, Top right: Food production plus fishing and foraging, Bottom left: Foraging and fishing, Bottom right: Foraging.*

The distribution of populations by latitude does not fit a normal curve (the ratios between skewness and kurtosis by the standard deviation are both greater than 2). In the recent past, there was a clear numerical dominance of *Food production plus fishing/foraging*, representing the mode of subsistence of 2,026 of the groups sampled (74.35 %). *Foraging* is the least frequent strategy, with only 121 populations (4.44 %). Complete dependency on *Food production,* i.e. without complementing the diet with foraging and/or fishing, occurs among 323 groups (11.85 %), while 255 populations (9.36 %) practice a mode of subsistence based on *Foraging/fishing*.

The distribution of each *Basic economy* along the latitudinal axis reveals the following patterns:

Food production - Most food producers are distributed between 10° and 20° N and between 0° and 10° S. In general, there are few food producers at very high latitudes.

Food production plus fishing/foraging – Observed at greater frequency in lower latitudes around the Equator, between 10° S and 10° N, but also present at higher latitudes.

Foraging/fishing - With a markedly skewed distribution, it is more frequent on the northern hemisphere. In both hemispheres, this strategy is present at very high latitudes, up to 70° N and 55° S.

Foraging – It has peaks around the Equator and around 30° both North and South. It is absent around 10° S, and reaches higher latitudes in the northern than in the southern hemisphere.

The results of the non-parametric Kruskal-Wallis analysis of variance show that categories of *Basic economy* occupy significantly different latitudinal ranges (X^2= 255.585, p=0.000***). Nevertheless, there are several exceptions to the pattern, and a box plot of *Latitude* per *Basic Economy* discloses outliers in the latitudinal distribution of populations whose economy is generalised, based on *Food production plus fishing/foraging.*

Distribution of Basic economy by Temperature

Figure 2 shows the distribution of each category of *Basic economy* according to *Mean Annual Temperature.* As expected given the relationship between latitude and temperature, the patterns revealed by the graph are compatible with those observed between latitude and economic strategy. Among these, the most relevant aspects are:

- *Food production* is not common where temperatures are too low; with the highest frequencies between 20° and 25°C. The maximum average monthly temperature at which food producers live (29.4 ^{o}C) is the highest observed among all *Basic economies*, while the minimum temperature at which sole dependence on food production is encountered (5.5 ^{o}C) is also the highest of all minimum temperatures found. This suggests that while a strategy that relies entirely on food production may be the most tolerant of high monthly temperatures, it is nevertheless unsustainable in areas where the minimum temperatures fall below a comparatively warm (5.5 ^{o}C) threshold.
- The addition of fishing and foraging to food production (*Food production plus fishing/foraging)* does not change significantly the spatial distribution, although its range stretches towards lower temperatures through a number of cases that, nevertheless, remain outliers of the main distribution.

- *Foraging/fishing* is the form of economy observed at the lowest temperature (-16.20°C), with a modal temperature value of *ca.* 10 °C. Nevertheless, there is also a high concentration of cases around 25°C, and the maximum values approach 28°C.
- *Foraging* distribution is within the range of other *Basic economies*.

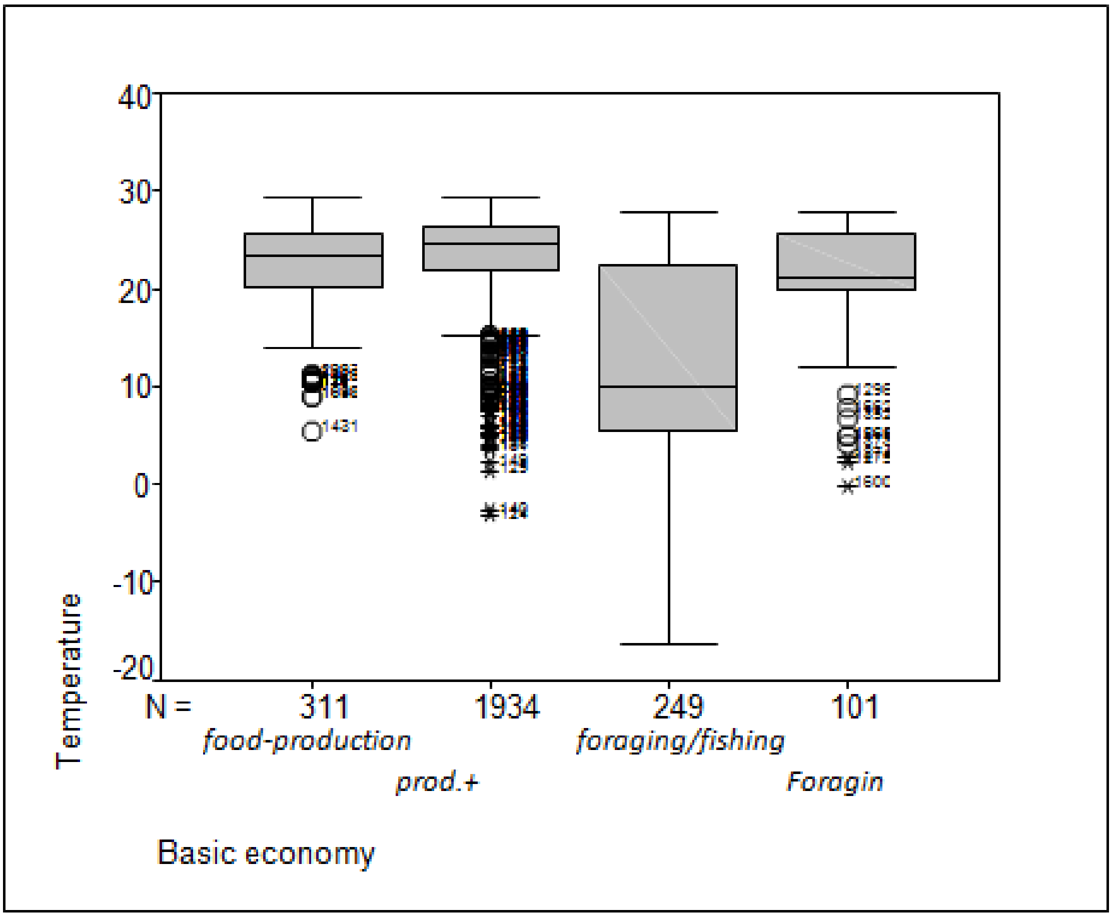

Figure 2. *Box plot showing the distribution, median values and outliers of temperature by basic economy.*

The results of a Kruskal-Wallis test suggests that the four categories *of Basic economy* differ significantly in relation to *Temperature* (X^2= 298.988, p=0.000***). Cases are mostly concentrated around the average, between 20°C and 25°C; *Foraging/fishing* is the exception, occupying a wider temperature range.

Distribution of Basic Economy by Total Annual Rainfall

Figure 3 shows the distribution of each category of *Basic economy* in relation to *Total Annual Rainfall.* The most relevant pattern is that, in general, all categories are concentrated at lower values of rainfall. This is especially the case for those which involve foraging, which are found in places with the lowest modal annual rainfall. *Figure 3* shows that all *Basic economies* have mean values of *Rainfall* between zero and 1,800mm/year, and that *Foraging/fishing* has the highest number outliers, including extremely high values of rainfall.

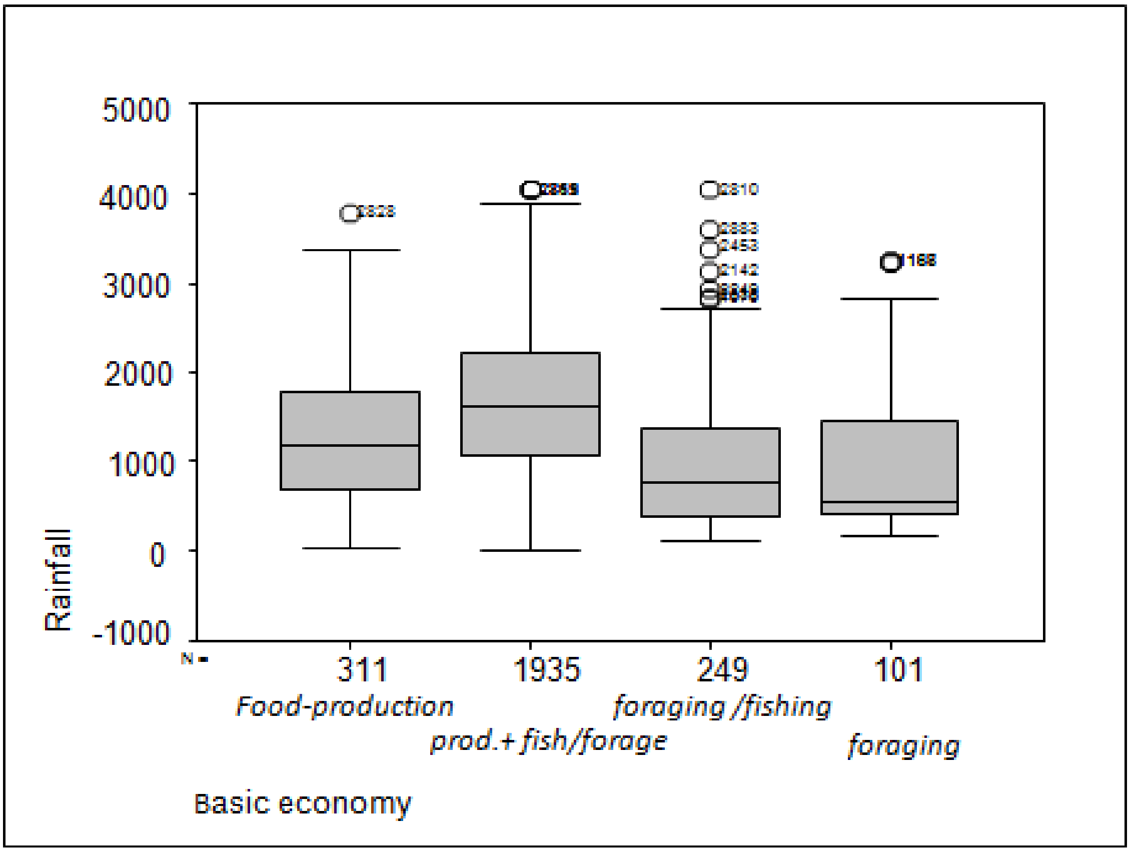

Figure 3. *Box plot showing the distribution, median values and outliers of rainfall by basic economy.*

Food Production is found across a wide range of rainfall patterns (from close to no rainfall to *ca.* 3400 mm/yr), but the majority of cases is restricted to low rainfall regimes between ~ 800-1800 mm/yr. The long tails to the distribution and outliers suggest that food producing may not be optimal, but is not limited by rainfall.

As the generalist strategy, *Food Production plus foraging/fishing* occupies the wider range of vales (from 6.40 mm/yr to 4038.10 mm/yr). However, it is interesting to note that although the range between the minimum and maximum rainfall in which 95% of cases of this subsistence is found is similar to food producers, the actual minimum/maximum limits differ, here being between ~1100 mm/yr and 2100 mm/yr. In other words, the complementation of food production with foraging and fishing does not widen the range of temperatures for the majority, but shifts the threshold of optimal tolerance.

Foraging/fishing has a markedly skewed distribution, with most cases at low values of rainfall, although a small number of outliers populate areas with rainfall that exceeds 3000 mm/yr.

Populations who depend solely on *Foraging* live in areas with the minimum average annual rainfall, while the highest rainfall value recorded amongst foragers is also the lowest maximum value among the four distributions (3234.80 mm/ yr).

The sample is skewed in relation to *Mean Annual Rainfall* (Skewness (0.456)/Std. Error (.046) > 2 (=9.9)). The results of a Kruskal-Wallis test again indicate that the variance of *Rainfall* is significantly different among the four categories (X^2= 234.406, p=0.000***).

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Discrimination of Basic economies by climatic variables

In order to investigate the potential interaction effects among the climatic and environmental variables, as well as to test whether it is possible to some extent to predict the form of economy from these variables, a discriminant analysis was performed. This analysis tests the capacity of climatic variables to discriminate among the *Basic economies* based on their global geographic distribution. Linear combinations of independent variables are formed (*Latitude, Temperature,* and *Rainfall*) to create predictive functions of cases within the categories of *Basic economy.*

Outliers were excluded, which makes results reliable even in the absence of normal distributions (Tabachnick and Fidell, 1996). We use Wilk's Lambda minimization and prior probabilities are calculated taking into account the differences in sample size between categories.

The results show that the three climatic/environmental variables combined are poor discriminators of human subsistence. The analysis employed all three variables; these have relatively weak patterns of covariation with the type of economy, with Eigenvalues well below 1 (N =2592) (*Table 2*).

Table 2. *Eingenvalues and other descriptive values of functions 1,2, and 3.*

Function	Eigenvalue	% of Variance	Cumulative %	Canonical correlation
1	.446	89.6	89.6	.556
2	.043	8.5	98.1	.202
3	.009	1.9	100.0	.096

Figure 4 illustrates the correlations between variables in Functions 1 and 2, which account for the highest proportion of the total variance (98.1%) that can be explained by these 3 variables. The horizontal axis represent Function 1, which shows a very strong positive correlation with temperature (>0.9), a strong negative correlation with latitude (>-0.7), and a relatively weak correlation with rainfall (<0.4). In other words, Function 1 expresses the fact that temperatures are highest at low latitudes and vice-versa, with rainfall tending to be higher in hot low latitude localities, but not particularly so. Function 2, represented by the vertical axis, is strongly influenced by *Rainfall* (>0.7), showing that very positive values of rainfall are to some extent inversely correlated with both *Latitude* and *Temperature*.

The fact that 89.6% of the variation explained by climatic variables is due to Function 1 expresses the relative importance of *Temperature* in shaping the distribution of types of subsistence strategy. Thus we observe that the type of basic economy of 77.9% of all cases was correctly classified through Functions 1, 2, and 3. At a first instance, this appears a strong result, suggesting that in 4 out of 5 cases the subsistence strategy of a population follows a distribution predictable through temperature, latitude and rainfall combined. However, a closer examination of the distribution of correctly and incorrectly classified cases across types of subsistence shows that this is not the case (*Table 3*).

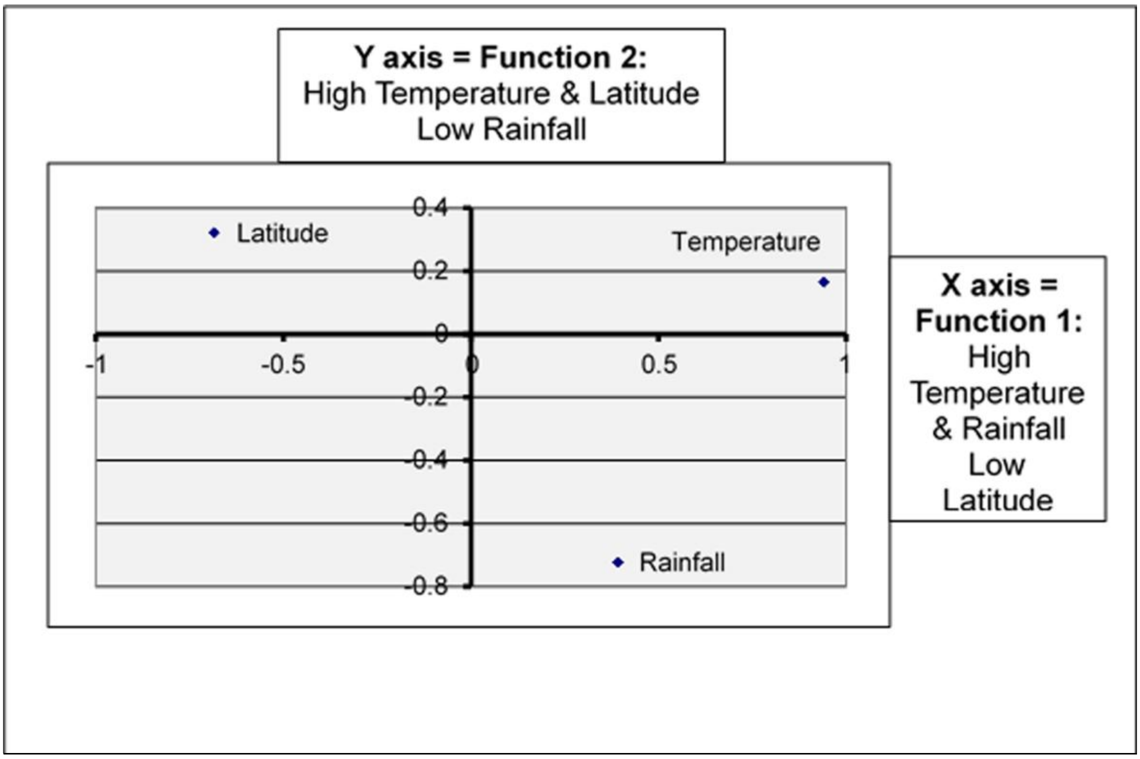

Figure 4. *Correlations between variables in Function 1, mostly influenced by temperature and latitude, and Function 2, determined mainly by rainfall.*

Table 3. *Percentage of cases that were correctly classified (in bold) through the discriminant analysis of Basic economies using climatic variables.*

		Predicted Group Membership				Total
	Basic economy	***Food-production***	***Food-prod.+ fishing/foraging***	***Foraging/ & fishing***	***Foraging***	
%	***Food-production***	**.0**	91.0	9.0	.0	100.0
	Food-prod.+ fishing/forage	.0	**96.7**	3.3	.0	100.0
	Foraging/fishing	.0	40.2	**59.8**	.0	100.0
	Foraging	.0	87.9	12.1	**.0**	100.0
	Ungrouped cases	.0	52.5	47.5	.0	100.0

The majority of correctly predicted cases corresponds to a single form of subsistence – the generalist *Food production plus fishing/foraging* which had nearly 100% of its cases correctly discriminated. Cases of *Foraging/fishing* also had moderately high levels of correct prediction at nearly 60% (*Table 3*). However, there were no correct classifications of *Food production* and *Foraging*, all of which were subsumed in the first two groups. In other words, the range of climatic and environmental conditions in which both *Food production plus fishing/foraging* and *Foraging/fishing* are observed fully encompass those in which some populations engage solely in food production and/or foraging. The small percentage of correctly predicted cases across all types of economy, together with the low Eigenvalues obtained, confirm that the combination of *Latitude, Temperature, and Rainfall* is not sufficient to predict the different types of subsistence strategies. The possible reasons for such a heterogeneity in the influence of climatic factors is the subject of our discussion.

Discussion

Our sample reveals an unbalance in the distribution of cases between the northern and southern hemispheres that is consistent with the distribution of the human population. Around 87.5% of the global population live in the North and only 12.5% in the South (Kummu and Varis, 2010). The northern hemisphere has more land than the southern, as well as being relatively warmer, with winter averages around -40°C and -60°C in the North and South Poles respectively. Only in the northern hemisphere are there human groups beyond 60° latitude. This highlights the extent to which the two halves of the world are not mirror images of each other, and that the use of "absolute latitudes" instead of real latitudes, could be misleading (as in Marlowe, 2005, for example).

The fact that the distribution of groups of food producers who complement their diet through fishing and foraging, and to some extent that of foragers/fishers, can be predicted on the basis of the variables used in this study reflects their distinctive range width as well as absolute numbers. The first type of subsistence strategy is clearly numerically dominant in the sample (~ 68% of cases); they appear to be more demanding in terms of temperature, but highly adaptable concerning rainfall. Foragers who also fish live at all possible temperatures. This is clear in *Figures 2* and *3*, and is compatible with the mis-classification of cases from the other two categories. Wide ranges and high numbers stress the importance of adaptability to adverse conditions and of adaptive success.

The economic category of *Food production plus fishing/foraging* represents the majority of cases and broadest latitudinal distribution, enclosing in its climatic range both *Foraging* and *Food production* economies. This is a common, flexible and generalist, rather than specialist, strategy. It challenges the commonly held perception that the comparative specialists economies that depend solely on *Food production* represent either the majority of cases, or the prevalent evolutionary tendency.

Horticulture and agriculture both constitute forms of subsistence intensification in relation to foraging (Boone, 2002). The generalist nature of *Food production plus fishing/foraging* comes not only from the mix of food extraction and production, but also from the diversified form of cultivation represented by less intensive agriculture. This implies a mixture of different crops, including trees, shrubs, herbs and tubers, many of which, although poorer in proteins than seed crops, are less labour-intensive (Grigg, 1977), while their combination can be used to offset environmental constraints. The time spared can be employed in the acquisition of protein by hunting or fishing, or indeed trading, complementing nutritional needs.

A fundamental principle of human health and nutrition is that diverse diets increase the overall health patterns by lowering infant mortality rates and increasing average life expectancy (Hockett and Haws, 2003). That the most widespread form of food production is an extensive and mixed strategy would account for the apparent paradox between the human necessity to buffer environmental fluctuations, best achieved by retaining a more generalist and flexible approach to food procurement, and the expansion of agriculture.

Despite the wide environmental range occupied by *Food production plus fishing/foraging,* most groups who depend solely on *Foraging/fishing* and *Foraging* live at the lower extremes of both *Temperature* and *Rainfall*. This could be accounted for by the fact that recent hunter-gatherers inhabit comparatively marginal environments (Kormondy and Brown, 1998; Milton, 2000; Marlowe, 2005). “Marginal ecosystems” refer to arctic, deserts, and rainforest habitats (Kormondy and Brown, 1998), and indeed both hot and cold deserts can be identified by low rainfall in our analysis.

In hot deserts, the distribution of *Food production* and *Foraging* overlaps, mainly reflecting the fact that the pastoralists of North Africa and West Asia represent most of the food producers in these places. Bedouins, for example, are known to acquire most of their water from milk and conserve it through behavioural adaptations (work schedule, robes, etc.) (Reader, 1988).

On the prevalence of foragers in hot deserts, our data show that hunter-gatherers are (or were) exclusive inhabitants of some of the world’s deserts, such as those of Australia, for example, where subsistence strategies exploited the particular nature of plants in those ecosystems. Hunter-gatherers of hot deserts rely mainly on the gathering of plants that have a great capacity to storage water. Among the G/wi San in the Kalahari Desert, plants provide each individual with an average of 4.5 litres of fluid per day in the hot dry season (Silberbauer, 1981). Over 90 % of the Kalahari San water requirement comes from plant sources (Tanaka, 1976). In contrast, most crops cultivated through intensive agriculture, such as rice, wheat, and maize, are not adapted to conserve water. Rather, their production is highly water demanding (FAO, 2011). This inevitably raises the need for irrigation or methods to control and retain natural water in dry regions, but even this depends on the reliable presence of a water source. So for example, in Egypt, intensive agriculture based on elaborate artificial waterways to use river flood cycles for irrigation is ancient and remains sustainable (Wild, 1994), while in the Wadi al-Ajjal of the Central Sahara, the successful irrigation system of *foggaras* in antiquity could not be maintained as aridification intensified. Therefore, such high technological systems and their complex socio-economic correlates that allow the establishment of plant food production in hot deserts have developed several times in human history but only in a few places have they represented stable strategies.

In cold deserts, like the Arctic and Patagonia, people who survive through *Foraging/fishing* are the exclusive inhabitants, which accounts for the easy prediction of the type of economy of human groups in those environments. This distribution is compatible with other findings showing that foragers rely on fishing in regions with low vegetal biomass (Kelly, 1999; Cordain et al., 2000; Binford, 2001). The results show that *Foraging/fishing* is, apart from trading, the only way to obtain food in “cold deserts”.

Foraging in the absence of fishing is present mostly in moderately low latitudes, with some groups also observed in boreal and temperate forests. For their distinct position in the group, these latter cases were identified as outliers. Considering both *Foraging* and *Foraging/fishing* as hunter-gatherers in the broader sense, foragers in cold deserts represent the strongest fit to the marginality concept.

A third type of marginal environments is tropical rainforests. According to the marginality theory, foragers should account for most cases in these environments. In our database, *Foraging* is concentrated in the rainforests of Southeast Asia, South America, Oceania, and Africa, but symbiotic relationships with agriculturalists are frequently observed (see De Souza, 2007; Milton, 1984, for examples). According to Bailey et al. (1989), considering carbohydrates as the most limiting resource in those habitats, the presence of foragers would only be possible in close proximity to food producers. Nevertheless, it is worth noting that certain groups of hunter-gatherers are known to have circumvented the absence of carbohydrates in rainforest environments in other ways (Hill and Baird, 2003).

Researches have discussed the difficulties imposed by rainforests over all forms of subsistence strategies. Low soil fertility represents a barrier to full reliance on agriculture (Meggers, 1954), while fresh water fishing is subjected to periodic scarcity, since fishes can become highly dispersed during the rainy season (Roosevelt, 1980). Interestingly, our data show the occurrence of all four strategies in the main rainforest complexes of the World. The existence of complex trading networks between groups is a possible explanation (Kelly, 1999), but the highly generalist strategy *Food production plus foraging/fishing* is by far the most frequent in the rainforests as in other regions of the World.

Conclusions

Human subsistence strategies are not equally distributed throughout the World. A generalist strategy composed of agriculture, foraging and fishing is the most adaptable and widespread form of food acquisition. Our findings suggest that such a combination was practiced by the first food producers and is responsible for the cooperative relations, reproductive success and expansion of agriculturalists to, virtually, everywhere. The exception are cold deserts, where foraging coupled with fishing is the only possible subsistence strategy in the absence of trade. Some degree of marginality occurs in hot deserts as well, where foragers co-exist with a small number of food-producers, mainly pastoralists. The marginality concept does not apply to rainforests, where the most abundant form of subsistence is, again, extensive food production complemented by the gathering of wild animals and forest products such as honey.

The World population is concentrated between 20° and 40° North (Kummu and Varis, 2010). In contrast, our sample has its greatest density between 15° South and 5° North, closer to the Equator. This suggests that the distribution of traditional/indigenous peoples is different from that of the world population as a whole, and in turn, that the marginalization concept might fit most ethnic minorities and not just foragers. Due to the intensification of commercial relations and technological improvements in food production, figures are probably very different today, and climatic parameters are, presumably, even weaker predictors of the distribution of contemporary subsistence strategies. Both environmental and historical factors have interacted to shape our physical and social world, with the latter playing an even stronger role in the last centuries.

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INTERACTION BETWEEN HABITAT CHARACTERISTICS AND INSECT DIVERSITY USING GROUND BEETLES (COLEOPTERA: CARABIDAE) AND ANTS (HYMENOPTERA: FORMICIDAE) WITHIN A VARIETY OF AGRICULTURAL HABITATS

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Abstract. Heterogenous agroecosystems have the capacity to maintain high insect diversity despite alterations due to human activities. The distribution of carabid beetles and ants within a variety of habitat mosaics was monitored at two climatically distinct locations. Both insect Families were monitored to compare community similarities between habitats, within and between the two sampling locations. Species occurrences were significantly different between the two locations ($p<0.05$), with distinct patterns of distribution, resulting in high dissimilarity between locations and habitats sampled. While the lowveld had highest populations and diversity of both ants and carabid beetles in unmanaged habitats, the middleveld had high carabid beetle diversity in managed habitat and populations in unmanaged habitat, while ant populations and diversity were highest in an unmanaged habitat. Although the two locations had no carabid beetle species in common, they had a few ant species in common. Due to their abundance, diversity and relation to management, both insect Families have the potential to be used as indicators in the locations assessed..

Keywords: *agroecosystems, carabid beetles, ants, habitat management, diversity, conservation*

Introduction

Land conversion for agricultural purposes affects large parts of terrestrial habitats so an understanding of its influence on biodiversity is critical (Tscharntke et al., 2005). A primary challenge in this is an understanding of how habitat alteration affects biodiversity, particularly since certain habitat characteristics and quality are known to be important drivers of local species richness (Daube et al., 2005; Weibull et al., 2003; Barragon et al., 2011). Variation in species of soil macroarthropods is related to a number of factors at habitat scale, such as land use and composition of the surrounding matrix. Habitat quality and soil conditions are of great importance to a number of epigaeic invertebrates including ants, beetles, butterflies and plants (Weibull et al., 2003; Daube et al., 2005). Differences in local species richness may be caused by processes at larger spatial scales and arthropod species, of differing size and mobility, can be regulated by different processes at the same spatial scale, e.g. carabids disperse by walking during foraging and egg-laying (although many species are capable of flight) while ants do so from mating flights of alates (Dauber et al., 2005). It is important to understand how heterogeneous landscapes may serve to maintain biodiversity and how this diversity may be affected by processes of

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transformation from traditional agroecosystems to modern ones (Perfecto et al., 1997). Since natural landscapes are usually embedded within managed agroecosystems nowadays, management practices in these can have an impact on the ability of the natural landscapes to sustain biological diversity at landscape levels (Perfecto et al., 1997). Diverse agroecosystems have the capacity to maintain high arthropod diversity despite transformation. Arthropods, which include insects, are good indicators of ecological condition since they are highly diverse and functionally important within ecosystems. Ground beetles (Coleoptera: Carabidae) and ants (Hymenoptera: Formicidae) are two invertebrates groups which have been studied within various ecosytems (Andersen et al., 2002; Gerlach et al., 2013). Ants are considered to be ecosystem engineers and are thus important components of ecosystems, constituting an important part of its animal biomass and have potential to be used as indicators (Fogariat, 1998; Peck et al., 1998; Anderson et al., 2002). This because they are capable of tracking environmental gradients and can be indicators of soil type. Carabids are also known to respond to different changes in landscape features and management (Weibull et al., 2003). Both carabids and ants are ubiquitous and abundant with known ecological relations, particularly in the northern hemisphere (Magura et al., 2000; Andersen and Majer, 2004; Underwood and Fisher, 2006). Research has been on the individual Families and this study was carried out to assess species distribution between different habitat mosaics at two distinct sampling locations for both insect Families. Since species richness is related to habitat characteristics and land use as well as the composition of the surrounding matrix (Weibull et al., 2003; Dauber et al., 2005) various habitat and soil characteristics were also measured to ascertain if this applied in this environment. Small-scale mosaics of land use types enable comparison of habitat patches differing in composition without geographical differences which would cause major bias (Dauber et al., 2005).

Materials and Methods

The study was carried out at two agricultural research stations in Swaziland. Both research stations have a variety of both natural and manmade habitat mosaics and are subsequently referred to as the sampling locations. Malkerns Research Station (MRS) is located in the middleveld region (26° 33.3S, 31° 9.93E; 739m asl) with mean temperatures ranging between 15.7 to 26.3°C and rainfall 87.6mm while the Lowveld Research Station (LES) is located in the lowveld region (26° 57.95S, 31° 31.52E; 89m asl), with mean temperatures ranging between 19.2 to 30°C and rainfall of 30.1 mm and during the sampling period. Maize is the primary crop grown at MRS while cotton is grown in the lowveld, thus the selection of these as the plots areas. Cotton is a regulated crop and has regulated pest management schedule which was followed. An attempt was made to match sample similar habitats at each location and four distinct habitat types were selected at each location depending on availability as indicated in *Table 1*.

Pitfall trapping was used to sample ground dwelling arthropods within the habitat patches. These are a convenient method of catching various epigaeic arthropods and provide information on the relative species abundance within the area sampled (Henschel et al., 2010). Within each sampling habitat, pitfall traps were placed 15m from edge and along three 20m long transects. For the maize and cotton plots, proportional distances were used since these had shorter dimensions than the unmanaged habitats. Each habitat thus had nine pitfall traps, resulting in 36 traps per location and 72 overall. Traps were half-filled with 70% ethylene glycol, which served as a preservative and were serviced fortnightly at each sampling locations. Sampling, carried out over one growing season (February to June), was begun four weeks after the maize had been planted and continued for 18 weeks, which was two weeks after the maize had been harvested.

***Table 1**. Decsription of sampling habitats within each sampling location*

MRS			LES		
Habitat	Abbreviation	Description	Habitat	Abbreviation	Description
1	MNG	Natural grassland, fallow	5	LNG	Natural grassland, fallow
2	MEB	Eucalyptus border. No plants for ground cover, no litter cover	6	LGB	Grass border between plots
3	MGB	Grass border between maize plots.	7	LCP	Cotton plot. Pesticide application as per recommended intervals (fortnightly) and weeding when needed.
4	MMP	Maize plots. No management thus increasing ground cover as maize matured	8	LPB	Pine border between sweet potato plots. Litter cover from dropped leaves.

Sampling procedure

Morphospecies were used in separating the specimen collected for both Families. The carabids were subsequently identified at the Ditsong Museum of Natural History and the morphospecies were all matched to the identification list. While the ants were also identified at AfriBugs, the initial morphospecies were maintained since a recount was not possible due to the high number involved and morphospecies also provide accurate biodiversity information (Anderson et al. 2002). With the exception of two morphospecies, the majority of specimen had been separated properly thus their scientific. Where more than one species was found by the expert, the species combination is indicated.

Habitat characteristics

Habitat characteristics were noted at three stages during the experiment (beginning, middle and end) and these were the litter cover and insolation (%) per square metre at each site. Standard soil sampling procedure was carried out, whereby 200g of soil was sampled at a depth of 10cm from different points at each habitat then mixed and analysed for various parameters at each site, i.e. pH, nitrogen (%), phosphorus (ppm), EA (me%) and Organic matter (%). Monthly rainfall and temperature for each location were obtained from the national meteorological services.

Analyses

Community analyses were carried to determine species distribution in all the habitats sampled at both sampling locations. Statistical analyses were carried out to assess differences in species dynamics between the two sampling locations and sampling habitats within each location as well as their interaction with the habitat characteristics measured. ANOVA (Statistix) was also used to determine differences between sampling occasions/weeks. Various modiles in PRIMER were used for community analyses, where the rank abundance curve, cluster analyses and ANOSIM were used. Simper was used to determine similarity between sampling habitats, by determining species typical of sampling locations and habitats. The same module also calculates levels of dissimilarity by determining species which are important for discriminating between the two sampling locations. Draughtsman plot was used to calculate Pearson’s correlation coefficients between environmental variables measured and the species. Single species occurrences within each location were excluded in the analyses to avoid vagrant species.

Results

Abundance and diversity of carabid beetles and ants

A total of 218 (19 species) of carabid beetles (*Table 2*) and 16080 (25 species) ants (*Table 3*) were collected from both sampling locations, with the middleveld location having significantly higher populations than the lowveld location ($R = 0.392$; $p = 0.001$). Due to the very significant differences in the numbers and species composition between the two sampling locations and insect Families ($P << 0.05$) subsequent analyses were carried out separately for these. *Abacetus* cf *percoides* was the dominant carabid species, making up 30.28% of all carabid beetles collected. This was also the dominant species in the middleveld, making up 38.69% of its carabid beetles while *Chlaenius notabilis* was the dominant carabid beetle species from the lowveld, making up 44% of its carabid beetles (*Figure 1a; Table 2*). Ants were dominated by the Morphospecies no. 1 at both locations (*Figure 1b; Table 3*).

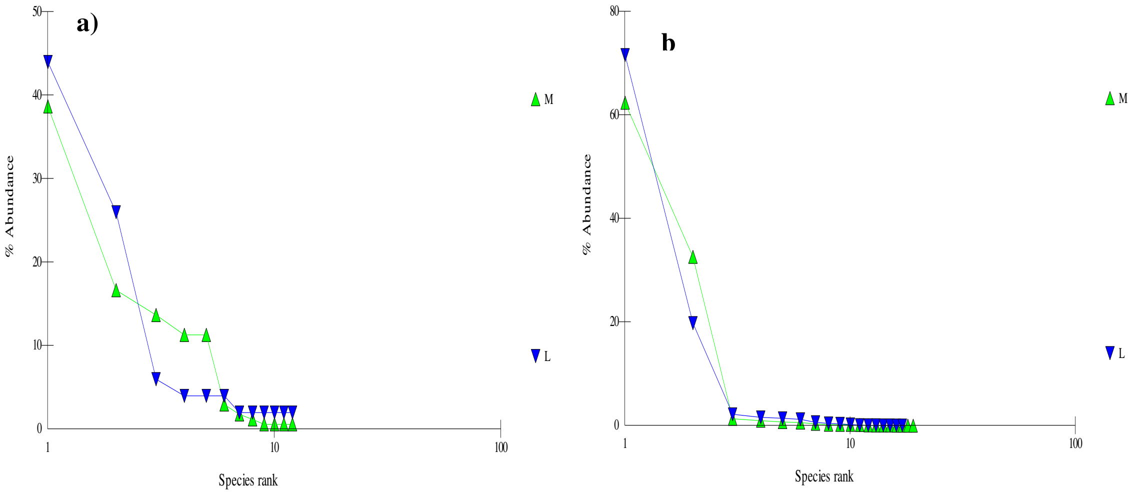

Figure1. *Species rank abundance curve for a) carabid beetles and b) ants collected from both sampling locations. M = MRS, L = LES*

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Table 2. *Total carabid beetles collected from all habitats in both sampling locations. See Table 1 for habitat abbreviations*

Species code	**MRS**						**LES**						**Total**	
	NG	EB	GB	MP	∑	% of sample	NG	GB	CP	PB	∑	% of sample	∑∑	Overall %
Abacetus cf. *percoides* Frm	5	0	10	50	65	38.69	0	1	0	0	1	2.00	66	30.28
Chlaenius cylindricollis	0	0	15	13	28	16.67	0	0	0	0	0	0.00	28	12.84
Parophonus tomentosus Dej.	1	1	2	19	23	13.69	0	0	0	0	0	0.00	23	10.55
Chlaenius notabilis Lafert	0	1	0	0	1	0.60	9	11	2	0	22	44.00	23	10.55
Unidentified *sp. #1*	0	0	4	15	19	11.31	1	0	0	0	1	2.00	20	9.17
Abacetus optimus Per.	0	0	18	1	19	11.31	0	0	0	0	0	0.00	19	8.72
Omostropus mandibularis Roth.	0	0	0	0	0	0.00	5	5	3	0	13	26.00	13	5.96
Scarites sp.	0	0	3	2	5	2.98	0	0	0	0	0	0.00	5	2.29
Progonochaetus sp.	0	0	0	0	0	0.00	2	1	0	0	3	6.00	3	1.38
Chlaenius dusaulti (L. Dufour)	0	0	0	1	1	0.60	0	1	0	1	2	4.00	3	1.38
Brachinus sp.	0	0	3	0	3	1.79	0	0	0	0	0	0.00	3	1.38
Chlaenius laetus Fab.	0	1	0	1	2	1.19	0	0	0	0	0	0.00	2	0.92
Harpalus hybridus Boh.	0	0	0	0	0	0.00	0	1	1	0	2	4.00	2	0.92
Dromica simplex	0	1	0	0	1	0.60	1	0	0	0	1	2.00	2	0.92
Callistoides cf. *pulchellus* Boheman	0	0	0	0	0	0.00	2	0	0	0	2	4.00	2	0.92
Lophyra fasciculicornis Barker	0	0	0	0	0	0.00	0	1	0	0	1	2.00	1	0.46
UnIC2	0	0	1	0	1	0.60	0	0	0	0	0	0.00	1	0.46
Amblyostoma sp. Erichson	0	0	0	0	0	0.00	0	0	1	0	1	2.00	1	0.46
Cypholoba graphipteroides Guerin	0	0	0	0	0	0.00	0	0	0	1	1	2.00	1	0.46
N	6	4	56	102	168		20	21	7	2	50		218	
S	2	4	8	8	12		6	7	4	2	12		19	
Richness d	0.56	2.16	1.74	1.51	2.15		1.67	1.97	1.54	1.44	2.18		3.34	
Evenness J'	0.65	1	0.83	0.68	0.72		0.82	0.72	0.92	1	0.70		0.76	
Shannon H'	0.45	1.39	1.72	1.42	1.78		1.47	1.41	1.28	0.69	1.74		2.23	

There were also significant differences in the number of carabid beetles and ants collected over the sampling period at both locations (p <<0.05) (*Figure 2*). Both Families had an initial increase during the second and third sampling occasions then subsequently declined.

There were significant differences in insect populations between sampling habitats at each location. In the middleveld, the grass border had significantly higher number of ants (R = 0.289, p = 0.001) while the same was true of the carabid beetles in the maize plot (R = 0.0557; p = 0.001). Comparatively, the lowveld had higher numbers of ants in the natural grassland and more carabid beetles in the grass border and natural grassland. A Principal component analysis (PCA) of the diversity indices for each sampling habitat was used to present the biodiversity information in a composite form for all the habitats sampled. For the carabid beetles, the PCA accounted for 91.6% of the information in the diversity indices where the first axis was of decreasing total species, diversity and number of beetles, while the second was of decreasing species evenness and richness (*Figure 3a*). The natural grassland in the middleveld had low richness and evenness, while the maize plot also had low evenness but the highest number of carabids collected. For ants, the PCA accounted for 87% of the information, where the first axis was of decreasing species evenness, richness and diversity while the second axis was of decreasing total number and species of ants collected (*Figure 3b*). The eucalyptus border had the highest species evenness despite low diversity and number of insects collected due to the absence of any dominant species while the maize plot at MRS had the lowest due to dominance by *Myrmicinae* ants, which also dominated the cotton plot at LES.

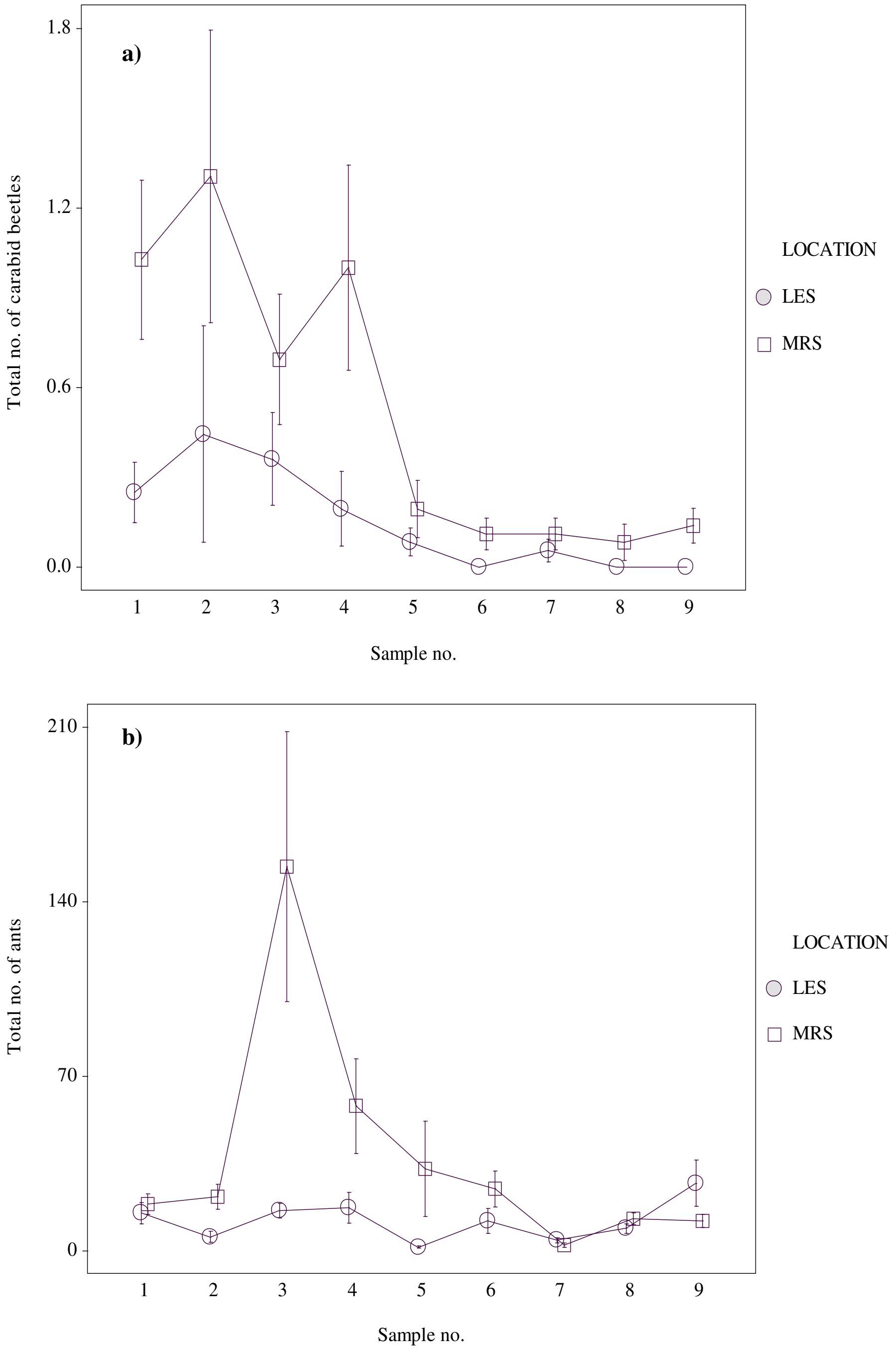

Figure 2. *Total number of a) carabid beetles (SE) and b) ants collected from both sampling locations over the sampling period*

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Table 3. *Total ants collected from all habitats in both sampling locations. See Table 1 for habitat abbreviations*

Species code	**MRS**						**LES**						**Total**	
	NG	EB	GB	MP	Σ	% of sample	NG	GB	CP	PB	Σ	% of sample	ΣΣ	Overall %
Morphospecies no. 1 (Myrmircinae)	399	170	5917	1108	7594	62.40	325	614	977	884	2800	71.61	10394	64.64
Anolepis custodiens	3872	73	26	0	3971	32.63	19	13	27	4	63	1.61	4034	25.09
Pheidole	52	32	2	2	88	0.72	672	45	21	37	775	19.82	863	5.37
Camponotus AFRC-za42	7	0	156	0	163	1.34	1	0	0	1	2	0.05	165	1.03
Leptogynys intermedia	110	5	0	3	118	0.97	0	0	0	0	0	0.00	118	0.73
Camponotus AFRC_za12	1	5	13	2	21	0.17	37	40	7	2	86	2.20	107	0.67
Polyrhachis schistacea	1	0	53	21	75	0.62	1	2	0	6	9	0.23	84	0.52
Aenictus AFRC_za03	0	0	1	1	2	0.02	34	8	0	14	56	1.43	58	0.36
Ocymyrmex fortior	0	0	0	0	0	0.00	37	9	1	1	48	1.23	48	0.30
Tetramorium notiale	38	7	0	0	45	0.37	0	0	0	1	1	0.03	46	0.29
Morphospecies no. 11	17	0	0	0	17	0.14	22	2	1	1	26	0.66	43	0.27
Technomrymex pallipes	13	11	0	0	24	0.20	4	3	1	6	14	0.36	38	0.24
Cerapachys wroughtoni	0	21	0	0	21	0.17	0	0	0	0	0	0.00	21	0.13
Odontomachus troglodytes	0	0	0	0	0	0.00	17	0	0	0	17	0.43	17	0.11
Nylanderia natalensis	9	0	0	0	9	0.07	0	0	0	0	0	0.00	9	0.06
Leptogynys schwabi	5	0	0	0	5	0.04	1	2	0	0	3	0.08	8	0.05
Lepisiota crinita	4	1	0	1	6	0.05	0	0	0	0	0	0.00	6	0.04
Camponotus AFRC-za53	1	0	1	2	4	0.03	0	0	0	0	0	0.00	4	0.02
Tetramorium sericeiventre	0	0	0	0	0	0.00	4	0	0	0	4	0.10	4	0.02
Cataulacus traegaordo	3	0	0	0	3	0.02	0	0	0	0	0	0.00	3	0.02
Camponotus mayri	0	0	0	0	0	0.00	3	0	0	0	3	0.08	3	0.02
Crematogaster AFRC_za01	2	0	0	0	2	0.02	0	0	0	0	0	0.00	2	0.01
Dorylus AFRC_za01	0	0	0	2	2	0.02	0	0	0	0	0	0.00	2	0.01
Pheidole AFRC_sz04	0	0	0	0	0	0.00	1	1	0	0	2	0.05	2	0.01
Cardiocondyla shuckardi	0	0	0	0	0	0.00	0	1	0	0	1	0.03	1	0.01
N	4534	325	6169	1142	12170		1178	740	1035	957	3910		16080	
S	16	9	8	9	19		15	12	7	11	17		25	
Richness d	1.78	1.38	0.80	1.14	1.91		1.98	1.67	0.86	1.46	1.93		2.48	
Evenness J	0.22	0.65	0.10	0.008	0.31		0.47	2.30	0.15	0.16	0.33		0.33	
Shannon H	0.62	1.42	0.22	0.18	0.91		1.28	0.74	0.28	0.39	0.95		1.05	

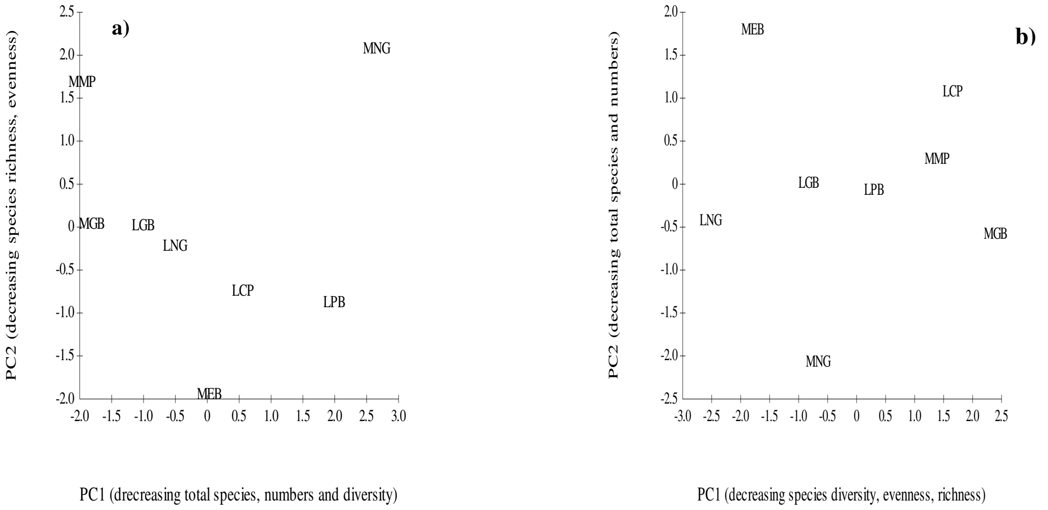

Figure 3. *PCA analysis of a) carabid beetle and b) ant diversity indices in al habitats*

Species distribution between habitats

Species composition for both Families was clearly distinct between habitats at both sampling locations (R = 0.860; p = 0.001), as observed from the respective cluster analyses carried for both Families. There was very low similarity in carabid beetle species between sampling habitats. *Figure 4a* shows separation of habitats from the two locations, with the pine border having only 3.34% similarity in carabid beetle species with the other habitats sampled. The next major branch in the dendogram is subsequently dominated by habitats from the respective locations, which had only 7.81% similarity, with the exception of the eucalyptus border habitat which was more similar to sampling habitats in the lowveld, probably due to similar habitat characteristics such as the low litter cover common between these.

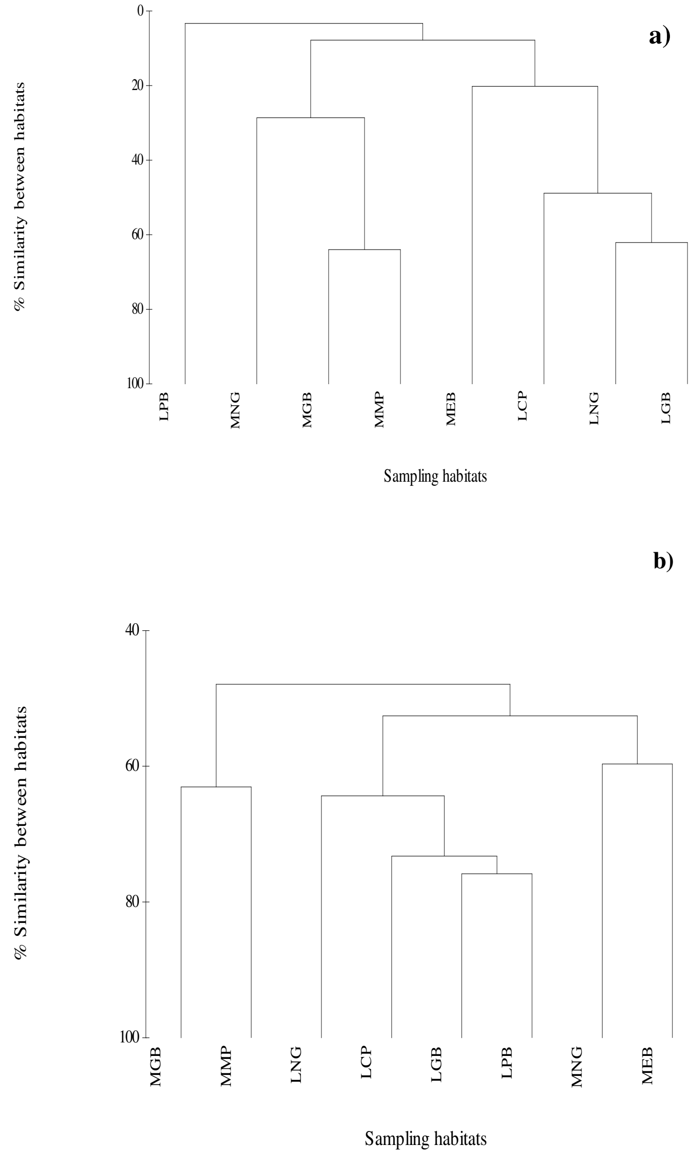

Figure 4. *Dendogram showing similarity in a) carabid beetles and b) ant species distribution between sampling habitats*

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A similar pattern was observed for the ants at the same location (*Figure 4b*) although similarity between habitats was comparatively higher, e.g. the maize plot and grass border shared 47.92% similarity with other habitats. The remaining middleveld and lowveld habitats had higher similarity at 52.56%. For both Families, habitats within each location were more similar and tended to cluster together.

Further analyses were carried in SIMPER to determine if any of the species collected were typical of the sampling habitats, thus leading to the dendograms obtained. Analysis of carabid distribution indicated that overall dissimilarity between the two locations was 95.23% and this was due to four carabid species, i.e. *A.* cf *percoides* and *P. tomentosus* were collected only from the middleveld location while *C. notabilis* and *O. mandibularis* were unique to the lowveld. Carabid species occurrences also varied within the respective sampling habitats at each location. Habitats in the middleveld had low similarity of 26.87%, and the dominant carabids at this location, i.e. *A.* cf *percoides*, *P. tomentosus* and *C. cylindricollis* were typical of only the grass border and maize plot, thus accounting for the low similarity with the other habitats. Similarity in carabid species between habitats was also comparable within lowveld habitats They had a similarity of 28.97%, with the dominant species, i.e. *O. mandibularis* and *C. notabilis* being typical of the natural grassland and grass border.

Compared to the carabid species, the two locations had a lower dissimilarity of 51.61% for ant species composition since they had more species in common compared to the carabid beetles. The dissimilarity observed was attributed to *A. custodiens*, which was overwhelmingly collected from the middleveld (3971) compared to 63 from the lowveld as well as *Pheidole*, which was collected mainly from the lowveld (775) compared to 88 from the middleveld. Middleveld habitats were differentiated by Myrmicinae sp. and *A. custodiens*, resulting in similarity of 47.65%. Myrmicinae sp. were predominantly from the grass border while *A. custodiens* was mainly in the natural grassland. Habitats in the lowveld had a higher similarity of 63.41%, with the discriminating species being Myrmicinae sp. and *Pheidole* sp., predominantly from the cotton plot and natural grassland respectively.

Draughtsman plot indicated low correlation between habitat and soil characteristics measured except pH and temperature (σ = 0.937) and EA and OM (σ = 0.986). PCA analysis of the habitat variables indicated that 72.7% of the variables was captured, with 49.5% in the first axis and 23.3% along the second axis. The first axis was dominated by decreasing OM, insolation and EA and increasing pH while the second axis was dominated by increasing N, decreasing P and rainfall (*Figure 5*).

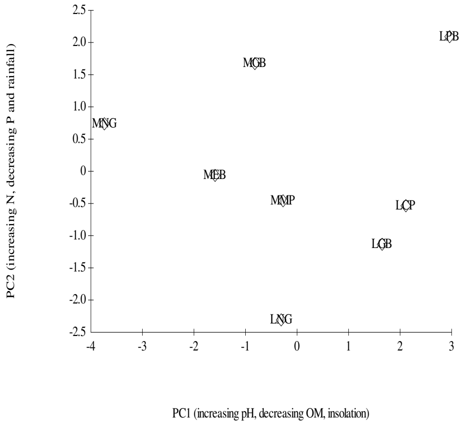

Figure 5. *PCA for environmental variables measured at the two sampling locations*

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The other variables measured did not feature significantly within the 2-dimensional plot. The carabid beetles had no significant correlation with any of the habitats variables measured, while the ants had a significant correlation with OM content ($\sigma = 0.6607$, $p = 0.0044$).

Discussion

Habitat characteristics

The habitats sampled supported a number of carabid and ants species, as expected of diverse agroecosystems. Diverse agroecosystems, as characterised by high heterogeneity, have the capacity to maintain high arthropod diversity (Peck et al., 1997). However, management practices have an impact on a habitat's ability to sustain its biological diversity. Fallow and unmanaged habitats have a positive effect on macroinvertebrate taxa within a heterogeneous landscape and this was observed in this study where the unmanaged habitats like the grass borders and natural grass had high numbers of individuals as well as species diversity despite being embedded in a matrix of managed habitats (Perfecto et al., 1997). For the two Families assessed, carabid beetle populations were highest in the maize plot and adjacent grass border while ant populations were highest in the grass border and natural grassland in middleveld habitats. At the lowveld, carabid beetle populations were highest in the natural grassland and grass border while ants were highest in the natural grass land and cotton plot. The differences in numbers and species between habitats sampled demonstrate that the various habitat patches assessed contribute to species diversity and conservation and therefore their importance as refugia for insect species. These habitat patches provide an abundant diversity of food, nesting and hiding places for ground arthropods such as ants and carabid beetles thus maintaining high diversity despite transformations of the surrounding landscape due to human activity (Perfecto et al., 1995; Goehring et al., 2002). Locally, similar results were observed in a similarly fragmented agricultural landscape, where coccinellid beetles occurred in higher numbers within managed citrus orchards, probably due to prey availability, while carabid beetles were higher in natural habitats (Magagula and Samways, 2001; Magagula, 2003). Such differences in species distribution relative to management practices emphasises the need to study more than one taxonomic group within landscapes modified by human activity.

The high numbers collected from the maize and cotton plots also emphasises that managed habitats also have a role to play in insect conservation despite anthropogenic transformation. Although habitats such as the eucalyptus and pine borders had low numbers and diversity, the majority of habitat variables measured had no correlation with the insect populations. In this study, only the ant populations had a significant correlation with OM content, as observed in other studies (Dauber et al., 2005). The primary determinant of the insect populations assessed is thus the type of management, with habitats where no management practises were carried out having higher populations and insect diversity, as in the natural grasslands and borders. It is the management practices within these habitats which determine their impact relative to invertebrates (Perfecto et al., 1997; Tsharntke et al., 2005), e.g. dung beetle communities are affected by intensified agriculture (Barragon et al., 2011). Comparatively, the cotton plot, which had weeding and pesticide applications, had the lowest total number of species and richness overall. This implies that the structure and character of a habitat are important in influencing the ecological value for ground dwelling predators, particularly with regard to shelter, microclimate and mobility

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(Melnychuk et al., 2003). The high numbers of carabid beetles within the maize plot could also have been due to the presence of shelter since various weed species, such as black jack, grass and *Desmodium* sp. were abundant in the absence of weeding (Weibull, 2003). Weeds provide high plant diversity and maintain arthropod diversity despite transformation for agricultural purposes (Perfecto et al., 1997; Melnychuk et al., 2003). The low correlation with the majority of variables measured implies that other factors not measured in this study may thus also contribute to insect species occurrences within habitats.

Borders between distinct habitat patches are known to be important for species movement between isolated habitats within a fragmented, heterogeneous landscape by serving as natural pathways between habitats (Magagula, 2003; Weibull et al., 2003). These remain important particularly in agroecosystems where habitats are divided into smaller, isolated remnants (Paolucci et al., 2012) thus acting as ecological corridors which reduce the detrimental effects of isolation between remnants in heterogeneous landscapes as well reducing isolation of populations within such landscapes. Borders and adjacent plots, like the maize plot and grass border in the middleveld, had species in common due that habitat islands usually have species in common with the surrounding matrix (Lovei et al., 2006). However, this study demonstrated that, while there may be movement between habitat patches, insect species can also be restricted within specific habitats, particularly carabid beetles, due to varying levels of adaptability within the landscape.

Species occurrences

Both sampling locations had species with variable levels of adaptability in the habitats sampled within the two Families surveyed. In the middleveld, carabid beetles were highly adaptable, whereby they established in recently disturbed habitats like the maize plot as well as undisturbed habitats like the grass borders, e.g. *A.* cf *percoides*, *C. cylindricollis* while other species like *A. optimus*, *Brachinus* sp. were restricted the natural grass border. In the lowveld, however, carabid beetles were less adaptable since they were collected primarily in undisturbed habitats, i.e. the natural grassland and grass border e.g. *C. notabilis, O. mandibularis, Progonochaetus* sp., *C.* cf *pulchellus*. Such differences between the locations emphasises the need for further study on carabid beetle ecology (Rainio, 2012) within the southern hemisphere as such studies have been mainly in landscapes in the northern hemisphere (New, 1998).

Alternately, ants illustrated generalist behaviour, with a few species in common at both sampling locations and similar habitat preferences, e.g. morphospecies no. 11 was only found in the natural grassland at both locations while *A. custodiens* was a generally found in all the habitats sampled at both locations. However, in the middleveld, this Family was less adaptable since they occurred in significantly higher numbers within undisturbed habitats, e.g. the grass border and natural grassland had significantly higher ant populations overall. Although generally widespread, the populations tended to be higher in certain habitats, e.g. morphopecies no. 1, *Camponotus* AFRC-za42 and *Camponotus* AFRC_za12 were found predominantly in the grass border while *A. custodiens, L. intermedia, T. notiale* were predominantly in the natural grassland. The natural grassland habitat had a few species unique to it, e.g. *N. natalensis, Leptogynys schwabi*, *Cataulacus traegaordo* and morphospecies no. 11 were not found in the other habitats sampled in the middleveld. Although the lowveld largely had generalist species, certain species also exhibited habitat preferences, e.g. *Camponotus* AFRC_za12 was

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found primarily in the grassland and grass border like in the middleveld, while *O. troglodyte*, *T. sericeiventre, C. mayri* were found only in the natural grassland.

These results indicate that the two Families had distinct patterns in species distribution at both sampling locations, including habitat loyalty, thus the high dissimilarity between locations and habitats observed. Carabid beetles were delineated by geographical location, with six species occurring only in middleveld habitats, three only in lowveld habitats and none (with more than a single occurrence) in common between the two locations. Ants had generalists as well as location specific species, e.g. Morphospecies no.1, *A. custodiens, Pheidole* sp. and *Camponotus* AFRC_za12 are some species these two locations had in common and those with restricted occurrence as described above. These results may suggest that ants could be better suited as indicator species since species which have a range of ecological states have been suggested to be more useful as indicators of environmental change than highly specific species with restricted distribution (Longino et al., 2002; McGeoch et al., 2002) such as the carabid beetles, particularly in agroecoystems where disturbances are not severe (Andersen and Majer, 2004). Ants are diverse and occur in any habitat type and although disturbed by agricultural practices, they recover quickly from these (Folgarait, 1998).

Conclusions

Habitat structure is an important component for many species and the spatial pattern of habitats has a major impact on maintenance of biological diversity and ecosystem function. Both Families assessed have important roles in ecosystem function, e.g. predation, seed dispersal etc. (Goehring et al., 2002; Gibb, 2012) and their conservation is of critical importance in agroecosystems thus the need for the maintenance of heterogeneous habitat patches. From this study, carabid beetle populations were affected by land use features while ants by habitat characteristics such as insolation and organic matter content (Dauber et al., 2005). There was no correlation between ants and carabids as observed in other studies (Andersen, 1995; Oliver and Beattie, 1996) where no correlation was observed between ants, beetles and spiders. Ultimately, the results suggest that habitat quality, as determined by land use, microclimate and soil characteristics need to be secured for insect species conservation, in addition to landscape heterogeneity (Dauber et al., 2005; Weibull et al., 2003) since they are important determinants of insect species occurrence, e.g. litter is significant in the maintenance of species diversity and richness (Magura et al., 2000). Depending on issues at hand, both Families thus have the potential to be used as environmental indicators due to their close abundance, diversity and close relation with habitat quality, (Peck et al., 1998; Magura et al., 2000; Andersen et al., 2002; Raino, 2012; Rosado et al., 2013). Determination of the use of these insect as indicators, however, needs further assessment over longer term to ascertain their sensitivity to change as well as their response to these changes within specific environments (Peck et al., 1998). The restriction to certain types of habitats illustrated by the species in this study, illustrates that habitat fidelity may be reflective of diversity of available habitats and human impacts within the landscape studied (Magagula and Samways, 2001). Considerations of more than one taxonomic group is thus essential since conservation strategies aimed at optimising species richness for one taxonomic group in agricultural landscapes will not automatically increase the species richness nor be representative of other groups (Weibull et al., 2003; Gerlach et al., 2013).

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APPENDIX

Electronic Appendix: Original Data

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PLANT GROWTH PROMOTING ENDOPHYTIC FUNGI ISOLATED FROM TEA (*CAMELLIA SINENSIS*) SHRUBS OF ASSAM, INDIA

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Abstract. The present paper deals with isolation of endophytic fungi from root, stem and leaves of tea (*Camellia sinensis*) shrubs collected from different tea gardens of Assam, India and evaluation of their plant growth promoting activities *in vitro*. Out of ten different endophytic fungi isolated the highest IAA (indole acetic acid) activity was observed for *Aspergillus niger* (36.49±1.17 µg/ml) followed by *Penicillium sclerotiorum* (36.35 ± 2.07 µg/ml). The highest GA_3 activity was exhibited by the fungus *Fusarium oxysporum* (12.46 ± 0.84µg/ml) followed by *P. chrysogenum* F1 (10.95 ± 0.37 µg/ml). Evaluation of insoluble mineral solubilization activities of endophytic fungi revealed *P. sclerotiorum* as most efficient phosphate solubilizer (215.98 ± 0.2 µg/ml), *A. niger* as the highest potassium solubilizer (solubilization index 1.7 4± 0.2) and *P. sclerotiorum* as the most efficient zinc solubilizer. It was also observed that plant growth promoting activities of the fungal endophytes increased up to a certain period of incubation and thereafter it decreased. As these fungal endophytes were found to be efficient in plant growth promoting activities they may have the potential to develop a biofertilizers consortium.

Key*words*: *endophytes, plant growth promoting activities, biofertilizers, mineral solubilization, IAA (indole acetic acid) production*

Introduction

Plant growth promoting microbes are heterogeneous groups of microbes associated with plants in diverse ways. The plant associated microbes colonize the rhizosphere (rhizospheric microbes), the phyllosphere (epiphytes) and inside of the plant tissue (endophytes). The word “endophyte” means “inside the plant” (derived from the Greek words “endon” meaning “within” and “phyton” meaning “plant”). Although there are diverse meanings for the term, endophytes are most commonly defined as those organisms whose “infections are inconspicuous, the infected host tissues are at least transiently symptomless, and the microbial colonization can be demonstrated to be internal” (Stone et al., 2000). In the last decade the study of endophytic microbes has become very important as these microbes are native to the host plants and most of the endophytes are beneficial to the host plants in terms of production of plant growth regulating hormones, solubilization of insoluble minerals and their antagonistic behaviour against plant pathogens and pests.

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Fungal endophytes are low in number in comparison to the bacterial endophytes. Though less in number, they are equally beneficial to the host plants. Most of the fungal endophytes including *Aspergillus flavus, A. niger, Fusarium oxysporum, Penicillium corylophilum, P. cyclopium, P. funiculosum and Rhizopus stolonifer* isolated from diverse kinds of plants were reported for their ability to produce different kinds of plant growth promoting hormones like indole acetic acid (IAA) and gibberellic acid (GA) Hassan, 2002; Waqas et al., 2012). Similar to the ability to produce plant growth regulating hormones, endophytic fungi were reported well for their mineral solubilizing activities including phosphate, zinc and potassium solubilization. Among the fungal groups *Aspergillus* and *Penicillium* are two important endophytic fungal genera having very efficient phosphate solubilizing activity (Wakelin et al., 2004; Souchie et al., 2006). Very few endophytic fungi were reported for their ability to solubilize the insoluble potassium and zinc salts. However, most of the endophytes resemble to their rhizospheric microbial groups and hence such endophytes could be assumed to have zinc and potassium solubilizing activities**.** Prajapati et al. (2012) isolated four different potassium solubilizing fungi from soils nearby ceramic industries and found that *Aspergillus niger* and *A. terreus* possess a greater potassium solubilizing activity.) *Aspergillus, Penicillium and Fusarium* were reported for their remarkable activity to solubilize different kinds of insoluble mineral salts in rocks including phosphates, zinc and potassium salts (Gaur, 1990; Simine et al., 1998). Lopes-Assad et al. (2010) reported that *Aspergillus niger* has a better ability to solubilize silicates of potassium and aluminium.

Present day agriculture is based on high inputs of agrochemicals. Over application of chemical fertilizers is one of the major causes of soil and water pollution. More over it is also responsible for depletion of soil quality. To minimize all these environmental problems modern day agriculture should concentrate on organic cultivation through biofertilizers. Though, soil is one of the most common sources for isolation of biofertilizers, adverse effects of soil microbes to plant health is not well known. As endophytes are generally harmless to the plant and have better plant growth promoting activities, manipulation of soil microflora by plant growth promoting endophytes may open a new area in the field organic agriculture.

Materials and Methods

Collection and preparation of samples

Root, stem and leaf samples were collected from mature tea (*Camellia sinensis*) shrubs from different tea gardens of upper Assam, India. Healthy and disease free young root, stem and leaf samples were collected in ice box, packed immediately in poly bags and stored at 4^0 C for further use.

Isolation of endophytes

To remove the debris root, stem and leaf samples were washed separately with tap water for seven times followed by distilled water for five times. After proper washing, about five grams of root samples were dipped in 70% ethanol for five minutes followed by 0.1% $HgCl_2$ for 1minute. However, equal amount stem and leaf samples were treated with 70% alcohol for five minutes followed by 0.05% $HgCl_2$ for 1minute. To remove the effect of surface sterilizing agents, samples were washed with sterilized distilled water for ten times (Bandara et al., 2006). After proper surface sterilization as well as removal of surface sterilizing agents, samples were homogenized in 50 ml of sterilized distilled water to prepare the stock tissue homogenate. From this tissue homogenate, proper dilution was done according to the standard process of serial dilution. Serially diluted sample tissue homogenates were spread on potato dextrose agar (PDA), Sabouraud Dextrose Agar (SDA) and Czapeck dox agar (CDA) medium (Tejera et al., 2006). Properly inoculated plates were incubated at 25ºC in an incubator for proper growth of fungal colonies. Single spore technique was used to isolate the pure fungal cultures (Choi et al., 1999). To avoid the unexpected contamination, whole process was done in front of laminar air hood.

Efficacy of the process of surface sterilization was confirmed by rolling a piece of leaf, stem or root on nutrient agar plate and incubated for 24 hours. The plate without any microbial colony after incubation period confirms the proper sterilization of the samples.

Estimation of IAA production

Quantitative estimation of IAA was performed according to Brick et al. (1991). For this purpose 100 ml of minimal salt (MS) medium were poured into 250 ml Erlenmeyer flasks, autoclaved and allowed to cool at room temperature. All the flasks were amended with filter sterilized L-tryptophan (1000 µg/ml). Flasks were inoculated with 1 ml of fungal spore suspensions (2 x10^6 spores/ml) and incubated at 25^0 C. The control flask was incubated without inoculation with spore suspension. Two, four, six and eight days old culture supernatants from each flask were centrifuged at 3,000 rpm for 10 minutes. For measuring the amount of IAA produced, 1ml of culture supernatant was pipetted into test tubes and mixed with 2 ml of $FeCl_3$-perchloric acid reagent (50 ml 35% perchloric acid + 1 ml 0.5 M FeCl3 solution) and 2 drops of *ortho*-phosphoric acid. After 25 mins development of pink colour was measured at 530 nm wave length by UV-VIS spectrophotometer (CECIL CE 7250).

Estimation of Gibberellic acid (GA3)

Spore suspension of 2 x10^6 spores per ml were directly inoculated into the flasks with 100 ml MS medium amended with 1000 µg/ml of tryptophan and incubated at 25º C up to 6 days. Amount of GA_3 present in the culture supernatant was determined according to the standard method forwarded by Uthandi et al. (2010). 30 ml of 6 days old broth culture from each flask were taken and centrifuged at 3000 rpm to remove the particulate matters including fungal hyphae. Twenty five ml of culture supernatant was taken in to a 40 ml test tube to which 2 ml of zinc acetate (1M) was added. After 2 minutes 2 ml of potassium ferrocyanide was added and centrifuged at 1000 rpm for 15 minutes. To 5 ml of this supernatant, equal volume of 30% HCL was added slowly and incubated at 20ºC for 75 minutes. An uninoculated broth was taken as reference and this blank sample was also treated in the same manner and the absorbancy was measured spectrophotometrically at 254 nm wave length.

Phosphate solubilization

Screening of fungal isolates for their phosphate solubilizing activity was done in Pikovskya's agar (glucose,10 g/L; $Ca_3(PO_4)$ 2, 5 g/L; $(NH_4)_2$ SO_4, 0.5 g/L; NaCl, 0.2 g/L; $MgSO_4.7H_2$ O, 0.1 g/L; KCl, 0.2 g/L; yeast extract, 0.5 g/L; $MnSO_4H_2O$, 0.002

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g/L; and $FeSO_4.7H_2O$, 0.002 g/L, agar 15 g/L) medium plates. Isolates showing clear zones around the growing colonies after 72 hrs of incubation at 25^0 C were considered as positive for P solubilization (Gour, 1990).

Quantitative estimation of phosphate solubilization in broth culture was performed according to the procedure of Jackson (1973). All the fungal isolates were grown seperately in 500 ml conical flasks containing 250 ml of Pikovskya's broth. . One uninoculated flask was used as control. Flasks were incubated at 25^0 C for ten days in a shaking incubator at 250 rpm. Five ml of four, six, eight and ten day's old cultures were centrifuged at 3,000 rpm for 10 mins. One ml of supernatant from each flask was mixed with 10 ml of chloromolybdic acid and the volume was adjusted to 40 ml with distilled water. To this mixture, 1 ml of chlorostannous acid was added. Final volume was made 50 ml by adding distilled water. Absorbance of the developing blue colour was measured at 600 nm wave length with UV- VIS spectrophotometer (CECIL CE 7250). The amount of soluble phosphate was calculated from standard curve of $KH2PO_4$. Periodic estimation of culture pH was performed by using digital pH meter (EUTECH pc 510).

Zinc solubilization

Screening of endophytic fungi for their zinc solubilization activity was done by using halo zone formation method in Basal medium (glucose-10.0 g/L; ammonium sulphate-1.0 g/L; potassium chloride-0.2 g/L; dipotassium hydrogen phosphate-0.1 g/L; magnesium sulphate-0.2 g/L; pH 7.0) plates containing 0.1% insoluble ZnO or ZnS (Venkatakrishnan et al. 2003). Solubilization index was calculated by using the standard formula:

$$\text{Zinc solubilization Index} = \frac{\text{Total diameter of the halo}}{\text{Diameter of the colony}}$$

Potassium solubilization

The isolates were screened for their potassium solubilizing activity by halo zone formation method in Aleksandrov agar medium (1% glucose, 0.5% Yeast extract, 0.05% $MgSo_4.7H_2O$, 0.0005% $FeCl_3$, 0.01% $CaCO_3$, 0.2% $CaPo_4$ and 3 % agar) containing o.2% insoluble potassium bearing mineral; mica (Hu et al. 2006). The plates were incubated at room temperature (30±1°C) for 3 days and the colonies exhibiting clear zones around them were considered as positive for potassium solubilization activity. Potassium solubilization index was calculated with the help of following formula.

$$\text{Potassium solubilization Index} = \frac{\text{Total diameter of the halo}}{\text{Diameter of the colony}}$$

Extraction of Fungal genomic DNA

Six days old fungal colonies were collected from PDA plates and ground to fine pastes in an 1.5 ml eppendorf tubes by adding small amount of sterilized sands and 400 µl of fungal lysis buffer (tris HCl-50 mM; EDTA-50 mM; SDS- 3%; ßmarcaptoethanol 1% just before use) to each tube. Centrifuged the mixture for 10 minutes at 12,000 rpm at 4ºC. Transferred the supernatants into new eppendorf tubes and mixed with 300 µl of phenol and 300 µl of chloroform: isoamyl alcohol (24:1). Shaken well and then centrifuged at 4ºC with 12000 rpm for 10 minutes. Supernatants were collected into new eppendorf tubes supplemented with 500 µl of 99.9% (v/v) chilled isopropanol; incubated the mixture at -20ºC (minus 20ºC) for overnight. Next morning, tubes were centrifuged at 12000 rpm for 10 minutes, discarded the supernatants slowly, washed the pallets gently with 70% ethanol, air dried for 30 minutes and dissolved the pallets in 30 µl tris- EDTA (1 mM tris HCl and 1 mM EDTA with pH 8.0) individually. Treated the solution with 2 µl of RNAse and loaded in 0.8% agarose gel electrophoresis to confirm the presence of DNA. DNA concentration in the solution was determined with the help of nano-drop DNA quantifier.

Amplification of fungal ITS region

Amplification of fungal ITS (internal transcribed spacer) was carried out in 25 µl PCR reaction mixture (Taq buffer- 2.5 µl; $MgCl_2$- 2.0 µl; 2.5 mM dNTP mix- 2.5 µl; forward and reverse primer 0.5 µl of each; sterilized distilled water- 14 µl; template DNA- 2 µl, Taq polymerase 1 µl) using universal ITS 1 (5′ CCG AAT TCG TCG ACA ACC TGG TTG ATC CTG CCA GT 3′ and ITS 4 (5′ CCC GGG ATC CAA GCT TGA TCC TTC TGC AGG TTC ACC T 3′) oligonucleotide sequences as forward and reverse primer respectively. DNA was amplified over 35 cycles of PCR profile with 94ºC for 3 min, 94ºC for 1 min, 50ºC for 30 s, 72ºC for 1 min, 72ºC for 7 min 4ºC for infinity. Amplification of ITS was confirmed by 1.2% agarose gel electrophoresis and the concentration of DNA was determined by using nano-drop DNA quantifier. The amplified PCR products were sequenced by Sanger dideoxy sequencing method in ABS 3700 sequencer system.

Results

IAA activity

Among 10 different fungal endophytes isolated from tea, 8 were found to be efficient IAA producers. Periodic estimation of IAA production of the fungal endophytes revealed that their activity increased gradually up to day 6 and thereafter, decreased (*Table 1*).

Table 1. *IAA activity of fungal endophytes of tea plant.*

Fungal endophytes	***IAA concentration in µg/ml***			
	Day 2	***Day 4***	***Day 6***	***Day 8***
P. chrysogenum F1	8.12±0.13	17.47±1.07	28.33±0.73	21.41±1.09
A. fumigatus	10.09±0.31	14.26±2.30	24.55±0.59	19.92±1.13
P. crustosum	8.06±0.22	18.35±1.00	30.62±1.78	22.94±1.10
A. niger	**11.25±0.9**	22.89±1.74	**36.49±1.17**	29.14±1.36
P. chrysogenum F5	9.176±0.36	19.9±1.57	28.33±1.97	23.32±0.86

P. sp F 7	8.73±1.15	15.76±0.80	25.51±0.68	18.58±0.95
F. oxysporum	**5.10±0.14**	**13.1±0.39**	20.56±0.75	17.42±0.21
P. sclerotiorum	10.17±1.08	25.29±0.89	36.35±2.07	33.07±1.78

Values are mean ± SD of three replicates

During the study period, IAA activity ranged between 5.10±0.14 and 11.25±0.9 µg/ml after 2 days; 13.1±0.39 and 25.29±0.89 µg/ml after 4 days; 20.56±0.75 and 36.49±1.17 µg/ml after 6 days and 17.42±0.21 and 33.07±1.78 µg/ml after 8 days of incubation. The highest IAA activity was shown by *Aspergillus niger* (36.49±1.17 µg/ml) followed by *Penicillium sclerotiorum* (36.35±2.07 µg/ml), *P. crustosum* (30.62±1.78 µg/ml) and *P. chrysogenum* F5 (28.33±1.97 µg/ml) after 6 days of incubation (*Fig. 1*).

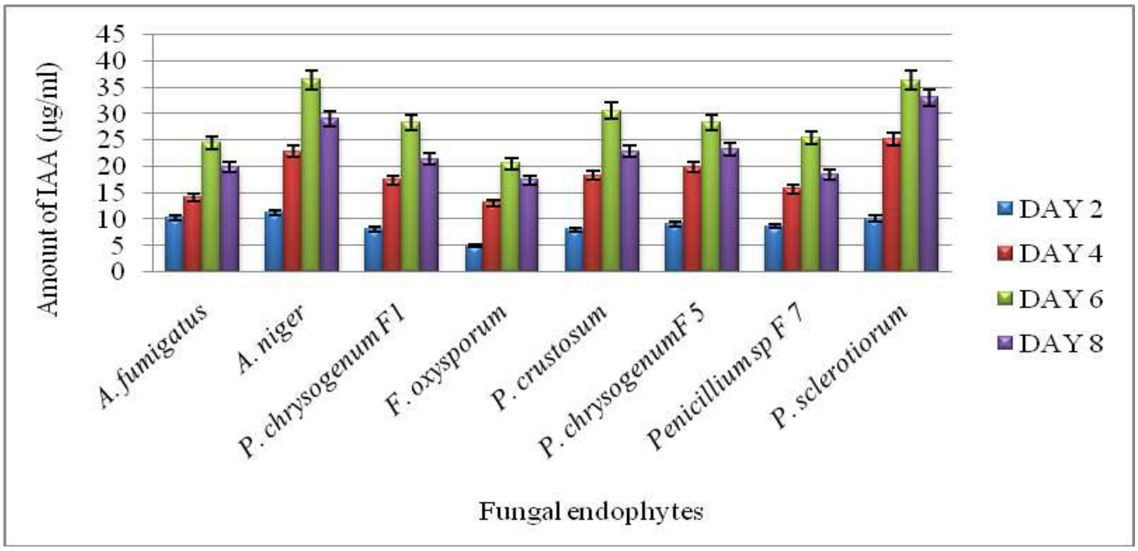

Figure. 1. *Indole acetic acid activity of fungal endophytes of tea plant.*

GA_3 activity

Estimation of GA_3 in the culture supernatant after 6 days of incubation showed that the activity ranged between 6.98 ±0.77 µg/ml and 12.46 ±0.84 µg/ml. The highest GA_3 activity was exhibited by *Fusarium oxysporum* (12.46 ±0.84 µg/ml) followed by *Penicillium chrysogenum* F1(10.95 ±0.37 µg/ml), *Aspergillus fumigatus* (10.04 ±0.20 µg/ml), *A. niger* (9.87 ±0.12 µg/ml) and *P. sclerotiorum* (9.86 ±0.30 µg/ml) (*Table 2; Fig. 2*).

Table 2. *GA3 activity of fungal endophytes of tea plant.*

Fungal endophytes	***Amount of GA_3 in µg/ml***
Penicillium chrysogenum F1	10.95 ±0.37
Aspergillus fumigatus	10.04 ±0.20
Penicillium crustosum	8.71 ±0.54

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Aspergillus niger	9.87 ±0.12
Penicillium chrysogenum F5	6.98 ±0.77
Penicillium sp F 7	7.17 ±0.23
Fusarium oxysporum	**12.46** ±0.84
Penicillium sclerotiorum	9.86 ±0.30

Values are mean ± SD of three replicates

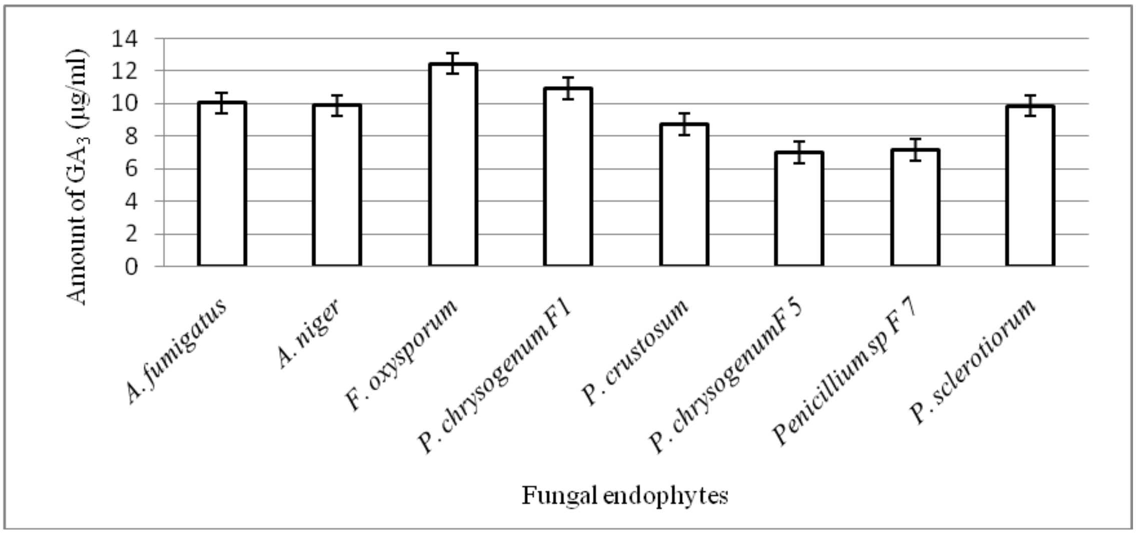

Figure 2*. GA$_3$ activity of fungal endophytes of tea plant.*

Phosphate solubilizing activity

Phosphate solubilizing enzyme activity of the fungal endophytes was carried out up to 10 days. It was observed that the enzyme activity increased and reached a maximum on day 8 thereafter, there was gradual decrease (*Table 3*).

Table 3. *Phosphate solubilizing activity of fungal endophytes of tea plant.*

Fungal endophytes	***Amount of phosphate released (in µg/ml)***							
	Day 4		***Day 6***		***Day 8***		***Day 10***	
	Soluble P	pH	Soluble P	pH	Soluble P	pH	Soluble P	pH
P. chrysogenum F1	49.90 ±2.7	5.2±0.1	63.28 ± 2.0	4.06±0.05	86.1±0,.9	3.3±0.1	80.55±0.7	4.50±0.3
A. fumigatus	67.83 ±1.9	5.11±0.1	132.75±2.0	5.32±1.05	167.35±1.9	3.49±0.2	134.5±2.1	4.21±0.1
P. crustosum	47.35 ±1.9	20.63±0.1	72.43 ± 2.1	4.55±0.1	84.23±1.2	3.53±0.1	75.25±1.2	4.6±0.01
A. niger	87.25 ±2.2	5.4±0.1	146.44±1.7	5±0.1	170.25±1.2	3.86±0.01	143.59±0.9	4.62±0.3
P. chrysogenum F5	72.55 ± 2.0	5.26±0.1	116.66±2.0	5.58±0.5	137.41±1.7	3.9± 0.02	116.36±1.8	3.29±0.2
Penicillium sp F 7	91.8 ± 3.8	5.63±0.05	157.50±0.7	4.52±0.1	193.02±1.5	3.99± 0.08	136.33±1.7	3.30±0.3
F. oxysporum	68.33 ± 1.6	5.8±0.1	125.56±1.2	4.73±0.1	146.26±0.8	3.415±0.4	128.82±1.6	4.84±0.9
C. gossypiicola	59.9 ± 1.9	5.63±0.2	123.72±1.0	4.05±0.05	155.43±1.8	4.35±1.2	142.49±1.0	3.82±0.8
P. sclerotiorum	100.33±2.0	5.01±0.01	177 ± 1.7	4.03±0.02	215.98±0.2	3.67±0.02	198.03±2.3	3.91±0.4

Values are mean ± SD of three replicates

Phosphate solubilization activity was measured in terms of amount of soluble phosphate in the culture supernatant. Phosphate solubilization activity ranged between 49.90 ± 2.7 and100.33± 2.0 µg/ml after 4days, 63.28 ± 2.0 and 177 ± 1.7 µg/ml after 6 days; 84.23±1.2 and 215.98±0.2 µg/ml after 8 days and between 75.25±1.2 and 198.03±2.3 µg/ml after 10 days of incubation. The highest phosphate solubilization activity was observed for *P. sclerotiorum* (215.98±0.2 µg/ml) followed by *Penicillium sp.* F 7 (193.02±1.5 µg/ml), *A. niger* (170.25±1.2 µg/ml) and *A. fumigates* (167.35±1.9 µg/ml) after 8 days of incubation. Other fungal endophytes also exhibited phosphate solubilization activity. It was also observed that pH of the medium decreased gradually till day 8 and then slightly increased thereafter. Hence, soluble phosphate in the culture medium increased with a decrease of pH (*Fig. 3*) due to the secretion of different organic acids into the medium (*Fig. 4*).

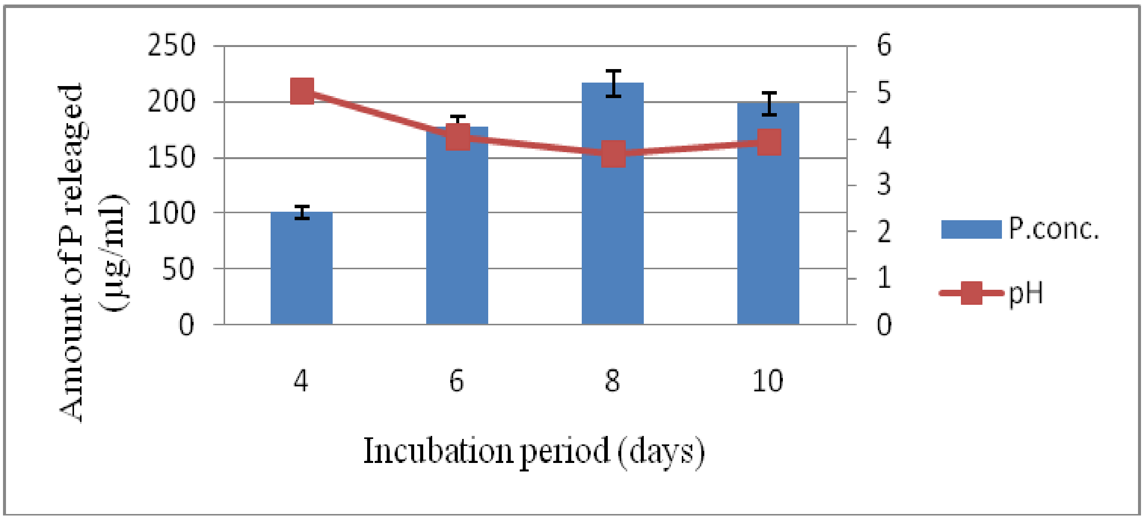

***Figure 3**. pH drop and phosphate solubilization for P. sclerotiorum*

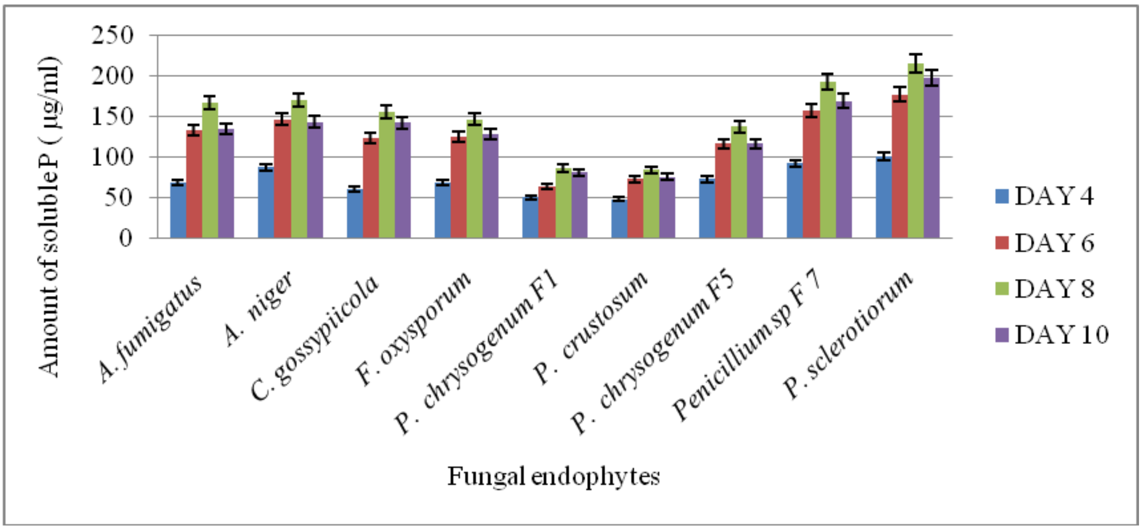

***Figure 4.** Phosphate solubilizing activity of fungal endophytes of tea plant.*

Zinc solubilizing activity

Fungal endophytes were screened for their ability to solubilize the insoluble zinc salts in terms of solubilization index through halo zone formation method. Periodic observation of zinc solubilization index for ZnO and ZnS revealed that the solubilization index increased gradually up to day 9 and then decreased.

For ZnO, the solubilization index ranged between 0.97±0.01 and 1.60±0.02 after 3 days; 1.21±0.01 and 2.36±0.04 after 6 days; 1.4±0.04 and 2.87±0.01 after 9 days and between 1.24±0.01 and 2.66±0.03 after 12 days of incubation (*Table 4*). The highest ZnO solubilization activity was observed for *P. sclerotiorum* (2.87±0.01) followed by *Penicillium sp. F 7* (2.22±0.02), *A. niger* (1.96±0.01) and *P. chrysogenum F 5* (1.87±0.005) after 9 days of incubation period (*Table 4; Fig. 5*).

Table 4. *Zinc solubilization (for ZnO salt) by the fungal endophytes of tea plant.*

Fungal endophytes	***Zinc solubilization index for ZnO***			
	Day 3	***Day 6***	***Day 9***	***Day 12***
P. chrysogenum F1	1.27±0.04	1.36±0.02	1.75±0.03	1.60±0.01
A. fumigatus	1.07±0.04	1.26±0.01	1.66±0.01	1.53±0.01
P. crustosum	1.05±0.06	1.29±0.005	1.44±0.01	1.24±0.01
A. niger	1.44±0.06	1.67±0.01	1.96±0.01	1.77±0.01
P. chrysogenumF 5	1.3±0.02	1.51±0.01	1.87±0.005	1.65±0.01
Unidentified	1.24±0.02	1.48±0.02	1.76±0.01	1.55±0.01
Penicillium sp F 7	1.47±0.04	1.74±0.02	2.22±0.02	1.94±0.02
C. gossypiicola	0.97±0.01	1.21±0.01	1.4±0.04	1.3±0.01
P. sclerotiorum	1.60±0.02	2.36±0.04	2.87±0.01	2.66±0.03

Values are mean ± SD of three replicates

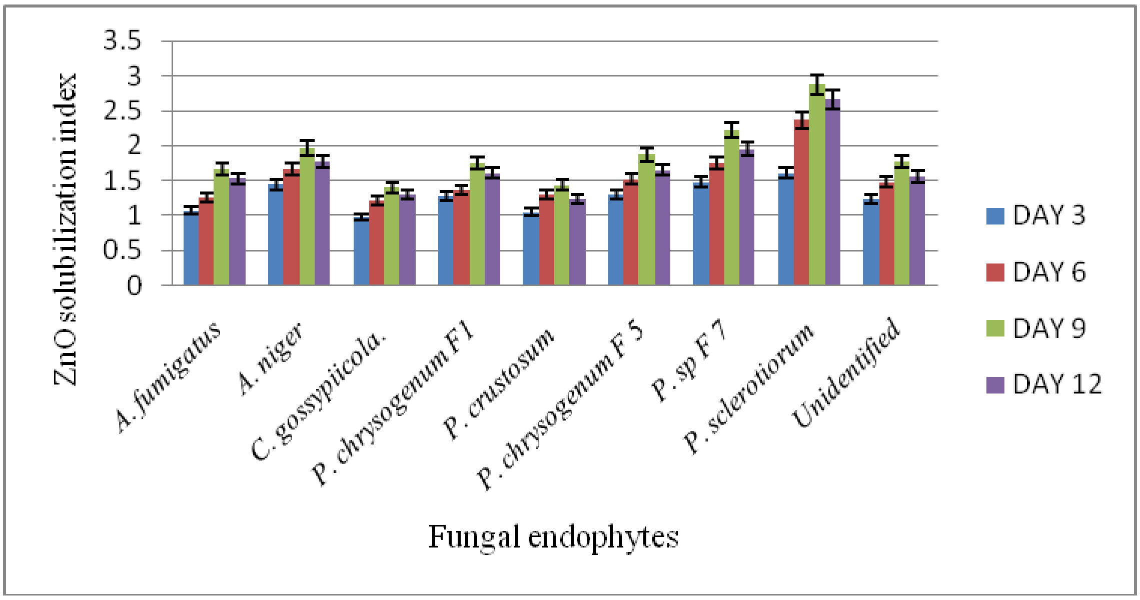

Figure 5. *Zinc solubilization (for ZnO) activity of fungal endophytes of tea plant.*

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The fungal endophytes were able to solubilize ZnS and the zinc sulphate solubilization activity of fungal endophytes ranged between 1.12±0.02 and 1.70±0.02 after 3 days; 1.28±0.01and 2.49±0.03 after 6 days; 1.56±0.01 and 3.29±0.02 after 9 days and between 1.3±0.02 and 3.04±0.06 after 12 days of incubation. The highest ZnS solubilization index was observed for *P. sclerotiorum* (3.29±0.02) followed by *Penicillium sp.* F 7 (2.51±0.04), *P. chrysogenum* F 5 (2.09±0.05) and *A. niger* (1.96±0.01) after 9 days of incubation (*Table 5, Fig. 6*).

Table 5. *Zinc solubilization (for ZnS salt) by the fungal endophytes of tea plant.*

Fungal endophytes	***Zinc solubilization index for ZnS***			
	Day 3	*Day 6*	*Day 9*	*Day 12*
P. chrysogenum F1	1.34±0.01	1.48±0.03	1.81±0.03	1.65±0.02
A. fumigatus	1.16±0.03	1.37±0.01	1.56±0.02	1.42±0.02
P. crustosum	1.12±0.04	1.28±0.01	1.56±0.01	1.44±0.01
A. niger	1.54±0.02	1.74±0.02	1.96±0.01	1.74±0.02
P. chrysogenum F 5	1.41±0.01	1.68±0.01	2.09±0.05	1.87±0.01
Unidentified	1.36±0.02	1.52±0.02	1.9±0.04	1.75±0.01
Penicillium sp F 7	1.67±0.02	1.87±0.02	2.51±0.04	1.64±0.5
C. gossypiicola	1.12±0.02	1.34±0.02	1.56±0.03	1.3±0.02
P. sclerotiorum	1.70±0.02	2.49±0.03	3.29±0.02	3.04±0.06

Values are mean ± SD of three replicates

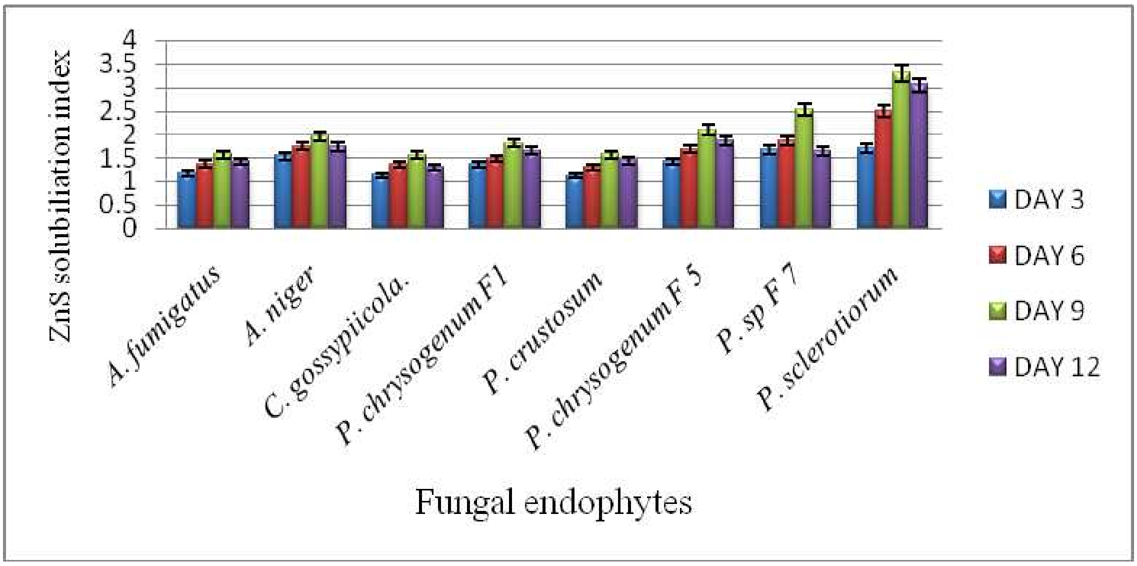

Figure 6. *Zinc solubilization (for ZnS) activity of fungal endophytes of tea plant.*

Potassium solubilizing activity

Potassium solubilization activity of the fungal endophytes was estimated by calculating the solubilization index (*Table 6*). The process of potassium solubilization is a very slow process as revealed by this study. Potassium solubilization index ranged

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between 0.28±0.05 and 0.76±0.06 after 5 days, 0.95±0.16 and 1.50±0.21 after 10 days and 1.27±0.21 and 1.74±0.2 after 15 days. The highest potassium solubilization activity was observed in *A. niger* (1.74±0.2) followed by *P. crustosum* (1.64±0.16) and *P. chrysogenum* F1(1.62±0.21) after 15 days of incubation. Other fungal endophytes have also exhibited K solubilization activity (*Fig. 7*).

Table 6. *Potassium solubilization by the fungal endophytes of tea plant.*

Fungal endophytes	***Potassium solubilization index***		
	Day 5	***Day 10***	***Day 15***
P. chrysogenum F1	0.53±0.06	1.27±0.12	1.62±0.21
A. fumigatus	0.45±0.04	1.47±0.34	1.45±0.24
P. crustosum	0.67±0.08	1.38±0.12	1.64±0.16
A. niger	0.76±0.06	1.50±0.21	1.74±0.2
P. chrysogenumF 5	0.52±0.08	0.95±0.16	1.30±0.18
Penicillium sp F 7	0.59±0.03	1.16±0.11	1.36±0.17
F. oxysporum	0.28±0.05	0.98±0.13	1.27±0.21
P. sclerotiorum	0.68±0.01	1.24±0.16	1.47±0.17

Values are mean ± SD of three replicates

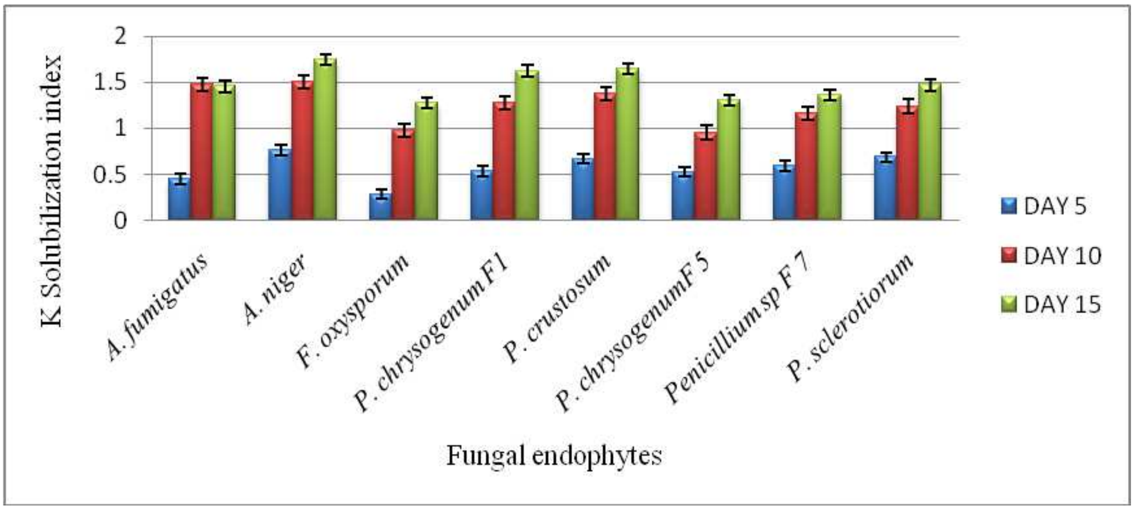

Figure. 7. *Potassium solubilization activity of fungal endophytes of tea.*

Phylogenetic analysis of the endophytic fungi

Fungal isolates were characterized at molecular level by amplifying and sequencing their ITS region (*Fig. 8*). The isolates were identified using similarity percentage through BLAST and sequences were submitted to genbank, NCBI Among the fungal endophytes, different species of *Penicillum* were found to be predominant. The phylogenetic relationship among them is depicted in *Fig. 9*.

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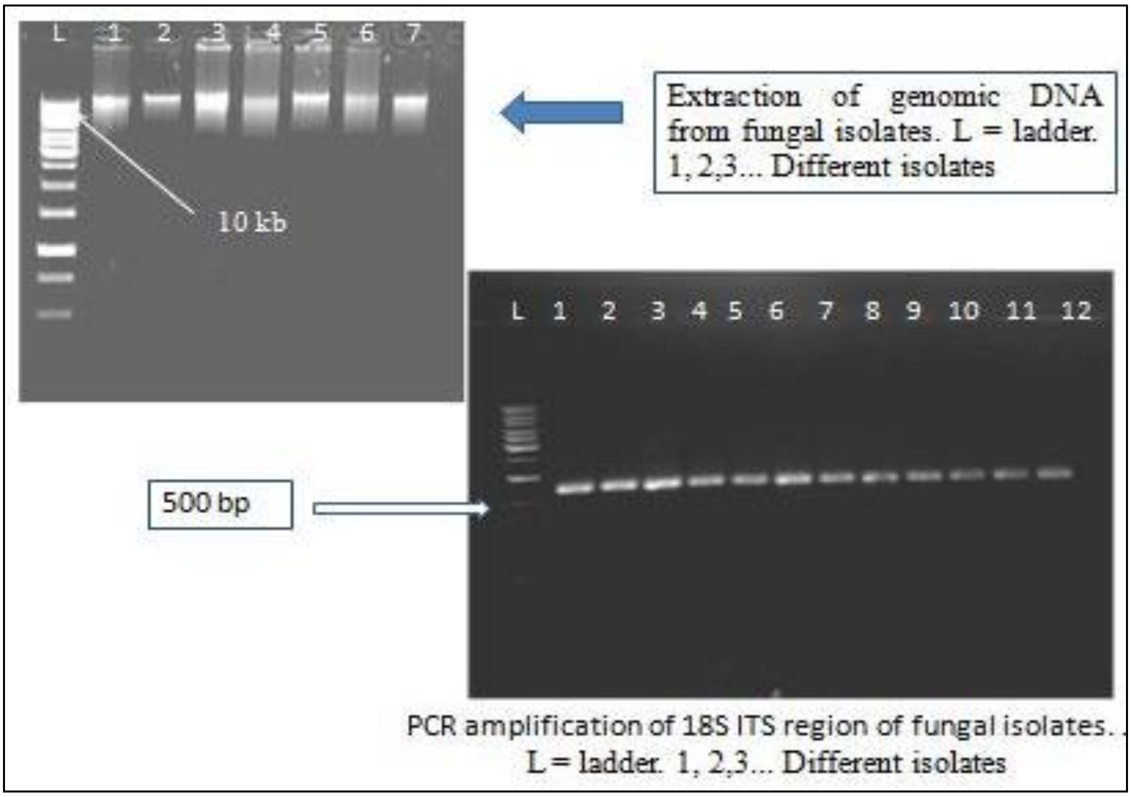

Figure 8. *Isolation of genomic DNA from fungal isolates and their ITS amplification in gel.*

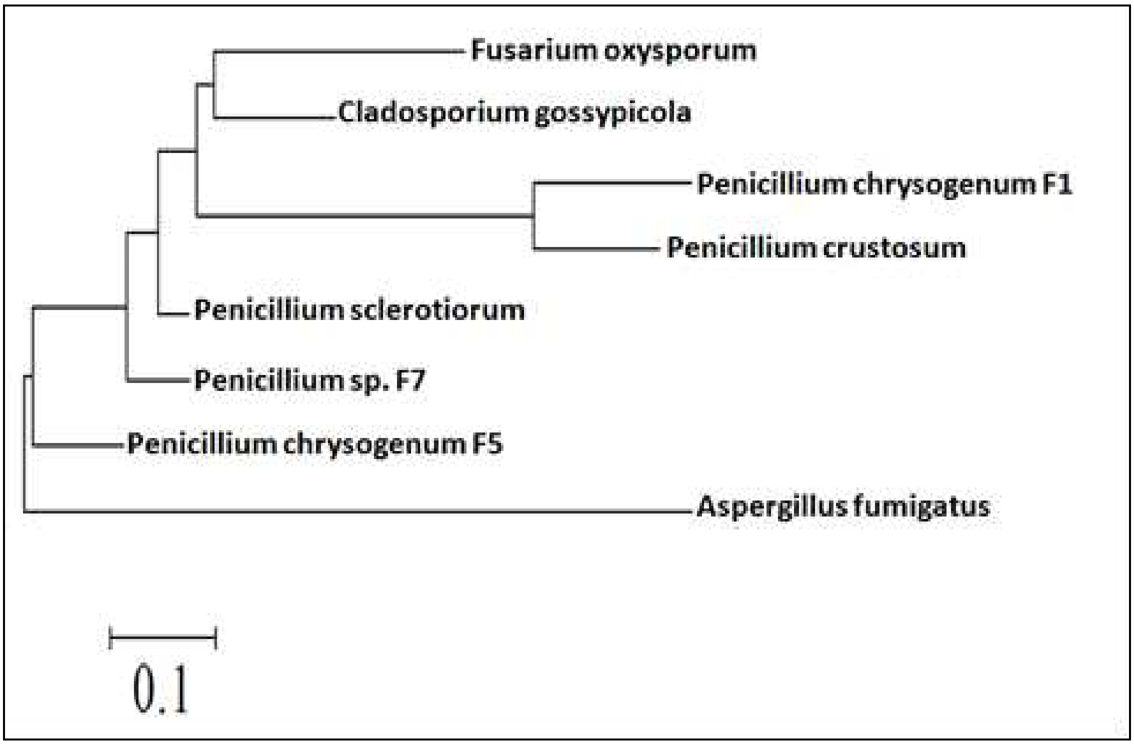

Figure 9. *Phylogenetic relationship of endophytic fungi isolated from tea plant*

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Discussion

Plant rhizosphere is the main store house for most of the plant growth promoting microbes. From rhizosphere some microbes which are best compatible to the plant internal environment enter in to the root cortex mainly through the cracks at the site of emergence of lateral roots and then migrate towards the apical parts of the plant body though xylem (Zachow et al., 2010). Most of these endophytes are not only compatible to the host internal environment but have higher plant growth promoting activities in comparison to their rhizospheric partners (Tsavkelova et al., 2007). Hence, exploration of endophytic microbes has a greater opportunity to develop biofertilizers superior to biofertilizers developed from other sources.

The main aim of the present study was to isolate and characterize the fungal endophytes of *Camellia sinensis* for their plant growth promoting activities *in vitro*. The results mentioned above depict efficient plant growth promoting activities of the isolated endophytic fungi. In the present study *Aspergillus niger* was found to be the most efficient in IAA production and potassium solubilization activities. The highest GA_3 activity was exhibited by *Fusarium oxysporum*. Zinc and potassium solubilization activity was observed to be highest in *P. sclerotiorum* . Hassan (2002) reported some species of *Penicillium, Aspergillus and Rhizopus* for their IAA and GA_3 producing activities. Similarly, Uthandi et al. (2010) reported *Fusarium fijikori* SG2 as one of the best GA_3 producer. Many *Trichoderma* associated with rhizosphere and endosphere of *Calophyllum brasiense* were also reported for their plant growth promoting hormone producing and mineral solubilizing activities (Resende et al., 2014). Many bacterial, fungal, yeast, and actinomycetes species capable of solubilizing sparingly soluble phosphorus in pure culture have been isolated and studied (Halder et al., 1991; Abd-Alla, 1994; Whitelaw, 2000; Goldstein, 1986). The main mechanism of phosphate and other mineral solubilization is the production of diverse kinds of organic acids that lowers the pH of the medium causing solubilization of insoluble mineral salts (Ahmad and Jha, 1968). Among phosphate solubilizing fungi species of *Aspergillus, Penicillium* and yeast have been widely reported for solubilizing various forms of inorganic phosphates (Whitelaw, 2000). *Aspergillus niger Penicillium simplicissimum*, *Penicillium expansum* and *Scopulariopsis brevicaulis* were reported to solubilize the insoluble potassium salts such as potassium aluminosilicates (Sterflinger, 2000). Lots of fungal genera including some ericoid mycorrhizal fungi (mycorrhizal endophytes of *Woollsia pungens*; Epacridaceae, *Hymenoscyphus ericae* and *Oidiodendron maius*) were reported to dissolve hydroxyapatite, Zn oxide and phosphate (Van Leerdam et al, 2001; Martino et al., 2003)**.** These results are in agreement with the results of the present study. Moreover, the endophytic fungi isolated from *Camellia sinensis* in the present study were found to be superior in plant growth promoting activities in comparison to the earlier reports. Hence, the fungal endophytes isolated from *Camellia sinensis* were efficient in plant growth promoting activities and are assumed to be a future source of biofertilizers consortium for *Camellia sinensis* and other crops.

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HABITAT TYPES – WHAT THEY CAN TELL US NOW AND IN THE FUTURE

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Abstract. Habitat classification systems utilize the relationship between the herbaceous layer and potential climax vegetation to classify forest vegetation. Habitat classification systems have been developed throughout the United States including Michigan. In 2010, ten years after the first sampling, 30 of the original 200 plots throughout the Western Upper Peninsula were resampled twice during the growing season. Exotic earthworm populations were also sampled in early September at all 30 plots. Nonmetric multidimensional scaling (NMS) ordination was used to discern differences in habitat types between years (2000 vs. 2010) and between seasons in 2010 (spring vs. summer). Overstory trees per hectare (TPH) decreased from 2000 to 2010, likely the result of forest management activities. A greater number of herbaceous species were observed in 2010; however, the majority of these new species were weedy or invasive. Exotic European earthworms were observed in all habitat types; earthworm densities generally increased with increasing soil richness and site quality, with herbaceous plant cover negatively associated with earthworm biomass. Continual monitoring of these plots will allow scientists and managers to assess how herbaceous community change through time and observe the effects of invasive species and changing climatic patterns on forest ecosystems of the Great Lakes region.

Keywords: *exotic earthworms, herbaceous species, forest dynamics, Great Lakes Region, forest management*

Introduction

Every forest ecosystem is distinct and requires individual consideration when developing management options. Scientists and managers often classify forests with similar attributes to help guide management decisions and to allow for comparisons when different variables are manipulated. Forest classification systems range from the use of one variable (e.g., dominant overstory vegetation to characterize forest cover type) to the use of many complex variables including climate, soils, geology, and vegetation cover (as used in the Terrestrial Ecological Unit Inventory (TEUI); Winthers et al., 2005). Habitat typing was first developed in the Western United States and relies on the presence/absence of particular herbaceous species (see Daubenmire and Daubenmire, 1968). This system of classification was later developed for the Great Lakes region by sampling plots across the Upper Peninsula of Michigan and Northern Wisconsin (Coffman et al., 1983). Further sampling of the Upper and Lower Peninsulas

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of Michigan allowed for the development of *The Guide to Forest Communities and Habitat Types of Michigan* (Burger and Kotar, 2003).

Early habitat classification systems utilized key concepts from both Clements' (1916, 1936) and Gleason's (1926) theories of vegetation development (Daubenmire, 1976). Habitat typing relies on the early stabilization of the understory, especially the herbaceous layer, compared to the overstory (Daubenmire and Daubenmire, 1968; Pfister and Arno, 1980; Kotar, 1986; Pfister, 1989). This stabilized understory is used to predict the potential climax overstory (resulting in the habitat type name), which is thought to reflect the growth potential of the site (Daubenmire and Daubenmire, 1968; Pfister, 1989). Habitat typing does not view succession as a unidirectional process (Clements, 1916; Clements, 1936) but rather, one that is affected by environmental variables and disturbance history (Daubenmire, 1976).

The concept of habitat typing contains many assumptions about sampling, species interactions, and how species respond to disturbance (see Daubenmire and Daubenmire, 1968; Daubenmire, 1976; Pfister and Arno, 1980; Cook, 1995 for a critical review). Studies within the Great Lakes Region have observed varying responses of the herbaceous layer to disturbance (Metzger and Schultz, 1984; Fredericksen et al., 1999; Jenkins and Parker, 1999; Scheller and Mladenoff, 2002; Zenner et al., 2006; Kern et al., 2006), suggesting potential implications to how this vegetation layer is used to classify site potential. Metzger and Schultz (1981) observed in a northern hardwood forest in the Upper Peninsula of Michigan no large differences in the herbaceous layer between different harvest intensities after 50 years. They did note that spring ephemerals may be more sensitive to repeated disturbances such as single-tree selection. However, Roberts and Gilliam (1995) observed in Northern Lower Michigan a greater change in overall diversity and species composition in disturbed mesic sites than dry/mesic sites when comparing them to undisturbed mesic and dry/mesic sites. The disturbed mesic sites had greater increases in weedy and early successional species. This trend of increasing weedy and early successional species was also observed in mesic northern hardwood forests receiving uneven- aged management compared with even-aged management and old-growth stands in Northern Wisconsin and the Upper Peninsula of Michigan (Scheller and Mladenoff, 2002).

Disturbance is only one factor that can affect herbaceous species composition. Native and exotic species interactions have also been shown to be important (Bohlen et al., 2004; Fisichelli et al., 2012). The introduction of European earthworms in the Great Lakes region, a region that developed without native earthworms following the last glaciation (James, 1995), has not only affected soil nutrient cycling (Scheu and Parkinson, 1994; Tomlin et al., 1995; Bohlen et al., 2004; Suárez et al., 2004; Hale et al., 2005b) but has also affected herbaceous species diversity (Gundale, 2002; Hale, 2006; Holdsworth, 2007).

Habitat typing is just one classification system that both scientists and managers can use to assess and classify forests. *The Guide to Forest Communities and Habitat Types of Michigan* by Burger and Kotar (2003) was developed along a moisture and soil nutrient gradient. The objectives of the resampling in this study were to monitor changes in summer herbaceous vegetation after 10 years, to gain a better understanding of possible shifts in herbaceous species communities, and to observe the potential impacts exotic earthworms may have on the herbaceous community. We hypothesized that the spring and summer herbaceous community would adhere to similar nutrient and moisture gradients that were observed ten years previously. We also hypothesized that herbaceous species composition would differ between years (2000 vs. 2010) and between seasons (spring and summer). We could not identify exact mechanisms that could lead to compositional shifts between years and seasons as these were not measured during the first sampling, but we did hypothesize that: 1) herbaceous species composition may be changing due to high earthworm densities which would lead to simplified plant communities between sample periods; 2) increased percent cover of a few invasive or weedy species may outcompete native species, reducing species richness and diversity; and 3) spring ephermals would decrease and summer herbaceous species would increase in percent cover and frequency as the canopy closed.

Methods

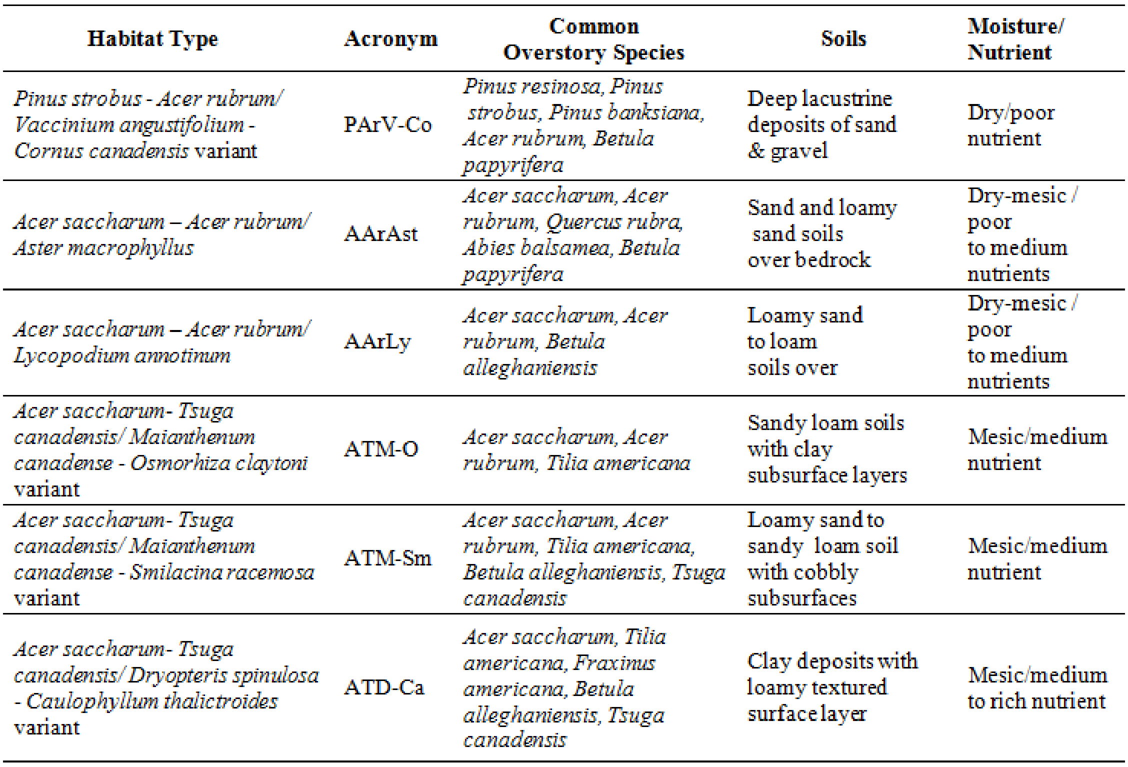
Study area

Within the Western Upper Peninsula of Michigan there is approximately two million ha of forested land. Of this, thirty-three percent (650,000 ha) is located within Houghton, Keweenaw, and Ontonagon counties. The vast majority (93%) of this 650,000 million ha is in young forests, under 100 years (Forest Inventory Data Online, 2010). The three most common cover types are the maple/beech/birch (57%), aspen/birch (19%), and spruce/fir (10%) (Forest Inventory Data Online, 2010). Previous glacial activity greatly influenced the surface geology occurring in these counties; common geological features include ground moraines, end moraines, outwash deposits, and glacial lake shoreline (Soil Conservation Service, 1991, 2006, 2010).

All three counties generally have a continental climate, with average daily maximum temperatures of -6.7°C and 23.8°C and average daily minimum temperatures of -13.9°C and 12.4°C for January and July respectively (recorded in Houghton County; Soil Conservation Service, 1991). Temperatures are generally warmer in the summer and cooler in the winter with increasing distance from Lake Superior. Average precipitation is 0.87 m with an additional 5.3 m of average snowfall (Soil Conservation Service, 1991, 2006, 2010). Normal season conditions occurred during the summer sampling of 2000 (personal communication, John Kotar). Abnormal seasonal temperatures and precipitation occurred during the summer of 2010, the year of our resampling. There were only trace snowfall events during the months of March and April; compared to March and April 2000, there was 7.70 cm and 2.21 cm, respectively, less precipitation in 2010 (PRISM Climate Group, 2004). Maximum temperatures during the months of April and May, 2010 were also 5.21°C and 0.79°C above maximum temperatures recorded for April and May, 2000.

Six different habitat types were selected for resampling during the spring and summer of 2010 in Houghton, Keweenaw, and Ontonagon counties. The six habitat types span a range of moisture and nutrient richness. General characteristics of each habitat type, along with the full and abbreviated name, can be found in *Table* 1.

Table 1. *Summary information of habitat types that were resampled in 2010. Habitat types are arranged from least productive to most productive. Additional information on each habitat type can be found in The Guide to Forest Communities and Habitat Types of Michigan by Burger and Kotar, 2003.*



Habitat Type	Acronym	Common Overstory Species	Soils	Moisture/ Nutrient
Pinus strobus - Acer rubrum/ Vaccinium angustifolium - Cornus canadensis variant	PArV-Co	*Pinus resinosa, Pinus strobus, Pinus banksiana, Acer rubrum, Betula papyrifera*	Deep lacustrine deposits of sand & gravel	Dry/poor nutrient
Acer saccharum – Acer rubrum/ Aster macrophyllus	AArAst	*Acer saccharum, Acer rubrum, Quercus rubra, Abies balsamea, Betula papyrifera*	Sand and loamy sand soils over bedrock	Dry-mesic / poor to medium nutrients
Acer saccharum – Acer rubrum/ Lycopodium annotinum	AArLy	*Acer saccharum, Acer rubrum, Betula alleghaniensis*	Loamy sand to loam soils over	Dry-mesic / poor to medium nutrients
Acer saccharum- Tsuga canadensis/ Maianthenum canadense - Osmorhiza claytoni variant	ATM-O	*Acer saccharum, Acer rubrum, Tilia americana*	Sandy loam soils with clay subsurface layers	Mesic/medium nutrient
Acer saccharum- Tsuga canadensis/ Maianthenum canadense - Smilacina racemosa variant	ATM-Sm	*Acer saccharum, Acer rubrum, Tilia americana, Betula alleghaniensis, Tsuga canadensis*	Loamy sand to sandy loam soil with cobbly subsurfaces	Mesic/medium nutrient
Acer saccharum- Tsuga canadensis/ Dryopteris spinulosa - Caulophyllum thalictroides variant	ATD-Ca	*Acer saccharum, Tilia americana, Fraxinus americana, Betula alleghaniensis, Tsuga canadensis*	Clay deposits with loamy textured surface layer	Mesic/medium to rich nutrient

Vegetation sampling

Thirty of the 200 plots used to create *The Guide to Forest Communities and Habitat Types of Michigan for the Western Upper Peninsula* (Burger and Kotar, 2003) were relocated and resampled during the spring and summer of 2010. Original plot locations were not permanently marked during the summer of 2000 but clear directions were recorded for each plot. Plots were relocated in spring 2010 and a GPS location was taken at each plot to document plot location and to relocate plots during the summer sampling period. Three of the thirty plots (10%) had to be offset slightly due to extreme changes at the original plot location such as a new trail or building. Even with these offsets and lack of permanent plots, we are confident that the sample locations are representative of the forest communities sampled during the summer of 2000.

Sampling in 2010 was modified slightly from the original sampling during the summer of 2000 (Burger and Kotar, 2003). Sampling in 2010 occurred during the spring (May 4th – May 24th) before full leaf on and in summer (June 24th – July 13th). Summer sampling coincided with phenological changes in plants, such as the ripening of *Vaccinium* spp. and *Rubus* spp. berries.

At each plot location, a 21 m x 14 m macroplot was established (Burger and Kotar, 2003). Modification of the original sampling included the use of 1-m^2 plots to measure herbaceous species (*Fig. 1*). Environmental data such as topography, configuration, slope, and time since last harvest activity were recorded. Topography was described as a ridge, upper slope, mid slope, lower slope, or bench. Configuration was described as convex, straight, concave, or undulating. Slope was categorized into four classes: 1) 0-10%, 2) 11-25%, 3) 26-50%, and 4) 51% and greater. Harvest activity was identified by the presence of stumps and small diameter harvest slash and was estimated as having occurred within the last five years, six to ten years ago, eleven to fifteen years ago, sixteen to twenty years ago, and greater than twenty one years.

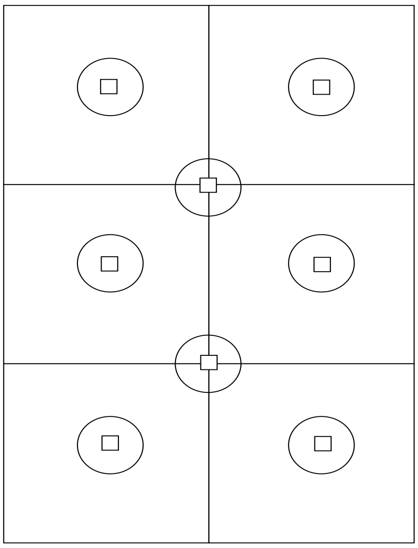


Figure 1. *Sampling design used during the spring and summer of 2010 was slightly modified from Burger and Kotar (2003). Within the 21 m by 14 m macroplot, eight 1-m^2 square quadrats were established to measure herbaceous species. Eight circular 0.0004-ha plots were used to measure seedlings and saplings.*

Inside each macroplot, all overstory species greater than 10 cm dbh were identified to species, and diameter was measured at breast height (1.37m). Overstory trees were divided into five canopy classes: open grown, dominant, co-dominant, intermediate, or suppressed (Oliver and Larson, 1996). Diameter at breast height was also measured on all snags. Overstory measurements were conducted last to decrease trampling of the herbaceous layer.

Within the macroplot, eight 1-m^2 quadrats were used to sample herbaceous species (*Fig. 1*). All herbaceous species within the quadrat were identified to species with the exception of grasses, sedges, mosses, and certain families where identification could only be made to genus. After identification, species were placed into a coverage class. The same coverage classes were used as the original sampling in 2000: 1) 0-1%, 2) 1-5%, 3) 5-10%, 4) 10-25%, 5) 25-50%, 6) 50-75%, and 7) greater than 75%. All estimates of percent cover in 2010 were done by one researcher to minimize bias. Using the quadrat locations, two opposite corners were selected to estimate canopy cover using a spherical concave densitometer for a total of 16 measurements per macroplot. The macroplot was then searched for any new herbaceous species that were not present in any quadrat; these species were recorded as present.

At the center of each of the eight quadrats, a 0.0004-ha circular plot was established to measure the seedling and sapling layers. Seedlings were defined as any woody tree species less than 30.5 cm in height. Saplings were subdivided into small saplings (30.6 cm to 1.4 m in height) and large saplings (1.5 m in height to 9.9 cm in diameter).

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Density and percent cover were measured for seedlings, small saplings, and large saplings by species. Percent cover was estimated using the same coverage classes used for the herbaceous species.

Earthworm sampling

Three 0.5-m^2 metal quadrats were placed randomly within the macroplot to sample earthworm populations by liquid extraction during the first two weeks of September (Sept 2-12, 2010) to ensure that leaf litter sampled was from the previous fall. The quadrats were placed into the soil to ensure that the extraction solution would not leak outside the sample area; leaf litter depth was measured to one-tenth of a centimeter and classified based on the major overstory species present. The leaf litter was then collected.

The liquid extraction solution consisted of 40 g ground yellow mustard mixed in 3.8 L of water. This solution has been shown to be a skin irritant and causes earthworms to surface (Hale, 2007). After the solution was poured, we collected earthworms for 3 minutes (no additional earthworms were found after 3 minutes). Earthworms were collected and later identified in the lab.

In the lab, the wet weight of the leaf litter samples were measured after each sampling day and then placed in a drying oven at 30°C for a minimum of seven days. Oven dried samples were then weighed to the nearest tenth of a gram.

Collected earthworms were identified within 48 hours. Due to the high amount of juveniles present, earthworms were only identified to genus with three genera collected throughout the sample location: *Aporrectodea* spp., *Dendrodilus* spp., and *Lumbricus* spp. All earthworms were measured to the nearest millimeter for their total length. For each macroplot, total earthworm biomass (ash-free dry mass) was determined for all earthworm species combined and each genus (Hale et al., 2004).

Data analysis

Herbaceous species were divided into different growth forms based on the USDA PLANTS Database (2011): 1) equisetum, 2) fern, 3) forb/herb, 4) graminoids, 5) lichen, 6) moss, 7) shrub, 8) sub-shrub, and 9) vine. Species were also classified as native or invasive using information about invasive species from the USDA PLANTS Database (2011). The definition used for invasive species was from Executive Order 13112, Appendix 1 (1999) where an invasive species is defined as "non-native (or alien) to the ecosystem under consideration and whose introduction causes or is likely to cause economic or environmental harm or harm to human health." Weedy plants were classified as invasive due to the potential decrease diversity. Species shade tolerance was also classified using the USDA PLANTS Database. When tolerance was not listed, the description of the habitat that the species was normally found in was used (Voss, 1985; Newcomb, 1989; Voss, 1996). Herbaceous species with habitat descriptions of woodlands or woods were classified as tolerant; open woods and thickets were classified as midtolerants; and waste places, roadsides, and meadows were classified as intolerant.

Data was summarized two ways due to slight differences in sampling methods between 2000 and 2010. Herbaceous species richness was summarized at the macroplot level between years and between seasons. Diversity and evenness could not be compared between years in the understory layer due to differences in area sampled. Overstory species were summarized by trees per hectare and basal area per hectare.

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Comparison of overstory basal area between summer 2000 and 2010 required overstory trees to be placed into 5.1 cm diameter classes with all trees greater than 55.6 cm in diameter (dbh) excluded to be consistent with the original data collection. Therefore, all overstory basal area measurements are underestimates as there is no way to calculate basal area of trees with unknown diameters above 55.6 cm. Diameter to the nearest tenth of a centimeter was used to calculate overstory basal area when comparing spring and summer data collected in 2010.

Trees per hectare within the macroplot was used to calculate overstory species richness. Trees per hectare was also used to calculate Shannon's Index of Diversity (Maguurran, 1988),

$$H = -\Sigma\ p_i/p_t * \ln(p_i/p_t) \qquad \text{(Eq.1)}$$

where H is Shannon's Index of Diversity, p_i is the cover of species i, and p_t is the total species richness for all species in the plot.

Evenness was also calculated using trees per hectare (Margurran, 1988)

$$EH = H/Hmax \quad Hmax = H/lnS \qquad \text{(Eq.2)}$$

where E_H is evenness, H_{max} is the maximum potential evenness, and lnS is the natural log of species richness.

Shannon's index of diversity and evenness for herbaceous species sampled in spring and summer of 2010 were summarized by averaging the percent cover of the eight 1-m^2 quadrats in each plot.

Repeated measures ANOVA in statistical interface R (R Development Core Team, 2011) was used to test for significant differences between habitat types and between either years or seasons. Tukey's Honest Significant Difference (Tukey, 953) was used when there were significant differences between years (2000 vs. 2010), seasons (spring vs. summer), and habitat types (see *Table 1*). Linear regression was used to explore relationships between exotic earthworms and percent cover of vegetation and environmental variables.

Nonmetric multidimensional scaling (NMS) ordination using PC-ORD Version 5 (McCune and Mefford, 2011) was used to compare the shift in the herbaceous layer between sampling periods and between habitat types. NMS has relaxed normality assumptions and does not assume a linear response to different gradients which is common in ecological data (McCune and Grace, 2002). Herbaceous species presence/absence data at the macroplot level was used to run the ordination. NMS was run on herbaceous species presence/absence data for summer 2000 versus summer 2010 and spring 2010 versus summer 2010. Autopilot mode (slow and thorough) was selected using Sørensen (Bray-Curtis) distance measurement and a random starting point for both datasets. Two hundred and fifty runs were completed for both the real data and randomized data to determine dimensionality for both data sets. Correlation analysis in statistical interface R (R Development Core Team, 2011) was used to test environmental variables used in the ordinations for significance.

Results

Herbaceous species composition

During the summer of 2000, 80 herbaceous species were identified across all habitat types with only four species (5% of all species) considered invasive. A total of 130 herbaceous species were sampled during both spring and summer of 2010 across all habitat types with 25 species (19% of all species) considered invasive. There were 69 new species observed in 2010 with 22 or 33% of these new species considered invasive. Seventeen species were only observed in 2000; none were considered invasive. A complete list of herbaceous species can be found in Appendix A (*Table A.1*).

A greater number of species, 114, were sampled during the summer 2010 period compared with 90 species sampled during spring 2010 across all habitat types. Sixteen herbaceous species were captured in the spring sampling period which would have been missed with only summer sampling. Habitat types are delineated on the basis of floristic differences. However, few species were unique to only one habitat type; herbaceous species overlap between habitat types ranged from 11-57%. Habitat types occur across range of nutrient and moisture conditions (*Table 1*). PArV-Co and ATD-Ca represent the least and most productive habitat types, respectively. These two habitat types, PArV-Co and ATD-Ca contained the fewest species in common (11%) in summer 2010. However, habitat types with more similar moisture and nutrient conditions shared more species in common with AArAst and AArLy sharing over half (57%) of the same species. Species such as *Dryopteris spinulosa, Maianthemum canadense, Maianthemum racemosoum* ssp. *racemosum, Polygonatum pubescens,* and *Trientalis borealis* exhibited a high frequency of occurrence within habitat types and between habitat types in both the summer 2000 and spring and summer 2010 sampling periods.

Comparison between summer 2000 and 2010

Overstory trees per hectare significantly decreased from 2000 to 2010 ($p< 0.001$); there was an average 31% decrease between all habitat types, with no significant difference between habitat types (*Table 2*). A similar trend was observed with overstory basal area per hectare ($p<0.001$) (*Table 2*) with a significant difference between habitat types; PArV-Co had greater basal area per hectare than AArAst ($p=0.03$) and AArLy ($p=0.03$). All habitat types had at least one plot that was surmised through the identification of recent stumps to have had management activities within the past 10 years. Habitat types ATM-Sm and AArLy had the greatest number of plots (three and two respectively) with management activity occurring in the last five years, while also experiencing the greatest decrease in overstory basal area (47% and 48% respectively).

Total herbaceous species richness significantly increased between years ($p<0.001$) with significant differences between habitat types ($p<0.001$; *Table 2*). Native herbaceous species richness did not vary between years but was significantly different between habitat types ($p<0.001$; *Fig. 2*). The most productive habitat type, ATD-Ca, had greater total herbaceous species richness and native herbaceous species richness than all other habitat types except ATM-O (*Table 2*). Invasive herbaceous species richness increased between years ($p<0.001$) but there was no significant difference between habitat types (*Fig. 2*).

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Table 2. *Response of overstory structure and herbaceous species richness to year of sampling, habitat type, and their interaction. All variables are summarized at the macroplot level The associated standard errors are in parentheses.*

	Habitat Types	Summer 2000	Summer 2010	Year		Habitat Type		Interaction	
				$F_{1,48}$	p-value	$F_{5,48}$	p-value	$F_{5,48}$	p-value
Trees Per Hectare	PArV-Co	945 (39)	898 (176)	4.67	<0.001	0.83	0.53	1.22	0.31
	AArAst	1007 (66)	660 (128)						
	AArLy	1034 (140)	537 (46)						
	ATM-Sm	959 (113)	510 (108)						
	ATM-O	980 (129)	694 (110)						
	ATD-Ca	850 (63)	653 (54)						
Basal Area Per Hectare (m^2ha^{-1})	PArV-Co	50 (3)	36 (5)	35.20	<0.001	3.25	0.01	0.52	0.76
	AArAst	42 (5)	24 (7)						
	AArLy	38 (4)	20 (3)						
	ATM-Sm	39 (5)	19 (3)						
	ATM-O	40 (5)	25 (4)						
	ATD-Ca	31 (2)	24 (4)						
Total Herbaceous Species Richness	PArV-Co	17.4 (0.8)	15.4 (2.4)	7.69	0.007	7.01	<0.001	2.24	0.07
	AArAst	15.8 (2.4)	16.0 (2.5)						
	AArLy	14.8 (1.8)	18.0 (2.0)						
	ATM-Sm	11.8 (1.1)	21.0 (1.3)						
	ATM-O	17.2 (0.4)	24.0 (2.2)						
	ATD-Ca	24.4 (2.2)	26.0 (3.0)						

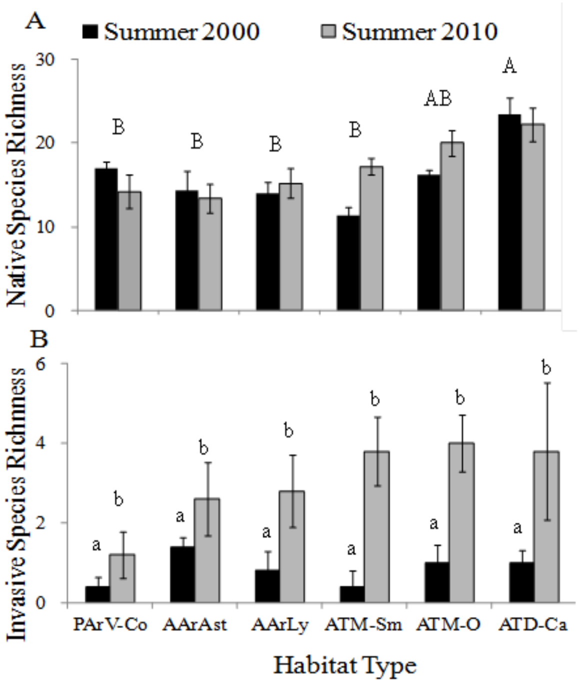

Figure 2: *Native (A) and Invasive (B) herbaceous species richness observed at the macroplot level in summer 2000 and 2010. Upper case letters that are different represent significant differences between habitat types at α=0.05. There were no significant differences observed between years for native herbaceous species richness. Lower case letters represent a significant difference between years at α=0.05. There were no significant differences between years across habitat types for invasive species richness.*

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Comparison between spring and summer 2010

Canopy cover increased from spring 2010 to summer 2010 (p<0.001) but did not differ significantly between habitat types (*Table 3*). Herbaceous species percent cover, richness, and evenness all increased significantly from spring to summer (p<0.001; p=0.01; p<0.001) (*Table 3*). The two least productive habitat types, PArV-Co and AArAst, had significantly lower herbaceous species richness than the three richest habitat types, ATM-Sm (p=0.006, p=0.02), ATM-O (p<0.001, p=0.03), and ATD-Ca (p=0.005, p=0.02) (*Fig. 3*). There were significantly greater diversity, using Shannon's Index of Diversity, in ATM-Sm than AArAst (p=0.03) and ATM-O and ATD-Ca than AArAst (p=0.07; p=0.04); herbaceous evenness was significantly greater in ATM-Sm than AArAst (p=0.02) (*Fig. 3*).

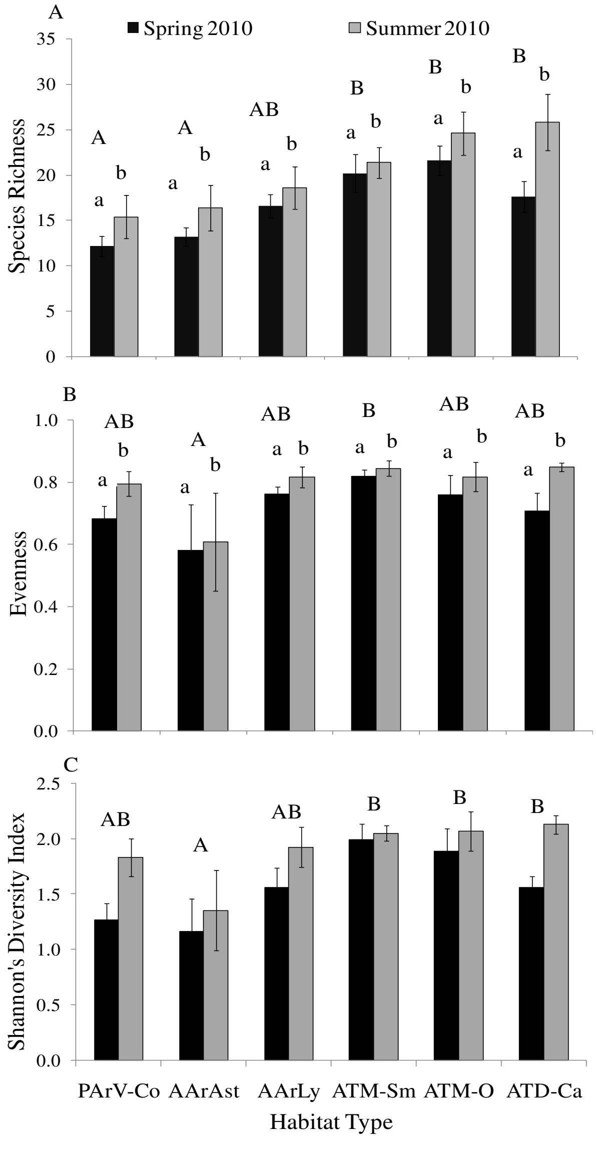

Figure 3: *Herbaceous Species Richness (A), Evenness (B), and Shannon's Diversity (C) for spring and summer 2010. Species richness was observed at the macroplot level. Herbaceous evenness and Shannon's diversity were calculated from 1-m^2 plots. Upper case letters that are different represent a significant difference between habitat types at α=0.05. Lower case letters that are different represent a significant difference between seasons at α=0.05. There was no significant difference between years for Shannon's Diversity.*

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Table 3. *Response of the canopy and herbaceous species percent cover to season, habitat type and their interaction. Herbaceous species percent cover was summarized at the subplot level (eight 1-m2 quadrats). The associated standard errors are in parentheses.*

	Habitat Types	Spring 2010	Summer 2010	Season		Habitat Type		Interaction	
				$F_{1,48}$	p-value	$F_{5,48}$	p-value	$F_{5,48}$	p-value
Percent Canopy Closure	PArV-Co	86 (0.7)	90 (0.9)	45.77	<0.001	0.71	0.62	1.19	0.33
	AArAst	73 (0.8)	92 (1.2)						
	AArLy	78 (1.0)	93 (0.5)						
	ATM-Sm	81 (0.6)	92 (0.8)						
	ATM-O	79 (1.0)	93 (0.5)						
	ATD-Ca	80 (1.0)	91 (0.7)						
Herbaceous Species Percent Cover	PArV-Co	25 (11)	61 (27)	21.06	<0.001	2.38	0.052	0.40	0.84
	AArAst	16 (7)	40 (18)						
	AArLy	25 (11)	55 (25)						
	ATM-Sm	28 (12)	73 (33)						
	ATM-O	33 (15)	66 (30)						
	ATD-Ca	38 (17)	67 (30)						

Herbaceous composition shifts

A three-dimensional solution was found through NMS with a final stress of 16.9 in the comparison between summer 2000 and 2010. The ordination explained 83% of the variation in the data; axis 1 and axis 2 explained the most variation, 18% and 67% respectively (*Fig. 4*).

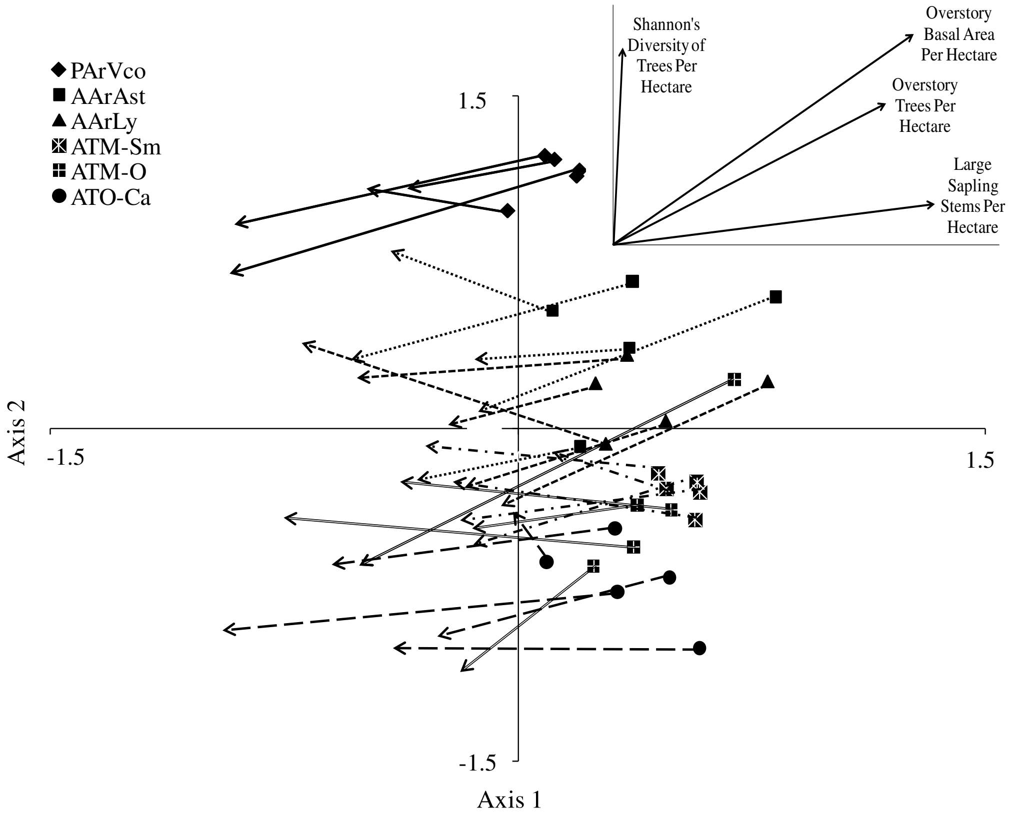

Figure 4. *Non-metric multi-dimensional scaling ordination of herbaceous species communities for six habitat types in the western Upper Peninsula for summer 2000 and summer 2010. Axis 1*

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explains 18% percent of the variation while Axis 2 explains 67% of the variation. Habitat type are displayed in order of productivity; PArV-Co the least productive to ATO-Ca the most productive. Markers represent summer 2000 conditions and arrows represent direction and magnitude of composition change. Where the arrows end represent summer 2010 herbaceous community composition. Longer arrows represent greater difference between years. The insert is the significant environmental variables (p=0.05) and their relation to ordination space.

Overstory TPH and basal area per hectare were both strongly associated with axis 1 and axis 2 (*Fig. 4*). Overstory Shannon's diversity calculated with TPH was strongly associated with axis 2; large sapling density was strongly associated with axis 1 (*Fig. 4*). There was generally strong and consistent movement in all habitat types to areas of decreased overstory TPH and basal area per hectare (*Fig. 4*). However, even with increasing invasive species and changes in overstory density, the productivity gradient between habitat types was still evident.

A three-dimensional solution with a final stress of 17.5 was also found in the comparison between spring and summer 2010. The ordination explained 77% of the variation in the data; axis 1 and axis 3 explained the most variation, 54% and 14% respectively (*Fig. 5*). Percent canopy cover is the main driving variable in this ordination and is strongly associated with axis 3 (*Fig. 5*). Species shifted from a more open canopy in the spring to a closed canopy in the summer. Percent down dead wood and seedling richness were associated with axis 3, while overstory TPH and basal area per hectare were associated with axis 1 (*Fig. 5*).

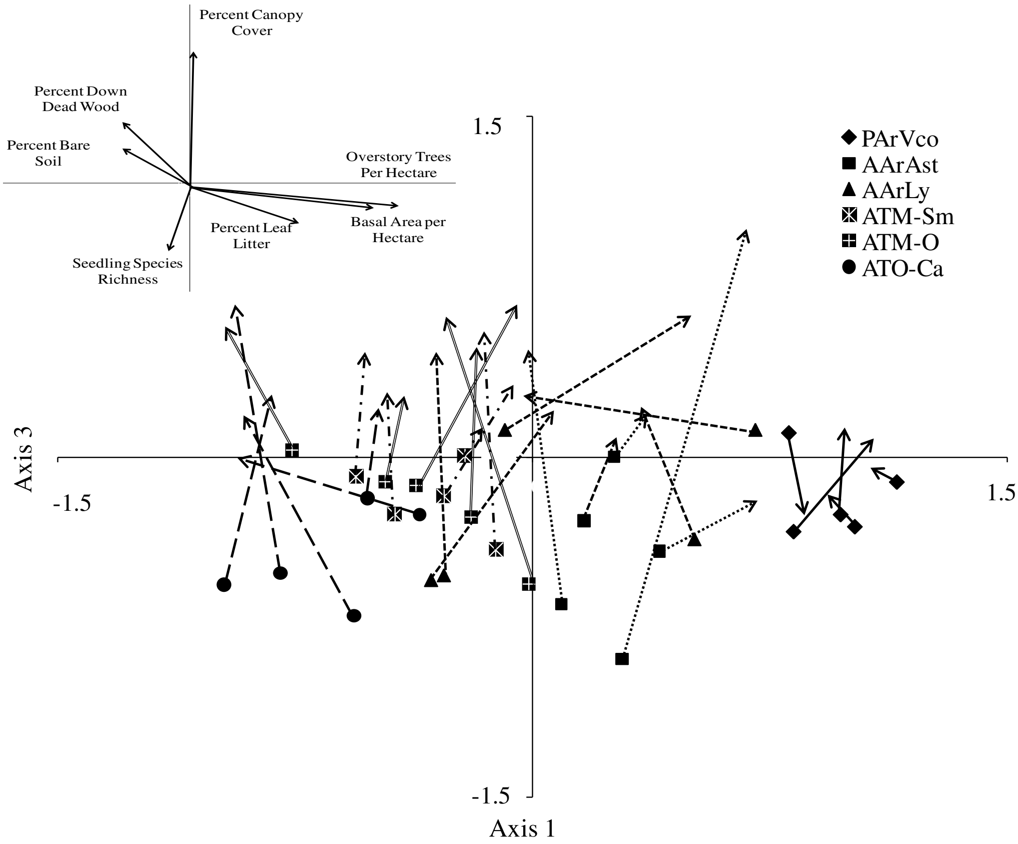

Figure 5. *Non-metric multi-dimensional scaling ordination of herbaceous species communities for six habitat types in the western Upper Peninsula for spring and summer 2010. Axis 1*

explains 54% percent of the variation while Axis 3 explains 14% of the variation. Habitat type are arranged in order of productivity; PArV-Co the least productive to ATO-Ca the most productive. Markers represent spring 2010 conditions and arrows represent direction and magnitude of composition change. Where the arrows ends represent summer 2010 herbaceous community composition. Longer arrows represent greater difference between spring and summer. The insert is the significant environmental variables (p=0.05) and their relation to ordination space.

Earthworm densities

Overall earthworm ash-free dry mass generally increased from less productive habitat types to more productive habitat types, with *Lumbricus* spp. generally following the same trends (*Fig. 6*). The PArV-Co and AArLy habitat types had significantly lower ash-free dry mass than ATD-Ca (p = 0.01; p = 0.03) (*Fig. 6*). There were significant negative relationships between ash-dry weight of *Lumbricus* spp. and both spring and summer herbaceous species percent cover (p=0.010, r^2=0.21; p=0.019, r^2=0.18). Total ash-dry weight of all earthworms followed this same trend (p=0.049, r^2=0.24; p=0.005, r^2=0.13). There was a significant negative relationship between ash-dry weight of *Dendrobaena* spp. and total herbaceous species richness, native species richness, and invasive species richness (p=0.003, r^2=0.27; p=0.031, r^2=0.15; p=0.006, r^2=0.23), respectively.

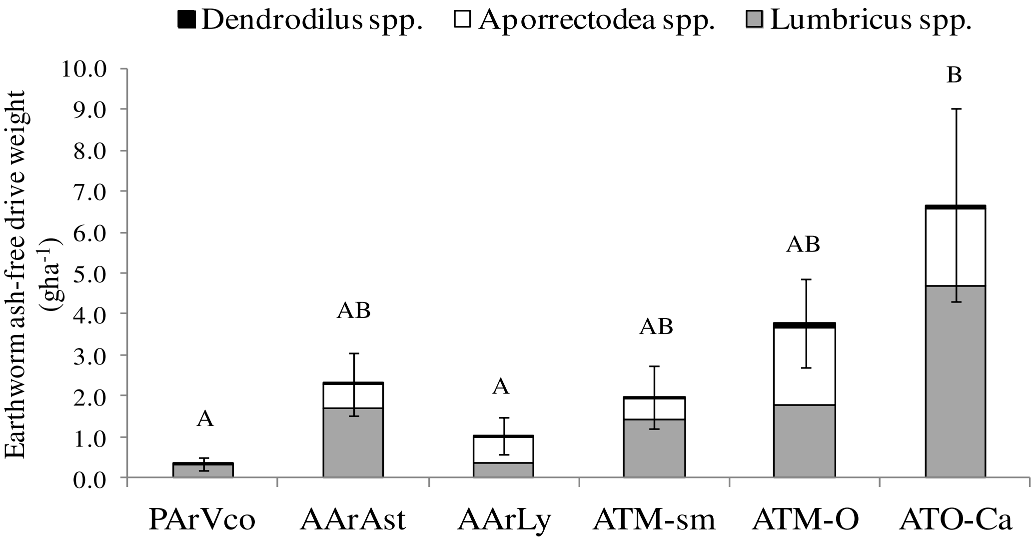

***Figure 6.** Earthworm ash-free dry weight (g ha-1) by genus for each habitat type. Letters that are different represent a significant (α=0.05) difference between habitat types comparing total ash-free dry weight of earthworms, not by individual species.*

Discussion

Even with changes in herbaceous species composition, overstory density, and canopy cover between years (2000 vs 2010) and between season (spring vs summer) there was still an identifiable productivity gradient between different habitat types (*Fig. 4 - 5*). Overstory density significantly decreased between 2000 and 2010, which we hypothesize is a result of forest management as all habitat types contained at least one plot where recent stumps and logging slash were observed. It is not known the exact intensity of past harvest activities or the season in which the harvests took place. Forest management has been shown to have a variety of effects on the herbaceous layer in a variety of forest types (Metzger and Schultz, 1981; Gilliam et al., 1995; Roberts and Gilliam, 1995; Fredericksen et al., 1999; Jenkins and Parker, 1999; Crow et al., 2002; Scheller and Mladenoff, 2002; Gilliam and Roberts, 2003; Zenner et al., 2006; Kern et al., 2006; Wolf, 2008; and others).

The Argonne Experimental Forest in northern Wisconsin observed no difference in the spring or summer herbaceous communities in even, uneven-aged, or unmanaged stands after 40 years of consistent treatment (Kern et al., 2006). However, at the Ford Forest (Michigan Technological University) differences in summer herbaceous community composition were observed between different harvest intensities after 50 years of management. After a recent harvest, the diameter-limit treatments generally had herbaceous communities with more weedy and invasive herbaceous species (Campione et al., 2012). Scheller and Mladenoff (2002) also observed this increase in early successional and weedy species in uneven-aged northern hardwood stands compared to even-aged or unmanaged stands.

The seasonality of logging can also affect herbaceous species composition. A study in the Chequamegon-Nicolet National Forest observed that herbaceous species that are more vulnerable to disturbance were observed more often in winter-logged sites than summer-logged sites (Wolf et al., 2008). Logging intensity and seasonality may be two unknown variables contributing to increases in invasive species richness between years in this and other studies (Wiegmann and Waller, 2000; Watkins et al., 2003).

Another group of invasive species, European earthworms, may also be having an effect on the herbaceous species composition. Exotic earthworm densities and ash-dry mass were estimated for each habitat type in the present study. There was a general increasing trend between total earthworm ash-free biomass and habitat type productivity. There is no way to estimate exactly how long earthworm populations have been present at each site. All habitat types except for PArV-Co (the habitat type with lowest productivity) had all three genera of earthworms. No earthworms from the *Aporrectodea* genus were collected during sampling at PArV-Co sites. *Aporrectodea* species are classified as endogeic, living in soil down to depths of 50 cm, and feed on mineral soil (Hale, 2007). Hale and others (2006) observed that *Aporrectodea* species were generally behind the leading edge of the earthworm invasion front. The lack of observed *Aporrectodea* species at the PArV-Co sites during sampling may be a result of their population size being too small to pick up in our sampling. Alternatively, the sandy soils and pine leaf litter of this habitat type may inhibit this genus of earthworms from establishing or creating large populations. The other two genera observed, *Dendrobaena* and *Lumbricus*, can be classified as epigeic and anecic or epi-endogeic respectively (Hale, 2007). The genus *Dendrobaena* is commonly the first earthworm genus to colonize new locations (Hale, 2007).

Our results of a negative relationship between total earthworm ash-free biomass and percent cover of herbaceous species are consistent with Hale and colleagues (2005). However, our results are different than Holdsworth (2007) in that plant species richness increased with increasing mass of the earthworm genera *Dendrobaena* and *Aporrectodea*. We observed decreases in total herbaceous species richness, native species richness, and invasive species richness with increasing mass of *Dendrobaena* ($p=0.003$, $r^2=0.27$; $p=0.031$, $r^2=0.15$; $p=0.006$, $r^2=0.23$), respectively. Invasive species richness also decreased with increasing mass of *Aporrectodea*. These differences may be due to the low total earthworm and individual species biomass that was observed.

The habitat type ATD-Ca had the highest mass of *Aporrectodea* which averaged 1.1 g/m^2 compared to an average of 4.8 g/m^2 observed in northern Wisconsin and 3.9 g/m^2 in northern Minnesota (Holdsworth et al., 2007).

These low earthworm populations may be one of the reasons we did not observe the same changes in species richness as other authors (e.g. Gundale, 2002; Hale, 2006; Holdsworth, 2007; Powers and Nagel, 2008). Both earthworm densities and herbaceous species richness generally but not significantly increased with increasing nutrient richness and moisture. We also did not observe simplified herbaceous communities dominated by *Carex pensylvanica* (Holdsworth et al., 2007; Powers and Nagel, 2008) which does grow in this area but at much lower abundance than in these other studies. Future climate conditions have the possibility to not only affect the current dynamics in forests but also the dynamics of invasive species (e.g. Walther et al., 2002).

These changing climatic conditions have already been observed in the Upper Peninsula of Michigan. Myers and colleagues (2009) observed an increase of approximately 2.1°C in the daily minimum and 0.42°C in the daily maximum temperatures from 1970 to 2007. Spring ephermals may be more sensitive to these changing conditions since flowering times are closely related to mean monthly temperatures (Miller-Rushing and Primack, 2008). These early flowering plants are an important functional component of ecosystems, reducing nutrient losses from soils (Muller and Bormann, 1976). However, spring ephermals may be more sensitive to repeated disturbances such as uneven-aged management (Metzger and Schultz, 1981; Scheller and Mladenoff, 2002) or may show no difference between managed and unmanaged forests (Kern et al., 2006). We observed a distinct shift in vegetation from spring to summer sampling. As canopy closure occurred at the end of spring/beginning of summer, the herbaceous community shifted from one dominated by spring ephemerals like *Claytonia virginica* and *Erythronium americanum* to a herbaceous community dominated by *Dryopteris spinulosa, Maianthemum canadense*, and *Trientalis borealis*. Increasing temperatures, especially daily minimum temperatures, may change the relationship spring ephermals have with overstory tree species. Spring ephermals may be important species to monitor and use as early detectors of changing conditions in forest ecosystems. Continual monitoring within these forest communities will allow scientists and managers to observe how a management activity, changing climate, and invasive plants and earthworms interact and influence forest composition.

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APPENDIX

Appendix A

Appendix A.1 Full list of species observed in 2000 and 2010.

Scientific name	Common Name	Family	Growth Form	Shade Tolerance
Acer pensylvanicum L.	striped maple	Sapindaceae	Shrub	Tolerant
Acer spicatum Lam.	mountain maple	Sapindaceae	Shrub	Tolerant
Achillea millefolium L. *	common yarrow	Asteraceae	Forb/herb	Intermediate
Actaea spp.	Baneberries	Ranunculaceae	Forb/herb	Tolerant
Adiantum pedatum L.	northern maidenhair fern	Pteridaceae	Forb/herb	Tolerant
Allium tricoccum Aiton	ramp	Liliaceae	Forb/herb	Tolerant
Amaranthus retroflexus L.	redroot amaranth	Amaranthaceae	Forb/herb	Intolerant
Amelanchier spp.	Serviceberry	Rosaceae	Shrub	Tolerant
Amphicarpaea bracteata (L.) Fernald	American hogpeanut	Fabaceae	Vine	Intermediate
Anemone quinquefolia L.	wood anemone	Ranunculaceae	Forb/herb	Tolerant
Antennaria neglecta Green	field pussytoes	Asteraceae	Forb/herb	Intolerant
Apocynum androsaemifolium L.	spreading dogbane	Apocynaceae	Forb/herb	Intermediate
Aquilegia canadensis L.	red columbine	Ranunculaceae	Forb/herb	Intermediate
Aralia nudicaulis L.	wild sarsaparilla	Araliaceae	Forb/herb	Tolerant
Aralia racemosa L.	American spikenard	Araliaceae	Subshrub Forb/herb	Tolerant
Arctostaphylos uva-ursi (L.) Spreng.	kinnikinnick	Ericaceae	Shrub	Intermediate
Arisaema triphyllum (L.) Schott	Jack in the pulpit	Araceae	Forb/herb	Tolerant
Asarum canadense L.	Canadian wildginger	Aristolochiaceae	Forb/herb	Tolerant
Athyrium filix-femina (L.) Roth	common ladyfern	Dryopteridaceae	Forb/herb	Tolerant
Barbarea vulgaris W.T. Aiton *	garden yellowrocket	Brassicaceae	Forb/herb	Intolerant
Berberis thunbergii DC. *	Japanese barberry	Berberidaceae	Shrub	Intolerant
Botrychium virginianum (L.) Sw.	rattlesnake fern	Ophioglossaceae	Forb/herb	Tolerant
Cardamine bulbosa (Schreb. Ex Muhl.) Britton, Sterns & Poggenb.	bulbous bittercress	Brassicaceae	Forb/herb	Tolerant
Caulophyllum thalictroides (L.) Michx.	blue cohosh	Berberidaceae	Forb/herb	Tolerant
Chimaphila umbellata (L.) W.P.C. Barton	pipsissewa	Pyrolaceae	Subshrub	Tolerant
Circaea alpina (L.)	small enchanter's nightshade	Onagraceae	Forb/herb	Tolerant
Circaea quadrisulcata (L.) Asch. & Magnus	broadleaf enchanter's nightshade	Onagraceae	Forb/herb	Tolerant
Cirsium arvense (L.) Scop. *	Canada thistle	Asteraceae	Forb/herb	Intolerant
Cladina mitis (Sandst.) Hustich	reindeer lichen	Cladoniaceae	Lichen	Intolerant
Claytonia virginica L.	Virginia springbeauty	Portulaceae	Forb/herb	Intermediate
Clintonia borealis (Aiton) Raf.	bluebead	Liliaceae	Forb/herb	Intolerant
Comptonia peregrina (L.) J.M. Coult.	sweet fern	Myricaceae	Shrub	Intermediate
Coptis groenlandica (L.) Salisb.	threeleaf goldthread	Ranunculaceae	Forb/herb	Tolerant
Cornus alternifolia L. f.	alternateleaf dogwood	Cornaceae	Shrub	Tolerant
Cornus canadensis L.	bunchberry dogwood	Cornaceae	Forb/herb	Tolerant
Cornus racemosa Lam.	gray dogwood	Cornaceae	Shrub	Tolerant
Cornus stolonifera Michx.	redosier dogwood	Cornaceae	Shrub	Intolerant
Corylus cornuta Marshall	beaked hazelnut	Betulaceae	Shrub	Tolerant
Crataegus spp.	Hawthorn	Rosaceae	Shrub	Midtolerant
Cuphea viscosissima Jacq.	blue waxweed	Lythraceae	Forb/herb	Intolerant
Cypripedium acaule Aiton	pink lady's slipper	Orchidaceae	Forb/herb	Intermediate
Desmodium glutinosum (Muhl. ex Willd.) Alph. Wood	pointedleaf ticktrefoil	Fabaceae	Forb/herb	Tolerant
Dicentra cucullaria (L.) Bernh.	dutchman's breeches	Fumariaceae	Forb/herb	Intermediate
Diervilla lonicera Mill.	northern bush honeysuckle	Caprifoliaceae	Shrub	Intermediate
Dirca palustris L.	eastern leatherwood	Thymelaeaceae	Shrub	Tolerant
Dryopteris spinulosa (O.F. Müll.) Watt	spinulose shield fern	Dryopteridaceae	Forb/herb	Tolerant
Epifagus virginiana (L.) W.P.C. Barton	beechdrops	Orobanchaceae	Forb/herb	Tolerant
Epigaea repens L.	trailing arbutus	Ericaceae	Subshrub	Tolerant
Epipactis helleborine (L.) Crantz *	broadleaf helleborine	Orchidaceae	Forb/herb	Midtolerant
Equisetum spp.	Horsetail	Equisetaceae	Equisetum	Midtolerant - Intolerant
Erigeron spp.	Fleabane	Asteraceae	Forb/herb	Intolerant
Erythronium americanum Ker Gawl. subsp. *americanum*	yellow trout lily	Liliaceae	Forb/herb	Tolerant
Eupatorium purpureum L.	green-stemmed Joe-Pye-weed	Asteraceae	Forb/herb	Intermediate
Eurybia macrophylla (L.) Cass.	bigleaf aster	Asteraceae	Forb/herb	Tolerant
Fallopia convolvulus (L.) A.Löve *	black-bindweed	Polygonaceae	Vine	Intolerant
Fragaria spp.	Strawberry	Rosaceae	Shrub	Midtolerant
Frangula alnus Mill. *	glossy buckthorn	Rhamnaceae	Shrub	Intolerant
Galeopsis tetrahit L. *	brittlestem hempnettle	Lamiaceae	Forb/herb	Intolerant
Galium boreale L.	northern bedstraw	Rubiaceae	Forb/herb	Intermediate

***Appendix A.1** Continued.*

Scientific name	Common Name	Family	Growth Form	Shade Tolerance
Galium triflorum Michx.	fragrant bedstraw	Rubiaceae	Forb/herb Vine	Tolerant
Gaultheria hispidula (L.) Muhl. ex Bigelow	creeping snowberry	Ericaceae	Subshrub Shrub	Tolerant
Gaultheria procumbens L.	wintergreen	Ericaceae	Subshrub	Tolerant
Gaylussacia baccata (Wangenh.) K. Koch	black huckleberry	Ericaceae	Shrub	Tolerant
Geranium maculatum L.	spotted geranium	Geraniaceae	Forb/herb	Intermediate
Grasses & Sedges	grasses & sedges	-	Graminoid	-
Gymnocarpium dryopteris (L.) Newman	western oak fern	Dryopteridaceae	Forb/herb	Tolerant
Hamamelis virginiana L.	American witchhazel	Hamamelidaceae	Shrub	Intermediate
Helenium nudiflorum Raf.	purplehead sneezeweed	Asteraceae	Forb/herb	Intolerant
Heliopsis helianthoides (L.) Sweet	smooth oxeye	Asteraceae	Forb/herb	Intolerant
Hepatica acutiloba DC.	sharplobe hepatica	Ranunculaceae	Forb/herb	Tolerant
Hepatica americana (DC.) Ker Gawl.	roundlobe hepatica	Ranunculaceae	Forb/herb	Tolerant
Hieracium aurantiacum L. *	orange hawkweed	Asteraceae	Forb/herb	Intolerant
Hieracium paniculatum L.	Allegheny hawkweed	Asteraceae	Forb/herb	Tolerant
Hieracium pilosella L. var. *pilosella* *	mouseear hawkweed	Asteraceae	Forb/herb	Intolerant
Hieracium venosum L.	rattlesnakeweed	Asteraceae	Forb/herb	Intermediate
Huperzia lucidula (Michx.) Trevis.	shining clubmoss	Lycopodiaceae	Subshrub	Intermediate
Hydrophyllum virginianum L.	eastern waterleaf	Hydrophyllaceae	Forb/herb	Intermediate
Hypericum perforatum L. *	common St. Johnswort	Clusiaceae	Forb/herb	Intolerant
Hypochaeris radicata L. *	hairy cat's ear	Asteraceae	Forb/herb	Intolerant
Impatiens capensis Meerb.	jewelweed	Balsaminaceae	Forb/herb	Tolerant
Juniperus communis L.	common juniper	Cupressaceae	Shrub	Intolerant
Laportea canadensis (L.) Weddell	Canadian woodnettle	Urticaceae	Forb/herb	Tolerant
Lapsana communis (L.) *	common nipplewort	Asteraceae	Forb/herb	Intolerant
Leucanthemum vulgare (Lam.) *	oxeye daisy	Asteraceae	Forb/herb	Intermediate
Linnaea borealis L. subsp. *americana* (Forbes) Hultén ex R.T.Clausen	twinflower	Caprifoliaceae	Forb/herb	Tolerant
Lithospermum canescens (Michx.) Lehm.	hoary puccoon	Boraginaceae	Forb/herb	Intermediate
Lonicera canadensis W. Bartram ex Marshall	American fly honeysuckle	Caprifoliaceae	Shrub	Intermediate
Lycopodium annotinum L.	stiff clubmoss	Lycopodiaceae	Subshrub Forb/herb	Tolerant
Lycopodium clavatum L.	Running clubmoss	Lycopodiaceae	Subshrub Forb/herb	Intermediate
Lycopodium complanatum L.	groundcedar	Lycopodiaceae	Subshrub Forb/herb	Tolerant
Lycopodium obscurum L.	rare clubmoss	Lycopodiaceae	Subshrub Forb/herb	Tolerant
Lysimachia quadrifolia L.	whorled yellow loosestrife	Primulaceae	Forb/herb	Intermediate
Maianthemum canadense Desf.	Canada mayflower	Liliaceae	Forb/herb	Tolerant
Maianthemum racemosum (L.) Link *ssp. racemosum*	feathery false lily of the valley	Liliaceae	Forb/herb	Intermediate
Maianthemum stellatum (L.) Link	starry false lily of the valley	Liliaceae	Forb/herb	Intermediate
Matteuccia struthiopteris (L.) Todaro	ostrich fern	Dryopteridaceae	Forb/herb	Tolerant
Medeola virginiana L.	indian cucumber-root	Liliaceae	Forb/herb	Tolerant
Melampyrum lineare Desr.	narrow-leaf cow-wheat	Scrophulariaceae	Forb/herb	Intermediate
Mitchella repens L.	partridgeberry	Rubiaceae	Subshrub Forb/herb	Tolerant
Mitella diphylla L.	twoleaf miterwort	Saxifragaceae	Forb/herb	Intermediate
Mitella nuda L.	naked miterwort	Saxifragaceae	Forb/herb	Tolerant
Monotropa uniflora L.	Indian-pipe	Monotropaceae	Forb/herb	Tolerant
moss				
Myosotis scorpioides L. *	true forget-me-not	Boraginaceae	Forb/herb	Intermediate
Myosotis verna Nutt.	spring forget-me-not	Boraginaceae	Forb/herb	Intermediate
Onoclea sensibilis L.	sensitive fern	Dryopteridaceae	Forb/herb	Tolerant
Osmorhiza claytoni (Michx.) C.B. Clarke	Clayton's sweetroot	Apiaceae	Forb/herb	Tolerant
Osmunda cinnamomea L.	cinnamon fern	Osmundaceae	Forb/herb	Tolerant
Osmunda claytoniana L.	interrupted fern	Osmundaceae	Forb/herb	Tolerant
Oxalis montana Raf.	common woodsorrel	Oxalidaceae	Forb/herb	Tolerant
Panax trifolius L.	dwarf ginseng	Araliaceae	Forb/herb	Tolerant
Parthenium integrifolium Britton	wild quinine	Asteraceae	Forb/herb	Intermediate
Parthenocissus quinquefolia (L.) Planch.	Virginia creeper	Vitaceae	Vine	Intermediate
Pedicularis canadensis L.	Canadian lousewort	Scrophulariaceae	Subshrub Forb/herb	Tolerant
Petasites frigidus (L.) Fr. var. *palmatus* (Aiton) Cronquist	northern sweet-colt's-foot	Asteraceae	Forb/herb	Intermediate
Phegopteris connectilis (Michx.) Watt	long beechfern	Thelypteridaceae	Forb/herb	Tolerant

***Appendix A.1** Continued.*

Scientific name	Common Name	Family	Growth Form	Shade Tolerance
Phryma leptostachya L.	American lopseed	Verbenaceae	Forb/herb	Tolerant
Plantago lanceolata L. *	narrowleaf plantain	Plantaginaceae	Forb/herb	Intolerant
Podophyllum peltatum L.	mayapple	Berberidaceae	Forb/herb	Intolerant
Polygala paucifolia Willd.	gaywings	Polygonaceae	Forb/herb	Tolerant
Polygonatum pubescens (Willd.) Pursh	hairy Solomon's seal	Liliaceae	Forb/herb	Tolerant
Prenanthes alba L.	white rattlesnakeroot	Asteraceae	Forb/herb	Intermediate
Prunella vulgaris L.	common selfheal	Lamiaceae	Forb/herb	Intermediate
Prunus serotina Ehrh.	black cherry	Rosaceae	Shrub	Intolerant
Prunus virginiana L.	chokecherry	Rosaceae	Shrub	Intolerant
Pteridium aquilinum (L.) Kuhn	western brackenfern	Dennstaedtiaceae	Forb/herb	Tolerant
Pyrola elliptica Nutt	waxflower shinleaf	Pyrolaceae	Subshrub	Tolerant
Pyrola spp.	Shinleafs	Pyrolaceae	Forb/herb	Tolerant
Ranunculus abortivus L.	littleleaf buttercup	Ranunculaceae	Forb/herb	Tolerant
Ranunculus recurvatus Poir. var. *recurvatus*	hooked buttercup	Ranunculaceae	Forb/herb	Tolerant
Ribes cynosbati L.	eastern prickly gooseberry	Grossulariaceae	Shrub	Intolerant
Rosa spp.	Rose	Rosaceae	Shrub	Intolerant
Rubus spp.	Raspberry	Rosaceae	Shrub	Intolerant
Rumex acetosella *	common sheep sorrel	Polygonaceae	Forb/herb	Intolerant
Sambucus canaadensis L. var. *canadensis*	American black elderberry	Caprifoliaceae	Shrub	Intolerant
Sambucus racemosa L. subsp. *pubens* (Michx.) House var. *pubens* (Michx.) Koehne	red elderberry	Caprifoliaceae	Shrub	Intermediate
Sanguinaria canadensis L.	bloodroot	Papaveraceae	Forb/herb	Tolerant
Sanicula marilandica L.	black snake-root	Apiaceae	Forb/herb	Tolerant
Senecio obovatus Muhl. Ex Willd.	roundleaf ragwort	Asteraceae	Forb/herb	Midtolerant
Smilax herbacea L.	smooth carrionflower	Smilacaceae	Vine Forb/herb	Tolerant
Smilax tamnoides L.	bristly greenbrier	Smilacaceae	Shrub Vine	Intermediate
Solanum dulcamara L. *	climbing nightshade	Solanaceae	Vine	Intermediate
Solidago flexicaulis L.	zigzag goldrenrod	Asteraceae	Forb/herb	Intermediate
Streptopus lanceolatus (Aiton) Reveal var. *Longipes* (Fernald) Reveal	rosey twistedstalk	Liliaceae	Forb/herb	Tolerant
Symplocarpus foetidus (L.) Salisb. ex W.P.C. Barton	skunk cabbage	Araceae	Forb/herb	Tolerant
Taraxacum officinale F.H. Wigg. *	common dandelion	Asteraceae	Forb/herb	Intermediate
Taxus canadensis Marshall	Canada yew	Taxaceae	Shrub	Tolerant
Thalictrum dioicum L.	early meadow-rue	Ranunculaceae	Forb/herb	Intermediate
Tiarella cordifolia L.	foamflower	Saxifragaceae	Forb/herb	Intermediate
Toxicodendron radicans (L.) Kuntza subsp. *negundo* (Greene) Gillis	eastern poison ivy	Anacardiaceae	Forb/herb Vine	Intermediate
Trientalis borealis Raf. subsp. *borealis*	starflower	Primulaceae	Forb/herb	Tolerant
Trifolium pratense L. *	red clover	Fabaceae	Forb/herb	Intolerant
Trillium cernuum L.	nodding trillium	Liliaceae	Forb/herb	Tolerant
Trillium grandiflorum (Michx.) Salisb.	white trillium	Liliaceae	Forb/herb	Tolerant
Uvularia grandiflora Sm.	largeflower bellwort	Liliaceae	Forb/herb	Tolerant
Uvularia sessilifolia L.	sessileleaf bellwort	Liliaceae	Forb/herb	Intermediate
Vaccinium angustifolium Aiton	lowbush blueberry	Ericaceae	Shrub	Intolerant
Vaccinium myrtilloides Michx.	velvetleaf huckleberry	Ericaceae	Shrub	Intermediate
Verbascum thapsus L. *	common mullein	Scrophulariaceae	Forb/herb	Intolerant
Veronica arvensis L. *	corn speedwell	Scrophulariaceae	Forb/herb	Intolerant
Veronica chamaedrys L. *	birdeye's speedwell	Scrophulariaceae	Forb/herb	Intolerant
Veronica officinalis L. *	Common Gypsyweed	Scrophulariaceae	Forb/herb	Intolerant
Viburnum acerifolium L.	mapleleaf viburnnum	Caprifoliaceae	Shrub	Tolerant
Viburnum lentago L.	nannyberry	Caprifoliaceae	Shrub	Tolerant
Viburnum rafinesqueanum Schult.	downy arrowwood	Caprifoliaceae	Shrub	Tolerant
Viola canadensis L.	Canadian white violet	Violaceae	Forb/herb	Tolerant
Viola conspersa Schrank	dog violet	Violaceae	Forb/herb	Intermediate
Viola macloskey F.E.Lloyd subsp. *pallens* (Banks ex Ging.) M.S.Baker	wild white violet	Violaceae	Forb/herb	Intermediate
Viola pubescens Aiton	downy yellow violet	Violaceae	Forb/herb	Tolerant
Viola sororia Willd.	common blue violet	Violaceae	Forb/herb	Intermediate
Vitis riparia Michx.	riverbank grape	Vitaceae	Forb/herb	Intermediate
Waldsteinia fragarioides (Michx.) Tratt. subsp. *fragarioides*	Appalachian barren strawberry	Rosaceae	Forb/herb	Tolerant

* Invasive species
woodlands, woods = tolerant
open woods, thickets = midtolerant
waste places, roadsides,meadows = intolerant

ELECTRONIC APPENDIX:

This article has an electronic appendix with basic data.