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A TULLGREN–TYPE EXTRACTOR FOR SAMPLING SPRINGTAILS POPULATIONS FROM SMALL VOLUME SOIL CORES IN HIGH SAMPLE SIZE

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Dombos, M. (2002): A Tullgren–type extractor for sampling springtails populations from small volume soil cores in high sample size. — *Tiscia 33, 3-7.*

Abstract. I investigated the accuracy and precision of a Tullgren–type extractor, modified to sample springtails populations from small volume soil cores in high sample size. Efficiency of the extractor was tested in two types of running procedures by putting known number of *Folsomia candida* (Willem) in the soil cores. The accuracy and precision depended highly on the running procedures, one of the loading types had sufficient reliability, whereas other conditions did make high variance in the efficiency. In the loading methods, when the temperature was slightly increased, both the accuracy and precision of the census technique was higher compared to that one, where temperature was enhanced abruptly. The construction of the extractor is detailed.

Key–words: sampling methods, Tullgren–type extractor, Collembola

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Introduction

There are several methods to estimate population size of microarthropods, among others of springtails. Like in all other measurements, the feasibility of these census techniques depends on its accuracy — how close a population estimate is to the true population size — and its precision — how close a population estimate is to its expected value. For sampling euedafic collembolan populations one of the most popular technique is the extraction method. The Tullgren-type extraction procedure (Tullgren 1918, Macfadyen 1953) is the simplest one, in which soil animals are forced by a temperature gradient to move from the soil cores to the vials. This technique is based on the behavior of soil animals, therefore it has a variability of its accuracy. Under different conditions the accuracy (efficiency in other papers) depended not only on the technical setting up, but on external factors, such as soil type, species and age (van Straalen and Rijninks 1982). There are some other works dealing with technical modifications (Hassal *et al.* 1988, Crossley and Blair 1991), which are improving both the cleanly of the samples, the practical laboratory serviceableness, the heating and cooling systems, as well.

According to the reviews of Edwards and Fletcher (1971) and Edwards (1991), although the extraction method has high accuracy compared to other techniques, the estimation of its precision has been neglected. The precision is reduced when springtails have to be sampled from small soil cores, like in analysis of spatial patterns of soil springtails. On the other hand, such an analysis requires relatively high number of samples at which precision is increased. My goal was to build up an extractor complying with such requirements.

The aim of this paper is (1) to present this extractor modified for the above demands with respect to its accuracy and precision under two different extracting procedure to estimate the sensitivity of the apparatus, and (2) to detail the materials used by the construction of the extractor, available in Hungary.

The extractor

The construction of the extractor is similar to that one built by Rijninks (van Straalen and Rijninks 1982).

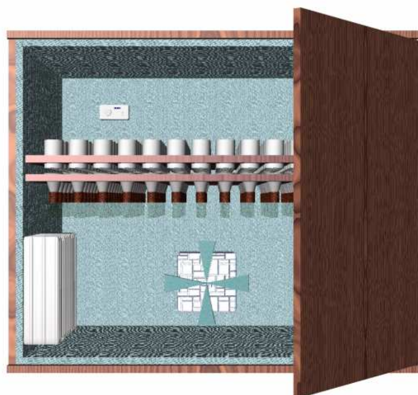

Fig. 1. The view of the extractor.

The cabinet made of plywood has 1.5 m^2 basic area (external dimension: 228x66x100cm) and is isolated on the inner side with polyurethane (thickness = 2cm, Fig.1). It can be opened by 2x2 doors, which split the frame into two sides. The inside of the extractor is also horizontally subdivided into two parts, one for the heating and one for the cooling system. The racks of cores and funnels are equipped in between the two parts, mounted on four sliding drawers. The upper drawers are made of polyurethane, which are hard enough to hold the 103 and 112 soil samples. They isolate the upper side from the lower one at the same time. These are perforated by a steel cylinder (Ø = 5.1cm) rendering the core holders to slide up from the racks possible. The core holder's dimensions are 5cm diameter and 8cm height, provided 137cm^3 inner volume and have a sieve at the bottom (mesh size: = 1mm). Under the sieve there are two perforated disks, which are twisted so that their holes do not overlap. This is an important detail, because these disks prevent the preservative samples from becoming dirty during the extraction. The core holders are covered with a fine-meshed gauze. The lower drawers consist of polyurethane too and are perforated like the upper ones to hold the funnels. The vials (Ø: 2.1cm, height: 5.6 cm) are joined to the ends of plastic funnels (upper Ø: 5cm, lower Ø: 2.1cm, angle: 31°C) with rubber tubes. They have to have the same diameter, for easy attaching, and because any obstacle for the moving of animals in this part would diminish the efficiency of the extractor (Merchant and Crossley 1970). This type of contact has other advantages, as it is easy and fast to work with, and it prevents the preservative material to evaporate from the vials during the extraction.

The heating system is equipped on the top of the inner side of the canister consisting of two 150W infra satin bulbs and a thermostat unit (IMIT, reliability: 0.2°C) to control the temperature. Below the bulb there is a plate to decrease the direct radiation of heat to the core samples.

The cooling system is mounted on the bottom of the cabinet. If the extractor works on room temperature, the cooling system is made up of a refrigerator unit, but if it works in cool room (10–15°C), it is enough to build in a simple ventilator. Other technical details are available on request.

Methods

Extraction

The soil cores with known number of animals (see below) was placed in the extractor. Two types of running procedure were completed. In the first experiment the temperature was set at 20°C the first day and was increased with 5°C the second and third days, so from the third to the sixth days the cores were extracted on 30°C. In the second one the thermostat unit was set at 30°C at start and remained on this temperature.

Measurements of temperature and humidity

Temperature was recorded with a thermistor (LOGIT) in the two compartments of the extractor and in the environment permanently throughout the extraction period.

Relative humidity of the soil samples was estimated by choosing randomly 5 samples from each drawer every day during the extraction and was determined according to the thermo-gravimetric method.

Testing accuracy and precision

Accuracy was measured by the efficiency, where efficiency [%] was defined as the number of collembolans in the soil core at the time/in the start]x100. The explicit efficiency was estimated by giving known number of *Folsomia candida* to the soil cores. 50 specimens were put in different age in each of the 50–50 cores on each rack. Precision was estimated by standard deviation and standard error of the number of springtails caught during the procedures. The soil used was defaunated by freezing at –20°C (Bengtsson *et al.* 1994).

The possible environmental heterogeneity in the cabinet can provide differences of the efficiency among the samples. Furthermore, differences in airing can also contribute to this systematic error. For this reason efficiencies were measured on the five different parts of racks in five groups and it was tested whether the extractor on different parts of the rack has different efficiency. Five parts were selected on each drawer, four in the corners and one in the middle of the drawers. Each group consisted of four core samples. The number of animals captured in the vials was counted every day.

Statistics were calculated using the software package StatSoft, Inc. (1995). Means ± standard deviations are presented, standard errors are indicated as SE

Results

Temperature profiles

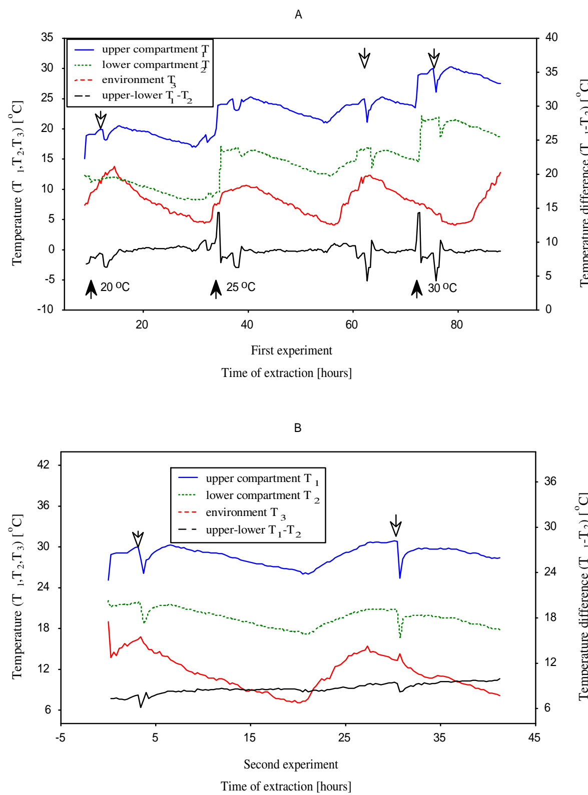

Fig.2. Temperature profiles. Footnotes: Thick arrows indicate setting time, Open arrows show, when the cabinet was opened.

In the first experiment the temperature was set at 20, 25, 30°C (Fig. 2a). The temperature of the environment ranged from 4 to 13.8°C with a mean 8.1±0.3°C, the inner temperature varied with the environment, but the gradient remained considerably stable. The difference between the upper and lower compartments of the extractor was 8.7±0.1°C.

Humidity profile

In the first experiment the cores were dried up more softly, compared to the second one, where after two days the relative humidity decreased sharply to 30% (Fig. 3). Higher values of the standard deviation in the second experiment indicated that the conditions were more uncontrolled. At the end of both experiment all of the soil cores, sampled from different core holders had low humidity (12.3 ± 0.8 % and 12.7± 2.2 %).

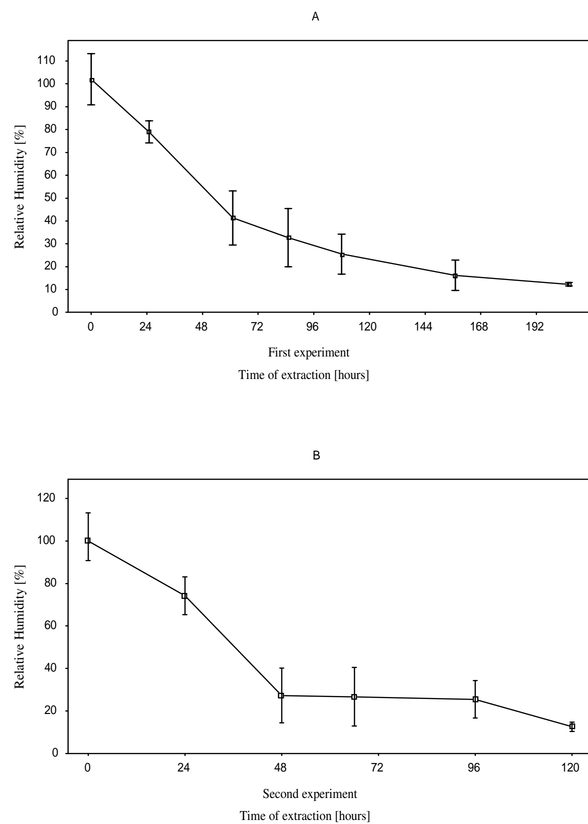

Fig. 3. Relative humidity of soil cores during the extraction. Footnotes: squares: means, whiskers: ± standard deviation

Accuracy and Precision

85.3±3 percent of the springtails has been recaptured in the first, and 72.1±9.4% in the second experiment. There was a significant difference

between the efficiency of the two procedures (t(38) = 2.8 (p=0.008)). There was no difference between the efficiency of the two last samples showing that no more animals would have been alive. The first procedure had not only higher efficiency then the second one, but provided lower and more stable variance during the experiment, compared to the second one, suggesting, that the first experiment had not only higher accuracy, but also it was more reliable, because it had higher precision. In the second procedure some soil cores could be found with extremely low efficiency (range = 68%).

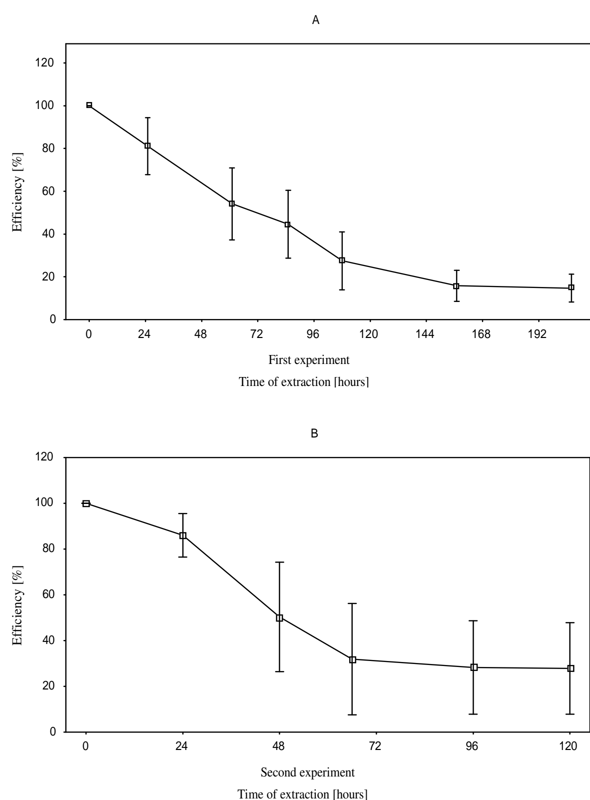

Fig. 4. The efficiency of the extractor during the extraction. Footnotes: squares: means, whiskers: ± standard deviation

There were considerable differences between the means of groups' efficiencies in both experiments (Table 1). In the first procedure the highest difference was 11%, whereas in the second one it was 26%. One way ANOVA demonstrated significant differences between the means of groups' efficiencies in the first experiment, but could not distinguish between the means of groups in the second one, because of high variances. If we regard the groups in both experiments, non of them behaved in the same manner, giving higher or lower efficiency consistently.

Discussion

Regarding the technical details we can conclude that the thermostat and the heating unit could not control the inner temperature with adequate sensitivity, because of the vulnerability of the heating unit. Because the compartments of the cabinet could keep approx. 8°C gradient, the isolation can be regarded proper. There have been many attempts to minimise the amount of soil and debris that falls into the collecting tube (von Torne 1962, Murphy 1962), but it always decreased the efficiency of the extraction. In our case the two perforated disks under the sieve had such a task, although we do not know how it reduced the efficiency.

In the second experiment not only the efficiency, but also the reliability of the extractor has to be regarded as insufficient. The cores could dry out immediately and therefore increased the probability of animals dying in situ. The results obtained in the first experiment has given an appropriate set of temperature and extraction time, non of the core's efficiency fell bellow 72 %.

The examination of the efficiency of the extractor was based upon giving known number of springtails to the soil cores, which technique is considered as a minimal estimate of efficiency, because laboratory animals are sometimes injured, or behave abnormally (Petersen 1978). The comparisons of different apparatus, given by van Straalen *et al.* (1982) suggested, that estimates of efficiency can vary between 62–90% and its efficiency is significantly lower, than passive technique, like hand–sorting or flotation–type technique.

The technical facilities available rendered possible to build up such a construction in that the heating and cooling system could provide relatively stable and homogeneous environment to the soil cores. Both accuracy and precision can be improved by further development, especially in heating system.

In ecological examinations, where high sample size employed sampling procedures require sampling error estimates. The extraction methods render possible to estimate absolute census or population number indexes on soil microarthropods, of which biases depend on the technique used. If the ecological analysis is more sophisticated, demographic, marking or other topics are investigated, further accuracy and precision estimates, for example age–specific aspect of efficiency have to be conducted.

Table 1. The efficiency of the extractor among the soil cores groups.

Experiments:	First			Second		
		Efficiency			Efficiency	
Groups	Mean	Std. Error	Range	Mean	Std. Error	Range
1	91	3.3	14	61.5	5.9	26
2	80	3.2	14	87.5	6.0	26
3	86	1.2	4	69	7.9	34
4	83	4.1	20	76.5	6.1	24
5	86.5	2.2	10	66	18.0	68

Differences in means between groups:

First: $F(4,15) = 3.68$; $p<.028$ — Second: $F(4,15) = 1.05$; $p<.414$

Acknowledgments

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TISCIA 33, 9-30

MOLECULAR MARKERS IN ECOLOGY

Zs. Pénzes, Gy. Csanádi, G. M. Kovács and Zs. Beer

Pénzes, Zs., Csanádi, Gy., Kovács, M.G. and Beer. Zs. (2002): Molecular markers in ecology. — *Tiscia 33, 9-30.*

Abstract. The aim of this paper is to encourage reluctant ecologists thinking more on the use of genetic markers. No training in molecular methods is supposed. The resistance may be explained by the confusion of the high number of methods, including laboratory techniques (named by up to four characters) and evaluation. Inevitably, field work needs some extra step to collect DNA source, but sampling strategy is the same or even less restrictive owing to the new, powerful statistical methods. Laboratory techniques develop very fast, many phases can be done automatically and/or many customers provide services on reasonable price.
Despite the financial and technical requirements, genetic markers provide high quality information that can be obtained hardly otherwise, or simply impossible. We try to overview the most important genetic markers and technology used recently. Some examples are given by studies of parentage, population structure (migration, fragmentation) or population history.

Keywords: DNA techniques, DNA markers, molecular markers

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Introduction

Until the mid-1960-s, population genetics was mainly a theoretical science. Now, this view is changed and the impact of population genetics increased considerably in many fields of biology (Sunnucks 2000). Suddenly, the mainly theoretical quantities of population genetics (percent polymorph loci, inbreeding coefficient, population structure etc.) are inevitably incorporated into the terminology of other fields of biology, including conservation biology (Smith and Waine 1996, Hedrick 2001) and ecology (Snow and Parker 1998, Baker 2000a). Population genetics studies the underlying processes of genetic variation, defined in samples of individuals from different populations and species. Nowadays, the genetic variation is not only a criterion for natural selection to work. Genetic variation is a huge source of information about the biology of individuals carrying a given variant. It also keeps track the history and spatial relationships of populations composed by these individuals. This information can be extracted using genetic markers.

The use of genetic markers is not limited to the age of modern genetics. They were essential tools also of the classical breeders who followed the inheritance of selected traits of their plants and/or animals. This aspect sheds light on the two main characteristics of a genetic marker: (1) it must be distinguishable and (2) must be inherited genetically. The genetic basis of having markers in an individual or in a population is the presence of different alleles on a given genetic locus, which results in a genetic variability or genetic diversity or genetic polymorphisms. These expressions are synonyms describing the same thing: there are genetic differences between individuals which can be used for making genetic analysis on them for different purposes like individual identification (genetic “fingerprint”), genetic mapping, breeding or to describe their genetic relatedness.

For centuries only visible phenotypic differences could be used as markers such as colours, spots, heights and weights and other morphological characteristics, which caused a serious limitation in genetic analysis, especially in plants. Despite of the

fact that Gregor Mendel produced his historical results in a plant system, these organisms have unfortunately low amount of characteristics, which can be used for genetic analysis. In addition, many of the visible and important characteristics are not represented by a single genetic locus but coded by multiple genetic loci (e.g. height, weight), which makes their use as genetic markers almost impossible. Therefore the appearance of molecular genetic markers was a revolutionary breakthrough in determining and using genetic polymorphisms for different purposes. The first such molecular markers were the protein polymorphisms which were based on the isoenzyme variations of the same polypeptide. Using this methodology the number of detectable polymorphisms increased significantly indicating that most of the available genetic differences remain invisible at the morphological level (Hubby and Lewontin 1966, Lewontin and Hubby 1966).

The discovery of DNA markers had really enormous effect on the field of genetic analysis unveiling the waste majority of hidden allelic variations. Application of solution-based DNA-DNA hybridisation technique is an important step in this process (Bledsoe and Sheldon 2000). The revision of bird phylogeny by Sibley and Ahlquist (1990) stimulated biologists to study DNA variation from a different point of view: the use of DNA markers. The methods are changed, but molecular phylogenetics is one of the biggest "end-user" of genetic markers today (e.g. Hillis *et al.* 1996, Page and Holmes 1998, Nei and Kumar 2000). The growing number of inferred phylogenetic trees provides the basis of the comparative methods, which is one of the most important tools in the evolutionary biology (Harvey and Pagel 1991).

Another important step was the introduction of the Southern-hybridisation based RFLP (Restriction Fragment Length Polymorphism) technology to eukaryotic genetics in the early 1980s which made possible for the first time to investigate the genetic differences directly at the genotype level in higher taxonomic level. This was followed by the elaboration of the polymerase chain reaction (PCR) technique at the early 1990s, which became an essential tool for modern geneticists because of its amazing simplicity, effectiveness and versatility. With appropriately selected piece of DNA sequence (the primers), small amount of DNA can be amplified to usable quantity. But the technical refinements are not yet finished. Highest variability can be obtained on the level of DNA sequence. Every single base accessed by automated methods of DNA sequencing could have high importance in an ecological analysis. On this ground, the number of markers useful for a given topic grows continuously. Recent success of highly variable microsatellites (SSRs, single sequence repeats) provides an excellent example. Molecular methods generated new data, discoveries and controversies that stimulated new theories and development of powerful statistical methods, available almost at once in user-friendly computer programs (e.g. Luikart and England 1999). These facilitated further the study of variation in DNA related to fundamental questions of biology. These key innovations together reveal otherwise unobtainable information at all level of biotic hierarchy (e.g. Avise 1994, Ferraris and Palumbi 1996, Smith and Waine 1996, Burke *et al.* 1998, Goldstein and Schlötterer 1999, Baker 2000a). Many efforts are made to standardise methodology and getting comparable results (e.g. EU Molecular Screening Tools project, see Karp *et al.* 1988).

To give a rigorous definition, genetic markers are simply heritable characters with multiple states at each character (Sunnucks 2000), where character means a given genetic locus and character state is defined by the alleles. Furthermore, individuals have many loci and separate loci can provide independent characters. Applying the same logic to lower organisation level, even nucleotides in any position of the DNA sequence may be interpreted as character with four states. This means that with sufficient variability, genetic markers may provide never seen discriminatory power.

The information can be obtained about loci defines two types of genetic variation. We speak about genotypic variation where the genotype is of interest. Genotypes of multiple single loci compose a genotypic array. Sometimes allelic correlations are also needed (linkage disequilibrium). In other words, the gametic phase or haplotype pair is identified in a given individual. Genic variation means that alleles or haplotypes are of interest, but not their combination into genotypes. On a pooled analysis carried out on population level, we may have frequency data from alleles of a given locus, even if we have no imagination about the genotype of individuals.

Every individual organism has unique combination alleles and DNA sequence. This gives unique set of markers on the lowest level of biological hierarchy and provides extreme sensitivity of the genetic markers. As Waser and Strobeck said (1998): "very few birds have bands but all have genotypes". But, according to the nature of genetic differentiation, the rate of change of the different kind of markers may vary. This rate is influenced by the processes of recombination, mutation and selective constraint. Of course, models of population genetics can be applied to DNA markers, too. In this

way, markers can be used for answering questions on different scale in space and time: different markers can be selected for different scales. On the other hand, genetic variation itself is organised hierarchically (irrespectively of species concept): alleles within individual, individuals within (sub)population, or even metapopulations. Besides the scale of study, appropriate marker choice depends also on the level of hierarchy.

The same conclusion can be drawn on a different way. The fate of a given individual both in space and time depends on its biology and its environmental interactions. These may be reflected in the DNA sequence (often referred as the "natural history of DNA") resulting in polymorphism. Fundamentally, this polymorphism is shaped by the known processes of mutation, recombination or selection and their connection, as exemplified by population genetics for at least eighty years. In this way, the theory is simple: by measuring the genetic variation and translating it to useful quantities with the models of population genetics, we can make inferences about the history and/or relationship of populations or the biology of organisms. Information can be obtained about the biology or many population level processes in space and time by examining genetic markers with appropriate rates of change. Just to mention some of them: mating structure, relatedness, population structure (e.g. migration, heterogeneity) or population history (e.g. bottleneck events). Of course, this logic can be extended to the scale of evolutionary processes as it is demonstrated by the enormous literature of molecular phylogenetics.

There are many factors should be weight for selecting DNA marker for a given problem. The classification of markers on the basis of these factors is detailed below. In less rigorous way, three levels of molecular change are generally separated (e.g. Sunnucks 2000). Levels are related to different aspect of population biology, claiming for different markers.

The most sensitive markers are the genotypic arrays (e.g. multiple microsatellite loci scored in individuals). The relevant time scale is the generation and the rate of change depends mostly on the frequency of recombination. Genotypic arrays are used in the shortest and finest scale studies. Some examples of usage are individual identification, parentage (paternity) and relatedness.

Genic analysis (e.g. microsatellite, mitochondrial DNA (mtDNA)): individual genes are considered and allele frequencies, geographic distributions are recorded. These properties change on larger scale. Typical examples are the studies of gene flow (migration) or reconstruction of population history.

Relationship of alleles (creation of new alleles by mutation) provides information on larger, evolutionary scale. It is used for studying long-term processes of phylogeography, speciation or phylogenetic reconstruction. This level is beyond the scope of this paper.

Our aim is to give a short overview, classification of the fundamental markers and techniques proven to be useful in ecology. This topic is covered by excellent books (e.g. Baker 2000a) or reviews published in journals of ecology (e.g. Snow and Parker 1998) written for non-molecular biologists. We try to highlight information from these on the basic level or sometimes update them by some new important results. Reviews are referred whenever possible as they are available more generally and further details and references can be found in them. We focus on how to collect and store sample or the reason why a given marker should be used for solving a special question. The advent of statistical methods and software is outside of our topic, mentioned shortly in the discussion. Furthermore, this paper is based primarily on our experience in using these methods even in ecological laboratory. That is why some of the important class of markers is not covered here (e.g. MHC) or mentioned shortly (e.g. mtDNA). Other markers are simply ignored as they have (more) interest in taxonomy or evolutionary biology (e.g. interspersed class of repetitive sequences, like SINEs and LINEs) but not in ecology. Abbreviations are given at the end of the paper. Finally, the importance of this field is proven also by statistical indices of its bibliography. The leader journal in this topic is the Molecular Ecology being among the most cited primary ecological journals (source: ISI, cited in Sunnucks 2000).

DNA sample

The success of molecular analyses depends upon collecting the most suitable samples and storing them in a proper way as to minimise damage. Properly handled DNA sample can be used for many different analyses. Molecular techniques require very small amounts of DNA (typically less then 5 μg) permitting non-destructive sampling of large number of individuals. In theory, one molecule of DNA template is enough for a PCR amplification. Not only the quantity but the quality of the DNA is important, depending on the method (see there). Properly prepared and stored samples are needed not only to carry out the present study but for the unpredictable

future possibilities. As new techniques are being developed, old samples can be reanalysed again with more efficient methods. This may happened many times in literature and it worth to keep in mind.

The logic followed in this chapter based primarily on Carter's (2000a) work. It provides also a review of sampling, preservation considerations and fundamental techniques not explained here. Good and continuously updated protocols are given in the cited reviews. We suggest also Karp *et al.* (1998) as it contains information about equipment and safety considerations. Shortly, the basic steps of molecular analyses are the followings:

1. Sample collection
2. Sample storage
3. Extraction of DNA
4. Preliminary modifications (e.g. digestion)
5. PCR or hybridisation
6. Visualisation (electrophoresis, sequencing)
7. Interpretation of the results.

In this section, sample handling (1-3.) and general techniques are overviewed in some details. Marker technologies with their special requirements are considered in the next section (4-6). Step 7 is outside of our scope, but some notes are given in the discussion.

Sample source

Almost any cells contain a copy of the genome and can be used to extract DNA. Owing to the advances of PCR, even the catching of individuals can be avoided: feathers of birds or hairs and faeces of mammals can be potential sources of DNA. These non-invasive techniques are of great interest (Taberlet *et al.* 1999), especially in conservation biology. For example, mtDNA and microsatellites markers can be assayed from faeces (for the topic of molecular scatology, see e.g. Kohn and Wayne 1997) or urine (Valiere and Taberlet 2000).

Nevertheless, not all cells and tissues are equally suitable sources of DNA. Secondary metabolites may interfere with the enzymes used to manipulate DNA: high concentration of melanin inhibits *Taq* polymerase used in PCR or polyphenols in plants inhibits many DNA modification enzymes. Bones or woody plant tissues are very hard and difficult to work with. In these cases some extra steps are needed to extract DNA.

Blood is one of the most convenient samples for DNA isolation in non-mammalian vertebrates, as they have nucleated red cells. As little as 1-2 µl of blood is sufficient, but usually much more is taken by venipuncture. For mammalians, 1-2 ml would be needed to work with easily. In some cases, tissues may be preferred, like skin biopsies. For insects, the whole individual or muscles may be used. Pigmented parts should be avoided. For plants, young leaves (or needles) are the most obvious sources (more cells per weight and less polyphenols and polysaccharides). "Ancient DNA", even thousands of years old may be isolated from bones and teeth and used for PCR amplification and sequencing (e.g. Hagelberg and Clegg 1991). Preserves used in museum collections (like formalin and Carnoy's solution) are not good for DNA preservation: usually large genomic DNA can not be isolated but PCR-based techniques can be used.

Sample preservation

DNA is very stable and robust, if it is handled properly. It is prone to damage by nucleases, chemical degradation, extremes of pH, mechanical shearing, excessive heat and strong light. It is a good idea to extract the DNA as soon as possible and store it –80 oC. Freshly collected samples are the best for DNA isolation but even these must be preserved until the extraction. There are many buffers suggested in the literature but they vary in efficiency. Generally true that the ratio of the preservative and sample must be kept.

The two basic methods of preservation are the chelation and dehydration. Agents such as EDTA are able to chelate the free magnesium ions (essential to activity of nucleases) and prevent the nucleases from degrading DNA. Detergents (SDS) are also included in cell lysing buffers. Disadvantages of these methods may be the some extra steps to remove proteins. Owing to the viscosity of the suspension, it can be difficult. Nucleases can also be inactivated by dehydration. One of the most generally applied ways is using several volumes of absolute ethanol (for example, suspending one drop of blood in 1 ml ethanol in an Eppendorf tube). Ethanol can be used also for preserving whole insects. Samples handled in this way can be stored for years even at room temperature without significant degradation, although alcohol contaminants may cause degradation.

There are many other possibilities for sample storage, like sodium chloride with 10 % DMSO used for DNA fingerprinting and mtDNA analysis from large tissues. Dried blood is also frequently used or one can also keep samples alive, like blood cells in Alseviers solution (for weeks only).

It worth to mention that samples should be stored away from strong light because of the photochemical degradation of the DNA. It is also a good idea to keep it in a cool place. Samples should be subdivided to avoid repeated cycles of freezing and thawing,

which causes loss in quality and quantity. In summary, we have plenty of possibilities. Appropriate preservation system should be chosen carefully and should be tested before the starting of the field season.

DNA extraction

The aim of the procedure is to produce sufficiently pure and adequate quantity and quality of total (nuclear and organelle) DNA for analysing markers. Protocols are straightforward: lyse the cells (e.g. SDS), remove the proteins and other cellular components (e.g. Proteinase K, phenol-chloroform extraction or salting) and recover DNA in the intact form. The concentration of the DNA (even as PCR template) may be important (e.g. Linacero *et al.* 1998). It should also be mentioned, that there are protocols for direct PCR from tissues without extracting the DNA directly. We had success using the "single fly" method for insects (Gloor *et al.* 1993).

For samples that are known to yield high quantities of DNA (e.g. blood and tissues) the standard phenol-chloroform-ethanol precipitation method may be used (Sambrook *et al.* 1989). The nuclear DNA could be easily extracted also from plant or fungal tissues. One of the most common is the CTAB method, which is widely used by botanists and mycologists (e.g. Štorchová *et al.* 2000). The simultaneous extraction of fungal and herbal DNA could make problems for example when natural samples are used with non-specific methods (e.g. RAPD) but these could be excluded with specific primers. Isolation of DNA from cellular organelles (chloroplasts and mitochondria) may need extra steps, to digest nuclear DNA. However, this can be avoided using PCR with specific primers.

Some techniques are developed to be fast, others to be cheap or to give high quality output. There are protocols to avoid phenol extraction, as special safety equipment is needed. The most time-consuming step is the extraction of DNA from hundreds of individuals. However, variety of commercial kits can decrease this time, used widespread in ecology (e.g. produced by Qiagen and Sigma). They are efficient and have reasonable cost, especially for unit time. They can be used for different sources and produces high quality and high yields of DNA. Based on our experience, kits (we used Sigma genomic DNA kit for insects) should be preferred. Phenol-chloroform extraction may yield higher amount, but kits are much faster, more robust and produce about the same quantity of DNA (quantification is not needed). Safety equipment can also be avoided in this way. Chelex with its simple protocol is also proven to be very useful.

Finally, protocols even may depend on the species. Genomic size and complexity may differ, so preliminary experiments should be performed to minimise data loss in the further studies.

Further steps, basic techniques

If we have the DNA in hand, further steps vary between marker technologies. Sometimes (minisatellite fingerprinting, generally for hybridisation based methods or direct sequencing) DNA must be digested by restriction endonucleases. For PCR, this step is not needed. Before going to details of markers, some words about the basic techniques are given. Any marker analysis results finally in a set of DNA fragment. These fragments may differ in size that must be separated and visualised. As already explained, even if fragment sizes are the same, or differences are not detectable by the method selected, DNA sequencing may provide the last solution.

Southern hybridisation

The basis of the method is the restriction enzyme digestion of the total genomic DNA isolated from the individuals. For this one has to use a laborious procedure because only the pure DNA can be digested properly. This digestion generates a huge amount of restriction fragments with different sizes. Approximately 10 μg of digested DNA is loaded on an agarose gel and separated according to their sizes. This extremely high amount of different fragments contains many overlapping sized fragments thus resulting in a continuous smear on the agarose gel. The high complexity of fragments can not tell us anything about genetic variance by itself. To be able to see the differences in the fragment sizes we have to use a radioactive- or fluorescent-labelled probe which is (Southern-) hybridised to this separated DNA. Because the probe DNA hybridises only to its complementary sequence(s) it can detect the fragment size of its complementers in the fragment population. If the probe hybridises to a single locus, it results a single-locus pattern, if it has many complementary sequences in the fragments we will see a multi-locus pattern after visualisation. For visualisation we detect the radioactive or fluorescent radiation of the probe by using X-ray film or digital imaging technology. Reliable and sensitive commercial kits may provide help us in this process (e.g. Digoxygenin DNA labelling and hybridisation kit, Boehringer-Mannheim).

Polymerase Chain Reaction (PCR)

The introduction of PCR technique (Mullis and Faloona 1987, Saiki *et al.* 1988) revolutionalised not only marker methodology but sampling strategy. Using PCR-based methods we need much less amount of isolated DNA (usually few ng is enough) and the detection procedure is also less laborious. The general PCR-procedure is the following: the genomic and/or organelle DNA is called as template DNA for the amplification. There are two short oligonucleotide sequences called primers that are specifically designed to be complementary to the two borders of the sequence on the template DNA which we want to amplify. These are for priming the DNA-strand elongation following the denaturation of the two strands of template DNA and the hybridisation (annealing) of the primer sequences to their complementary sequences on the template DNA. The repetition of the denaturation/ annealing /elongation steps results in exponential increase of the DNA amount bordered by the two primer sequences. It means that after thirty cycles the few fg DNA sequence can be increased to several µg (even 1000 0000 0000x increase!). The length differences between the amplified fragments of different individuals can be visualised on agarose or acrylamide gels after adequate staining or the amplified fragments can be sequenced.

There are many variations of methods applying this simple logic. Some of them are explained below, related to a given marker. See e.g. Birt and Baker (2000) for a recent overview, including variants and optimisation of PCR reaction.

DNA Sequencing

In supraindividal studies often resolution given by differences in fragment length is enough. However, sometimes we need the nucleotide sequence of a certain DNA region even for the analysis or to design adequate markers for further studies. The base of the sequencing used generally nowadays is the polymerase chain reaction with dideoxy nucleotides with which the products of the reaction are stopped at certain bases along of the whole target sequence so with appropriate separation of these products the sequence of the target could be determined. The productivity of the automatic sequencing — particularly with the capillary technique — has improved to such a level which is nearly incomparable with the early methods, however manual sequencing is still used — although not often — for short target regions (e.g. Milot *et al.* 2001). As the technique has developed the cost of the sequencing decreased and — although the equipment is expensive — the sequencing conducted in the frame of co-operations or even as a customer of the sequencing services of plenty of biotechnological companies is relative easy to pay.

Although high fidelity enzymes are available, sequencing of several quasi-independent samples from one region is particularly important when the template is a PCR product. The cloning of the PCR products and sequencing different clones could give a solution for this problem. Although this is more time and equipment consuming method, the samples could be kept as reference material and can be used for further studies and on the other hand using of cloned material could be one of the cheapest ways of sequencing. See e.g. Palumbi (1996) for technical notes related to different questions.

Fragment separation and visualisation

Nearly all the DNA methods with which data can be obtained to answer ecological questions after all based or connected somehow with the size-based separation and detection of the nucleotide fragments. One of the easiest methods — with which however very important and usable data could be obtained (see e.g. RAPD) is the agarose-based electrophoresis of the DNA visualised by ethidium-bromide. After the samples were run on the gel with appropriate agarose concentration using the electric charge of the DNA molecules and staining the gel in ethidium-bromide solution, the fragments can be visualised by ultra-violet light and documented by photo or by special gel documentary systems. Although this is a lightly manageable and relative cheap method and a lab could be easily supplied with the equipment necessary for collecting analysable data, one of the biggest limitations of the technique is its relative poor resolution. Small differences (typically less than 10 nucleotides) — even with using high concentration agarose gel — between fragments are hardly detectable.

The acrylamide-based gel electrophoresis with silver staining has much better resolution – one nucleotide differences of fragments could be detected — and although the method is more time and practice consuming it is commonly used in molecular works in ecology (see below) and the necessary accessories are relative not expensive. Isotope-based techniques are still also used for visualisation and documentation of DNA fragments but the work with them needs special safety equipment and permissions beside the special experience.

Very large fragments (e.g. chromosome sized) are separated by different methods, such as pulsed-

field gel electrophoresis (PFGE). Actually, efficient electrophoresis methods are developed to detect minimal differences as small as one nucleotide (e.g. SSCP).

Markers

As it was mentioned before there are two main types of molecular markers such as protein and DNA ones. However, they include several “subgroups” which can differ essentially from each other. Before going into details of their characteristics and grouping we have to consider some important characteristics of the different marker methodologies.

Genetic background

The basic background of genetic polymorphisms in organisms is the differences occurring in the DNA sequences due to mutations accumulated in their genomes. It is important to know what kind of mutations can occur because the type of a certain mutation significantly determines the selection of the most useful marker methodology for its detection. The main types of mutations are: (1) insertion or deletion of a certain DNA-fragment, (2) gene duplication or multiplication, (3) variable number of repetitive sequences such as mini- or microsatellites, all of them resulting in altered fragment lengths (4) or exchange of multiple or single nucleotides, the latter one called single-nucleotide polymorphisms (SNPs), without alteration of fragment length.

The mutations occurring in coding genes can influence the expression of them causing differences in visible phenotypes. Others are non-visible but result differences at protein level. All the others are so-called "neutral" mutations either in coding genes without affecting them or in non-coding regions such as repetitive or heterochromatic regions (e.g. “satellites”). These alterations are not represented even in the protein pattern therefore their existence can be demonstrated only at DNA level. This allows us to draw several important conclusions to be considered when selecting a right tool for analysis of genetic variability. First, the expected number of variations is the highest when using DNA markers and lowest when following visible phenotypic characters. This is because only very small portion of the total genomic DNA is coding for genes, which have phenotypic effect, therefore most of the genetic variations are not represented in the phenotype. Second, the less influenced therefore the most reliable markers are also the DNA markers because they are not influenced by the so-called "penetration-effect": i.e. some genes are not expressed, or suppressed by other gene's products, therefore they are not represented at protein or morphological level. Third, the use of visible markers is the most misleading because they can be influenced also by environmental factors, which effect is completely eliminated at DNA level.

It must also be mentioned here that because there may be a strong evolutionary pressure on the protein coding genes which doesn't allow them to mutate very frequently because sometimes they are essential for the survival of an organism. Therefore most of the mutations are concentrated mainly on the non-coding regions of the genome. According to this there are so-called "mutation hot-spots" which are hypervariable regions such as short repetitive sequences or microsatellites. It means that if we want to detect high level of polymorphisms we have to search them in the hypervariable regions of the genome.

Two classes of tandem repeat sequences are of great interest in the hypervariable region. These are called VNTRs (Variable Number of Tandem Repeats) including mini- and microsatellites (called as SSRs and sometimes STRs). Nowadays, VNTR tends to be maintained for minisatellites only. The function and evolutionary significance of these sequences is unknown. They are repeated many times in the genome and extremely variable in length. SSRs consist of the so-called core sequences from 30-150 bases, where the length of the repeat units is 1-8 (10) bases (that is 10-50 copies), most often 2-5. Minisatellites has more than 10 bases length unit and core sequence size much bigger. Minisatellites also have lower number of copies in the genome. The underlying genetics of high mutation rate are more or less known: unequal crossing over or strand slippage during replication may cause changes in the number of repeat units (Jeffreys *et al.* 1998) and consequently the size of the fragment we detect.

It must also be mentioned that if we investigate different regions of the genome we can not find the same mutation rate which can be misleading by the evaluation of results. Higher mutation rate of a genomic region results in higher genetic variability, which can be interpreted as higher genetic distance. Nevertheless, the regions where the selection works in the direction of keeping polymorphism at high level (called diversifying selection) can also be used as a powerful tool in population studies (e.g. MHC).

Mitochondrial (and chloroplast) DNA has different characteristics than the nuclear one. Most important ones are that they are haploid and can be considered as haplotypes. Furthermore, they are transmitted intact through generations owing to the (assumed) lack of recombination and inherited

usually maternally. Nevertheless, the lack of recombination is questioned today (e.g. Lunt and Hyman 1997, Hagelberg *et al.* 1999, Wallis 1999). These characteristics provide special importance for mtDNA: “The genome of mitochondria has been the workhorse of this molecular revolution.” (Randi 2000).

Classification of marker methods on different basis

The grouping of the different marker approaches can be performed based on different aspects. The most widely applied classifications are based on the methodology (A) and genetic information content (B) but the other ones can serve as important source of information, too (C-E). Of course, they can be classified on their use in ecology, generality (connectibility) in taxonomic scale or variability (sensitivity). These are not highlighted here but explained in the description of methodologies. (Full names of the markers are given at the end of the paper.)

A. Classification on technical basis:

1. Protein markers:
a. isoenzyme
b. total protein (SDS gels)
2. DNA markers:
a. Southern-hybridization based:
RFLPs of single or low copy number loci
mini- (VNTRs) and microsatellites (SSRs)
multilocus fingerprint hybridisation (e.g. multicopy genes, loci or transposons)
b. PCR-based:
(i) random primed methods (no need for sequence information):
RAPDs, DAFs,
AFLPs
(ii) specific primer-methods (need for sequence information):
specific gene amplifications (mtDNA, scnDNA)
VNTRs (rarely)
SSRs

Notes: referring mtDNA as marker means usually its sequence, although mtDNA RFLP is also used. scnDNA actually a pool for single locus nuclear markers (excluding repeated sequences and sometimes introns) but it can be evaluated many different way including solution DNA-DNA hybridisation. See below.

B. Classification on genetic information content

1. markers detecting single or low copy number loci (codominant inheritance):
a. isoenzymes
b. RFLPs
c. SSRs
d. scnDNA
2. markers detecting multiple loci (dominant/recessive inheritance):
a. total protein analysis
b. Southern hybridisation fingerprints using repeated sequences
c. RAPDs, DAFs
d. AFLPs

Note: mitochondrial and chloroplast DNA are haploid, so (single locus) mtDNA sequence and RFLPs are codominant by definition.

C. Classification on effectiveness and reproducibility/reliability

1. isoenzymes: needs low amount of protein, laborious, highly reproducible and reliable
2. total protein: needs low amount of protein, less laborious, middle reproducible and reliable
3. RFLPs: needs high amount of DNA, very laborious, highly reproducible and reliable
4. AFLPs: needs low amount of DNA, laborious optimisation and use, good reproducibility, high reliability
5. SSRs: needs low amount of DNA, very laborious to develop, but middle-laborious use, very high reproducibility and reliability
6. RAPDs, DAFs: need low amount of DNA, less laborious, low reproducibility and reliability
7. scnDNA: needs low amount of DNA, laborious to develop, but middle-laborious use, very high reproducibility and reliability
8. mtDNA: needs low amount of DNA, but middle-laborious use, very high reproducibility and reliability

Note: developing markers is related to the possibility of the transfer to new taxa. It varies. Furthermore, type of assay including separation (classification D below) may depend on the scale of question/variability.

D. Classification on separation and visualisation methods:

1. protein markers: acrylamide gel electrophoresis & protein staining
2. RFLPs: agarose gel electrophoresis & radioactivity/fluorescence
3. AFLPs: acrylamide gel electrophoresis & silver staining, radioactivity/fluorescence
4. SSRs: acrylamide gel electrophoresis & silver staining, radioactivity/fluorescence
5. RAPDs: agarose gel electrophoresis & EtBr staining
6. DAFs: acrylamide gel electrophoresis & silver staining

For scnDNA and mtDNA, see Table E.

E. Specific methods for detecting SNPs when using PCR amplification based methods

1. DGGE: Denaturing Gradient Gel Electrophoresis
2. SSCP: Single Strand Conformation Polymorphisms Both methods use acrylamide gel separation under denaturing conditions thus detecting single nucleotide alterations
3. Sequencing (mtDNA)

There are strong correlations between different classifications as it is explained below. On the other hand, the classification may suggest strict boundaries, which is not true. Methods can be combined on many different ways. For example, RAPD or AFLP fragments can be converted to single locus markers (isolating from the gel, sequencing and designing a primer pair), in which case they can be used as scnDNA. Another example can be the method for searching for microsatellites using AFLP (Hakki and Akkaya 2000).

Description of the different marker techniques

Protein markers

The isoenzyme-variation analysis (Wedel and Weeden 1989) is based on the knowledge that the same enzyme can have different subunit-composition. These subunit differences can be visualised after running of protein samples on acrylamide gels and staining them with a definite substrate resulting in a colour product thus indicating the presence of a certain protein allele. This method has the serious limitation by finding the right substrate and conditions for enzymatic reactions. The main advantage is its high reproducibility and reliability. Despite of the wide-range use of DNA markers there are examples for using it as efficient tool of genetic relatedness (Rouamba *et al.* 2001) and studying population structure (e.g. Meglécz *et al.* 1999).

The other way of using proteins as genetic markers is the analysis of total protein content of a given tissue of an individual. In this case the total protein content is visualised after acrylamide gel electrophoresis of the proteins under denaturing conditions at which protein subunits are separated by Coomassie or silver staining method. Using this technique we can follow the presence or absence of a certain subunit of different anonymous proteins and these differences can be characteristic for the individuals or groups (Vollmann *et al.* 2000).

Application. They are not detailed here. Isosymes are single locus markers and may show sufficient variability. The methodology is well founded and cheaper than the DNA methods. Nevertheless, studying protein polymorphism requires high quality samples and gives limited genealogical information comparing to the relevant DNA methods. Collection and storage of DNA sample is usually much easier. See e.g. Baker (2000b) for more technical details and usage.

DNA markers: non-PCR-based methods

The most widely used methods are the DNA-based genotyping techniques. According to the classification used before we should see first the Southern-hybridisation based methods like RFLP and hybridisation-based fingerprints.

The RFLP method was developed and first used in human genetic mapping (Botstein *et al.* 1980, Lander and Green 1987, Lander and Botstein 1989). We call **RFLP** in narrow sense if we follow the pattern of low number of loci at one hybridisation, and it is called hybridisation fingerprint if we use highly repetitive sequences as probes for hybridisation. The use of fingerprint methodology became highly significant by the exploration of the variable number of tandem repeats (**VNTR**, Horn *et al.* 1989) or minisatellites which are highly repetitive and polymorphic DNA-sequences capable to detect a very high number of different loci at the same time (Jeffreys 1985, 1990). Using this approach we can distinguish up to 10-30 loci parallel in one experiment.

If we isolate DNA from many different individuals and use them for Southern-hybridisation we can expect that there will be differences in the

length of restriction fragments between these individuals. We can detect fragment length differences if there was an insertion/deletion event in the region we can visualise or the recognition site of the enzyme had been changed between the individuals. The limitations of this technique are coming from two facts. First, only the genetic differences generated by the selected restriction enzyme can be detected. This means that if we have a polymorphism e.g. in the *Eco*RI site of the individuals and we use *Bam*HI enzyme we will not see the differences. The second is, if we use a given enzyme but our probe hybridises not to a polymorphic but to a non-polymorphic fragment. In this case the genetic differences remain also hidden. That is, selecting the right enzyme with a probe needs some optimisation.

Application. RFLP is expensive and time-consuming. Even it is true for its PCR analogue, not detailed here (see e.g. Snow and Parker 1998, but see ascnDNA below). Although RFLPs can be useful for studying population structure, they are rarely used today. It is replaced by more efficient PCR based single locus methods, such as mtDNA sequence analysis or microsatellites.

In contrast, the hybridisation based fingerprinting using repetitive sequences, such as multilocus and single locus minisatellites are still widely used. Owing to their variability, they are used especially for individual identification ("fingerprint") and testing parentage (paternity exclusion) but can be used to estimate diversity on population level. Multilocus fingerprint gives one of the most easily accessible (but not the cheapest) way for individual identification in higher vertebrate taxa, as it can be done by one hybridisation using universal probes for wide range of species (Burke and Bruford 1987). Nevertheless, it is very time-consuming and suffers of drawbacks of multilocus techniques (see below).

Single locus minisatellites would be an ideal tool for individual identification, owing to the advantages of one locus techniques and the use of agarose gel. However, development of probes is very time-consuming and expensive (involves library construction) and probes usually can not be used for other taxa. Single locus minisatellites could also be assayed by PCR. However, owing to the large fragment size, it is rarely used today and has some disadvantages comparing to microsatellites.

Microsatellites can be assayed with hybridisation on a multilocus manner and they have the same usage as minisatellites. The more common PCR based assay is detailed below. Some more notes of fingerprinting are given in the application section.

DNA markers: PCR-based methods

As explained before, for most of the PCR-based methods we need less amount of isolated DNA and the detection procedure is also less laborious. PCR-based assay can be used both of single and multilocus manner. For single locus methods, specific primer pair is needed similarly to the specific probes in Southern-hybridisation. The length of these primers is typically 20 bases at least.

Multilocus techniques

One of the most widely used PCR-technique in genetic variance analysis is the **RAPD** analysis (Random Amplified Polimorphic DNA; Williams *et al.* 1990, Welsh and McClelland 1990). In this case the primer is relatively short (10 base oligonucleotide) and only one sequence is used. The sequence is randomly selected, thus there is no need for prior sequence knowledge. These 10mer oligonucleotides will hybridise to their complementary sequences, which are dispersed randomly in the genome. Therefore many different fragments will be amplified at the same time due to the random distribution of the primers on both strands of the template DNA. The location and the sequence of these fragments are unknown but they can represent fragment length differences between different individuals, if any deletion/insertion happened in the regions they amplify. It can also be supposed that the primer-binding site was altered by mutations in some individuals resulting also differences in the amplification pattern. The amplified fragments are loaded on a concentrated agarose gel and underlain to electrophoretic separation, which is followed by an EtBr-stained visualisation on UV-lamp. The results are usually documented by photography. By this method usually 5-10 loci can be detected in one experiment.

Comparing the protocols of specific primed PCR and RAPD, the length of the oligonucleotide primers is significantly different and the annealing temperature of RAPD is much lower. These two characters are correlated: the shorter the sequence is the lower the hybridization (annealing) temperature must be (because there are much less amount of hydrogen bonds). The GC-nucleotide content has also very important effects on amplification. The higher the GC-content is the higher the annealing temperature can be (because there are 3 H-bonds between G and C nucleotides in contrast to A and T ones). Specificity of primer bound also depends on the temperature (because of the number of hydrogen bounds).

A slightly modified version of the random PCR-amplification methods is the **DAFs** (DNA Amplification Fingerprint, Caetano-Anolles *et al.* 1991). This is also based on the random primed PCR methodology but uses 8mer primers instead of 10mers and uses acrylamide gel electrophoresis for fragment separation instead of agarose. For fragment visualisation the silver staining technique is used instead of EtBr. This is because the shorter primer sequence allows lower specificity in annealing and therefore much higher number of possible homologue sequences in the genome. As a result, much more fragments will be amplified by this method, which can not be separated on simple agarose gels only on acrylamide. The silver staining method is a more sensitive way of fragment visualisation as ethidium bromide, so at the end much higher number of amplified fragments can be seen on the gel compared to the RAPD technique. Using this method it is possible to detect more than 20 fragments in a gel. DAFs can be used on the same manner as RAPD, but not detailed here.

The most recent PCR-based fingerprinting technology is the so-called **AFLPs** (Amplification Fragment Length Polymorphism, Vos *et al.* 1995, Mueller and Wolfenbarger 1999). The name resembles the RFLPs which is not accidental. Namely this method combines the advantages of both RFLPs and PCR methods. The theoretical/technical basis of the methodology is the following. The first step is the template DNA isolation which is then followed by a restriction enzyme digestion like in RFLPs. But the fragment length differences will not be visualized by the use of DNA-DNA hybridisation but will be amplified by PCR primers. For this specifically designed primers are used which are complementary to the oligonucleotides ligated to the ends of the digested DNA fragments. It is easy to ligate the adapters to the fragments because the restriction enzyme we used for digestion will result so-called "sticky ends" which can be used for ligation of adapters. So the sequence of the protocol is: restriction digestion of template DNA, ligation of specific adapters to the ends of the fragments containing the complementary oligonucleotide sequences of the primers, then PCR amplification using primers complementary to the ligated adapters. We can imagine if all the restriction fragments will be amplified, the gel separation of them will result in a very complex, useless pattern. Therefore specific "selective nucleotides" are planned to the ends of the primers allowing the amplification only a smaller portion of the digested fragments. The fragments will then be separated on polyacrylamide gels and visualised either by silver staining or radioactive/fluorescent reactions. As a result we will get a serious number of amplified fragments with different lengths and can check them for genetic polymorphisms. The genetic background of polymorphisms detected by this method is the change of nucleotides at restriction enzyme sites (like RFLPs), or the insertion/deletion events resulting in altered fragment sizes between individuals. By this method the number of distinguishable fragments is over 50 in some cases.

Application (RAPD, AFLP). At this time, both RAPD and AFLP are applied for plants more intensively (see e.g. Ritland and Ritland 2000). AFLP is relatively new method, but at least in plants, it replaces RAPD almost everywhere. Although, highly variable microsatellites are available for plants, even in the chloroplast genome. Contrary, in animals, where we have a choice, single locus microsatellites are preferred. If no primers are available, RAPD is the first to try. The difference in preferred markers between animals and plants can be explained rather by tradition and earlier experience than efficiency. It is probably result of the great use of RAPDs in plant genome mapping, as controlled crosses with large number of progenies are much easier. Of course, genomic composition also differs between animals and plants, giving rise to a preference of a given type of marker (see discussion).

Nonetheless, the explanation of wide use of RAPD is clear, irrespectively its theoretical drawbacks: it is cheap, simple and can be used without any preliminary sequence knowledge after some optimisation for any species. Furthermore, level of polymorphism may be tuned using different primers. Nevertheless, the price of simplicity is paid at the evaluation. It is a multilocus marker with dominant inheritance (such as AFLP) even if theoretical results make possible to use models of population genetics in a restrictive manner (Lynch and Milligan 1994). Furthermore, high quality DNA is needed for reproducible result (but see notes below).

RAPD and AFLP can be used in a wide range of studies owing to the high range of variability it can show. Sometimes it used for studying relatedness (like the fingerprinting) or mating structure, but much common use is the study of population structure. Linkage mapping and quantitative trait locus (QTL) mapping must also be mentioned. RAPD is also used in taxonomy because plenty of characters can be obtained easily to compare species. See also the application section for further details.

Single locus techniques (Sequenced-tagged-sites, STS)

By definition, STS markers are those that reveal codominant polymorphisms in specifically targeted sequences, including scnDNA, introns, microsatellites and mtDNA sequence. The main difference between the previous methods and these ones is based on the genetic information obtained. Alleles of loci can be identified (codominance) providing a huge advantage in many situations. It means that the full power of theoretical tools piled up by population genetics in 80 years can be used together with the advantages of specific-priming PCR technique provided in sampling.

Specific, conserved primers have also a disadvantage: the target sequences must be obtained usually from genomic library, which needs a lot of work and high laboratory requirements. Furthermore, flanking regions used for priming are usually species specific. In same cases, where conservative sequences — kept unchanged in evolutionary time scale — can be found, universal primers can be constructed that works for a large number of taxa (see e.g. bird sexing, below). But generally, moving even to a related species needs new primers. Fortunately, owing to some recent studies and technical refinements, these disadvantages seem to decrease at least for microsatellites (see below).

scnDNA (single copy nuclear DNA), as suggested by its name, means that alleles of an unique nuclear locus is amplified and visualised. It is the PCR analogue of RFLP. It has two types. When target locus in the nuclear genome is actually unknown but polymorphic, we may have a useful marker termed as anonym scnDNA (ascnDNA). Anonymous scnDNA has the advantages of technical convenience and may yield variable region. Its obvious advantage is the random priming in the genome (see RAPD) but on a (multiple) single locus manner. The disadvantage of ascnDNA is the lack of generality, it can be used only in closely related taxa. However, when the flanking region of a variable target locus is evolutionarily conserved, we can design specific PCR primer pairs for that. This marker is termed as specific scnDNA. It may be used in many taxa, depending on how much the flanking region (i.e. the primers we designed) is conserved. Commonly, scnDNA is sequenced but all methods of detecting SNPs can be used. These are recent methods, their full advantages are not known (Palumbi and Baker 1996, Karl 1996).

Nuclear **introns** are predicted to be of greater importance in the future (Friesen 2000). Introns are widely used target regions of sequencing based molecular ecological studies. It can also be evaluated using electrophoretic methods of detecting SNPs (SSCP, TGGE), so it can be screened routinely. Introns are transcribed but not translated, so the mutations at these regions are presumed as neutral for selection. It means also that it has higher mutation rate than other scnDNA markers. Furthermore, it is more representative for the genome than mtDNA which of special inheritance. The functional regions (exons) framing them make possible to design PCR primers for introns. This step is based usually on date from sequence databases. Sequences submitted to databases may contain introns that can be recognized easily. This fact itself suggests future success of intron-based methods. Conservative exon regions also provide wider range of species where primers can be used as explained before.

Application. The ribosomal genes are widely used in molecular phylogenetics and taxonomy, like their intron regions or the internal transcribed spacer (ITS) between genes coding for the small and large subunit of the ribosome (e.g. Kovács *et al.* 2001). Another use is the gender identification (Griffith 2000). As an example, two sets of universal primers are available for sexing non-ratitae birds (Griffith *et al.* 1998, Fridolfsson and Ellegren 1999). It is based on the highly conservative, sex-linked gene of the chromo-domain-helicase DNA binding (CHD) protein. Intron length of the CHD gene copies in the avian W and Z chromosomes differ that can be visualised on agarose gel with EtBr staining. Females — the heterogametic sex in birds — produce two bands (W and Z) while males do one (Z). This method is used routinely today starting from a drop of blood, even for sexing offsprings or individuals of species without sexual dimorphism (but see Dawson et al (2001) for a critique). Nevertheless, recently introns are used mainly in phylogenetics and evolutionary biology. There is not enough data to evalute its advantages in the mainstream of ecology.

As explained before, **microsatellites** (SSRs, sometimes called STRs, short tandem repeats) belongs to the class of tandem repeat sequences, with usually less than five bases length of repeat units. Owing to the supposed mechanism of mutation already mentioned, alleles differ in size of the repeat unit: larger unit size means easier differentiation. For microsatellites, smaller differences must be recognised comparing to minisatellites. That is why acrylamide gel and silver staining are needed, but often fragments are simply sequenced owing to the automated methods. Actually, expected polymorphism depends on the characteristics of the loci (like length), it is not detailed here.

Microsatellite loci are assayed on the standard way: target region is amplified by specific primer-pair. Different primers are needed for different loci, which means that PCR condition may also differ. Resolution is increased by analysing many loci consecutively from the same individual. Using specific primers has the disadvantage of the limited applicability to a new species. However, for microsatellites this problem tends to decrease.

Nowadays, according to the high interest for microsatellites, screening for useful markers in the genome is less and less laborious. There are techniques available to find microsatellite-enriched sequences efficiently. AFLP can also be used without cloning and screening, as mentioned before. But the primary source of information is the literature and the sequence databases, such as NCBI-GENBANK (http://www.ncbi.nlm.nhi.gov) and EMBL (http://www.embl-heidelberg.de). Further useful sources are the species specific databases or microsatellites can be queried in many different places (Scribner and Pearce 2000).

Specificity is much better as it was supposed earlier, at least for birds. For example, microsatellite primers developed for a species of swallow detected polymorphic microsatellite markers for 32 of 39 other species within the same order and 6 of 19 bird species within different order (Primmer *et al.* 1996). We are also using primers developed for house sparrow (*Passer domesticus*) in tree sparrow studies (*Passer montanus*), some of them seems to be polymorphic (Pénzes *et al.*, unpublished data). It means that if no primers are available for a given species, we have a chance that primers developed for a related species may work.

The efficiency of SSRs resolution is also demonstrated in plant samples e.g. in soybeans in searching for genetic polymorphism and using them for genetic mapping studies (Csanádi *et al.* 2001). Finally, technical advances can be shown best by a human example. First, owing to the human genome project, screening for any sequence means actually database search (which is much faster and cheaper, of course). So target sequences can be selected much easily, keeping in mind of their independence (e.g. lack of linkage: different chromosomes) and different size range (can be scored on one gel). Commercial kits are available for microsatellites (used for paternity test in courts, they are very expensive, e.g. Genetic Analyzer, Applied Biosystems). Nine independent microsatellite loci (plus one locus for sex identification) can be analysed in the same time — 10 pairs of different primers are used in one PCR reaction. Furthermore, fragments are separated by acrylamide based capillary electrophoresis, where samples of individuals are put in different capillary. Alleles of a given locus are recognised by its size range and/or labelling. As an example, we used these microsatellite loci for a population structure study of humans (Beer *et al.* unpublished data).

There are numerous recent reviews of microsatellites according to its importance. See e.g. Scribner and Pearce (2000) for a general overview with many useful information and we suggest Ritland and Ritland (2000) for applying them in plants. Mitochondria and chloroplasts contain also microsatellites, not detailed here.

Application. Microsatellites are extremely useful markers for studies on whole scale of ecology interested in, from individual level to populations. First, it is assayed by PCR, using specific primer with the full set of advantages on sampling. Microsatellites may be highly variable, in a single locus as many as 50 alleles can be examined. More loci are used generally together composing a multilocus pattern where both alleles of every locus can be identified (codominant inheritance). These provide sufficient statistical power for individual identification and parentage determination or infer relatedness. By extending it to more generation, pedigree or population level patterns can be constructed. We can have insight into the mating structure or estimate effective population size. Numerous studies apply microsatellites to determine the magnitude of differences between populations or understanding population subdivision and gene flow. Taxonomy and evolutionary biology also uses microsatellites (speciation, hybridisation). Results of different population studies can also be combined using the models of molecular evolution, opening the way for metaanalyses. Finally, besides its practical importance, there are many opened questions about microsatellites not detailed here: it is "an active arena for theoretical and empirical work" (Scribner and Pearce 2000).

Mitochondrial DNA has importance in many fields (Randi 2000). As it was mentioned earlier, it can be considered as a haplotype and inherited mainly maternally. It evolves faster than nuclear genes (but not the hypervariable sequences), at least on average — owing to the less efficient DNA repair mechanism, resulting in higher level of variation. Furthermore, its special transmission provides unique tool for reconstructing genealogy in a wide timespan, from populations to phylogenetics. The conservative protein coding regions can be used to trace phylogenetical relationships back to million years. Besides this, the main noncoding sequence, the control region (called D-loop in vertebrates and AT-rich region in invertebrates) is much more variable (it

regulates the replication and transcription of the whole mitochondrial genome) targeted in many studies. mtDNA is assayed by RFLP or most frequently PCR amplification followed by gelelectrophoresis (in any form) or DNA sequencing. For vertebrates, there are universal primers for PCR-based sequencing the genome (Sorensen, 1999). Some examples of application are the studying population variability and gene flow, hybridisation or phylogenetics and conservation biology. We emphasise its use in intraspecific phylogeography or historical biogeography, which has incredible importance in recent understanding in evolutionary (or simply general) biology (Avise 1994, Burke 1998).

Advantages and disadvantages of different marker techniques: which marker to use

Numerous reviews have been born to compare the efficiency and usage of different marker technologies (e.g. Lu *et al.* 1996, Powell *et al.* 1996, Jones *et al.* 1997, Milbourne *et al.* 1997, Russell *et al.* 1997, Sunnucks 2000 and others). To compare these techniques we can take into account the aspects of classifications explained above. On this basis we can compare them on the basis of technology, genetic and efficiency aspects.

The first decision should be about the **sensitivity** of the marker needed. Lots of data accumulated for today to help us in this decision, but pilot studies may make the picture cleaner. Less sensitive marker does not give polymorphism on the level of our interest (like mtDNA or ITS for studying paternity). Or, using sensitive marker, like microsatellite, for a question of large scale, e.g. taxonomy, would give a random pattern. Some methods can be scaled to give the desired level of polymorphism (e.g. selecting RAPD primer, different mtDNA regions). Among the markers with suitable resolution, choices can be made on more practical bases.

On **genetic basis** we can take into account two main aspects: the **information content** and the **robustness** of the technology. The information content can be described by the dominant/recessive or codominant heredity of the alleles on the locus. All the marker types inherit codominantly if all the alleles of the individuals can be distinguished. In this case in a diploid individual if one allele gives longer fragment and the other a shorter one, the heterozygote will have double fragments. This is true for RFLPs and SSRs. The other methods distinguish only the "presence" or "absence" of a certain fragment (such as RAPDs, DAFs and AFLPs). In this case if the fragment is not present we can say it is a homozygous individual for the allele but if it is present, we cannot decide if it is homozygous for the presence of both fragments or heterozygous because only one allele is present. This basic difference can cause information loss in the case of dominant markers compared to the codominants, because the genotypization is not so exact as by them. This suggests using rather codominant than dominant marker types.

On the other hand RAPDs, DAFs and AFLPs produce much higher number of visible fragments at the same time, so they are the most robust ones even if they are dominantly inherited. The higher amount of fragments can compensate for the loss of information content by the dominant inheritance.

With other words the information content of a given marker type can be numerically characterised by the expected heterogeneity (H_{av}, number of polymorphisms detected) and by the effective multiplex ratio (E, the number of effective bands) (Powell *et al.*, 1996). The distinctive capacity (characterised by marker indices, MI) of the marker system is the product of E and H_{av}. This means that the efficiency of a given marker system can be enhanced by increasing the value of E and/or H_{av}. The higher the Marker Index is the better the marker technique for a given species. According to the recent data in the literature the AFLPs proved to be the most and RAPDs the less efficient technology regarding both heterogeneity and multiplex ratio (Powell *et al.* 1996, Lu *et al.* 1996, Bohn *et al.* 1999).

The **reproducibility and reliability** of the different techniques is also widely investigated. In a very detailed analysis, the reproducibility of the different marker technologies was tested in several different European labs (Jones *et al.* 1997). It was found, that RAPDs are poorly reproducible in contrast to other marker techniques and besides AFLPs, SSRs proved to be the best in this comparison. This is very important in the evaluation and interpretation of the results obtained. Taking into account that AFLPs are the most robust technique between all genotyping methods one can conclude to use this methodology for checking genetic variance. Why most of the people choose RAPDs most preferably? To answer this question we have to check a different aspect of the molecular genetic methods. This is the cost- and labor-efficiency of a marker methodology.

The **efficiency** of the marker methodologies is depending on several different parameters. The best way to measure it when we count the polymorphic bands/ invested time and energy for each case. At the first glance RAPDs seems to be the less laborious,

moderately robust and not very expensive method. Comparing the cost of RAPDs and RFLPs with their efficiency, Ragot and Hoisington (1993) found that RAPDs are most cost-efficient when using small sample sizes in contrast to RFLPs. But basically it cannot be concluded which one is generally better in this aspect. When checking the efficiency we have to take in account several aspects of the different methods:

1. Price of the equipment
2. Price of the chemicals used
3. The number of working hours
4. The amount of polymorphic bands expected

1. Price of equipment:

RFLPs: hybridisation oven, tubes and sometimes documentation tools

RAPDs: usually require a PCR-machine, an electrophoresis system

and some documentation tools

SSRs: PCR machine, electrophoresis system and documentation tools

AFLPs: PCR machine, sequencing acrylamide gel system and documentation tools

From these the most expensive investment is related to the AFLPs. This is one of the reason why it is not so widely used as RAPDs

2. Chemicals:

RFLPs: hybridisation membranes, polymerase for probe labelling, radioactive or non-radioactive chemicals for detection

RAPDs: primers, nucleotides, polymerase, agarose and ethidium-bromide

SSRs: primers, nucleotides, polymerase, acrylamide and silver staining chemicals or radioactive / non-radioactive chemicals for detection

AFLPs: chemicals in kits: restriction enzymes, primers, adapters, polymerase,

nucleotides, radioactive or non-radioactive chemicals for detection

It can be clearly seen from this list, that most expensive investment is related to AFLPs again. The less expensive is the RAPDs.

3. Number of working hours

In this aspect to standardise one method can take for different times because sometimes it is very hard job to optimise even the simplest RAPD reaction. In general the more steps the method has the more time is necessary for optimisation and working. For a routine analysis one RAPD or SSR reaction can be performed in 4-5 hours, while RFLPs take for several days similarly to AFLPs. This would suggest working rather with the first two methods.

4. Number of polymorphic loci expected in one experiment

RFLPs: up to 10 loci

RAPDs: up to 10 loci

SSRs: up to 5 loci

AFLPs: up to 50 loci

In this case it would be optimal to work with AFLPs.

We can draw some conclusions from the above aspects. In general if we do not want to analyse a huge amount of samples or we do not have enough money for molecular analysis we can start with RAPDs. It is the less laborious and money-consuming method when using low number of samples, but it is the less reliable and reproducible, too. If we decide to analyse a huge amount of individuals which are relatively close to each other but we do not have much money for investment it is reliable to start with SSRs, or if they are not developed for the species — with RFLP fingerprints using random probes. We can also try to use DAFs after a certain optimisation procedure. If we have enough money for investment and want to generate a huge amount of polymorphic samples we should invest into AFLPs technology because of its highest productivity, reliability and reproducibility.

Besides these aspects there are also some other points to be considered. First ones are the source of DNA and its quality. For RFLPs, the most critical point is the isolation and purification of proper amount of source DNA. The isolation can be automatised by using modern extracting methods but it will increase our costs. Because of the restriction digestion step, the low amounts of source DNA must have high quality when using the AFLP technique: incomplete digestion can generate false positive results for genetic analysis. In addition, all PCR-based methods have the risk to be contaminated with foreign DNA, which is completely excluded in RFLPs analysis. In summary, high quality, non-degraded DNA is generally needed for multilocus methods. Again, this is less important for using short, single (multiple) locus markers assayable by PCR.

Second one is the automation possibility of a certain technique. The automation of RFLPs is possible and solved but only at industrial level: it is so expensive. The use of radioactive and non-radioactive stains for SSRs and AFLPs makes their detection easier but a bit more expensive. This pays out in longer times if we can detect significantly higher number of polymorphisms as by other methods.

It is worth to summarise the trade-off between multilocus (with dominance) and single locus (with codominance) methods. Most of the time, single

locus markers assayed by PCR are preferred. Besides the advantages in sampling, it gives genealogy and comparable results (called "connectivity", Sunnucks 2000). Thus, data from many different studies are comparable directly in meta-analysis. For studies including many species the universality of primers may be also important. In this, single locus methods vary considerably. Multilocus methods are usually more convenient but limited connectivity. Sometimes multilocus methods considered being more economical but it can be questioned. Inevitably, recent advances based on genotypic arrays are provided by single locus methods of codominant markers (Sunnucks 2000).

Rate of evolution in plant and animal DNA is often very different claiming for different markers for the same kind of question. Chloroplast in plants is uniparentally inherited like mitochondria and its DNA (cpDNA) evolves slow rate. It is useful for deeper studies in phylogenetics, like ribosomal RNA genes in nuclear DNA. New advances of chloroplast microsatellites may provide useful tools for population level studies in plants (Provan *et al.* 2001). Differences between nuclear and organelle DNA must also be kept in mind. For example, it is a bad practice to use mtDNA because of the availability of universal primers. mtDNA is inherited mainly maternally and this must be considered in conclusion. mtDNA has also a lower effective population size resulting in more sensitivity for keeping past events.

In summary we can suggest when selecting an ideal method for the analysis the first choice must be made on the sensitivity but we have to take into account the efficiency, reliability and productivity of a certain method and this must be related to the aim of our genetic work.

Applications

The most important general use of different markers is mentioned in the previous section. Now, a different classification is given: the topic of interest. The aim of this section is contrasting markers in some highlighted topics of ecology without rigorous overview. Molecular markers in ecology are used both on individual and population level. One of the main interests is the estimation of demographic parameters that would be difficult to obtain otherwise: reproductive success of individuals, dispersion patterns in space and time or population growth and fluctuation of effective population size. Furthermore, these may include the knowledge of gender, genetic relatedness of individuals or individuals must be assigned to a given group (population).

Five main areas can be outlined: (1) individual identification, (2) identification of sex, (3) testing parentage including pedigree or mating structure reconstruction, (4) population structure (dispersion) and (5) population history. These categories overlap both in theory and practice. Individual identification and testing parentage needs highly variable markers, discussed as DNA fingerprinting. Pedigree construction means subdividing populations into families, at least in time, giving rise to population structure. It also provides data for determining mating structure. Population history reconstruction is mentioned briefly here, although it is one of the most important fields as it provides completely new information for us.

Individual identification and testing parentage: DNA fingerprinting and profiling

This field needs the most sensitive markers. DNA fingerprinting was developed to detect individual-specific patterns for humans (Jeffreys *et al.* 1985). In its original form, it was a multilocus method, minisatellites were detected by Southern blot hybridisation. Using a 33 bp repeated sequence from a human intron, Jeffreys and co-workers isolated two probes, named 33.6 and 33.15, from a human genomic library. These are the most widely used probes today, not only in humans. They have extreme discriminatory power even in birds (Burke and Bruford 1987). Fragments are generated by restriction enzymes with 4 bp recognition site resulting in a set of bands with extreme allelic length variation. Important feature of the bands that they are generally inherited in Mendelian manner (half of the bands are inherited from each parent). In this way, close familiar relationships can be analysed, especially parentage: decision is based on the band sharing, calculated between offspring and supposed parents.

Multilocus fingerprinting detects many loci simultaneously using universal probes, usually by Southern blot hybridisation at low stringency (i.e. less specificity). However, allelism between bands is not known without complex segregation analysis, although sometimes codominant inheritance is suggested. It is a multilocus technique, without the knowledge of the number of loci. High quality and high molecular weight DNA is essential for successful analysis in standard concentration (it should be assayed). Even different gels can be compared carefully, but for this internal size markers are needed. It means that all potential parents must be analysed simultaneously. In summary, universal

probes have advantage, but technical requirements are high.

Single locus fingerprinting is the detection of alleles at a given minisatellite locus using a single locus probe, usually by Southern blot hybridisation at high stringency (high specificity). Offspring can be examined for the presence of non-parental alleles, as evidence for multiple paternity or maternity. Offspring exclusion can be done even if all supposed parents are not known. Alleles can be organised into a database and identification of individuals can be done routinely (e.g. Wetton *et al.* 1995). Analysis of different seasons and years can be performed more readily using single locus method but not with multilocus one. Its main drawback is the lack of general probes. Probes are often synthetic oligonucleotides as it has been realised that simple tandem repeats (microsatellites) detect fingerprint-like patterns. Good commercial kits are available for hybridisation for non-radioactive labelling (e.g. Boehringer's digoxygenin based kit). DNA profiling term is coined for single locus fingerprinting because single locus pattern is not individual specific: it has less discriminatory power. This can be solved when different loci are hybridised consecutively. In both type of fingerprinting, fragments are separated on agarose gels. Usually maxi gel (20x25 cm) is needed. In summary, as a single locus technique, it has many advantages, however, probes are not available generally.

Minisatellites are rarely used in PCR. Even if used, usually not as many locus are available as in microsatellite and primers seems to be more specific resulting in more restricted applicability for different taxa (Sunnucks 2000). Use of microsatellites for testing parentage is equivalent to minisatellites in theory: offspring must carry parental alleles in each locus. Non-parental alleles suggest e.g. extra pair paternity or maternity. For microsatellites, acrylamide gel separation is necessary.

To mention one of the numerous examples of their usage (e.g. Carter 2000b, Scribner and Pearce 2000), studies of extra pair paternity of birds is analysed in this way (e.g. Griffith *et al.* 2002). Using single locus method for analysing pedigree provides not only information about the reproductive success of individuals but the mating structure in the population or effective population size. The classical example is given by Craighead and co-workers (1995) studying 30 family groups of grizzly bears.

Population structure

Population structure, that is difference between populations or subdivision can be studied using many different markers: proteins, RFLPs, RAPDs, mtDNA sequences or microsatellites (minisatellites are rarely). It is related to relatedness studies, at least in methodology. But instead of single individuals (as in relatedness) our main interest is on the group of individuals, populations. Traditionally, Wright's F-statistics are calculated from allele frequencies (or its modern analogues for different markers) and/or variance components are estimated using a tuned analysis of variance (AMOVA, Analysis of Molecular Variance, Excoffier *et al.* 1992). Again, the advantages of single locus methods are clear as they provide allele frequencies. RAPD and AFLP may also be used although it has less power (see Lynch and Milligan (1994) for theoretical background). Methods used to analyse data are very similar to statistics familiar to ecologists (analysis of variance, spatial statistical models and multivariate methods).

Population structure study starts with partitioning variability into different levels, like within and between population components using e.g. phenotypic similarity indices of ecology (e.g. Nei and Li, 1979). This can be used in cluster analysis or PCA. The following approach is more reliable. Bands may provide allele data directly (single locus) or scored for presence (multilocus pattern) that can be analysed further using AMOVA. Correlation between genetic (e.g. Nei's) and geographical distances can also be tested using Mantel test. This is a common list of steps in studying genetic structure in fragmented landscape or gene flow. Of course, different markers may be useful for different scales, as explained before.

Population history, recent advances

Most of the codominant markers (mtDNA sequence, mtDNA RFLP, microsatellites and scnDNA) are extremely useful as they can provide information about gene genealogies. This property adds the time dimension to allele frequency distributions (Sunnucks 2000). Nowadays, clear tests can be carried out about history and spatial patterns. Some examples are: relative timing of past events, like range expansion and differences in gene flow, bottleneck events can be inferred. Genealogies may provide information from population processes to phylogeographic events and have of great importance today. Theoretical advances must also be mentioned, like coalescence approach (Kingman 1982, see e.g. Nordborg 2001, Posada and Crandall 2001, Stephens 2001) and nested clade analysis (Templeton 1998) related to history. Although comparative phylogeography has incredible contribution to ecology and

conservation, we can not detail this field here (see e.g. Burke *et al.* 1998).

Discussion

Molecular tools are inevitable incorporated into the methodology of ecology. To access molecular markers is more complex than the evaluation of the morphological ones. Using DNA techniques need special equipment. Field methods might be changed. However, we believe, this investment will be recovered soon. Students of biology at most of the universities learn these DNA techniques and may have some practice using them. Therefore, the taste of uniqueness will disappear soon. Some of the DNA work can be done even in ecological laboratories (e.g. DNA isolation using kits and PCR with specific primers as in molecular sexing of birds). The most laborious steps (e.g. sequencing) are provided by many companies for reasonable price. Collaboration between ecological and molecular laboratories is also a potential solution, this is how this paper has been born: both sides has same interests. See also the notes of Snow and Parker (1998). It must also be mentioned that various local, national or international laws may regulate the collection and use of biological samples.

New approaches, including markers and statistical methods are also useful in studying nonequilibrium situations. These are in the heart of conservation biology or useful in studies of invasions, population foundations (Davies *et al.* 1999, Waser and Strobeck 1998). The importance of molecular methods in conservation biology increases continuously (e.g. Smith and Wayne 1996, Karp *et al.* 1998), not only on the level of the researches but also on the level of arrangements and acts. It is not accidental that for example a non-profit high-tech was recently established within (till 2003) the Max Planck Institute (Laboratory for Conservation Genetics, LCG, Leipzig, www.raredna.com) to serve the technical and experimental possibilities for applied conservation.

The subject of the unit of the conservation provides an example for the meeting of the results of the molecular methods and conservation biology. The term of the adaptive evolutionary conservation was born from the operativisation of the definitions of the evolutionarily significant unit (ESU) (Fraser and Bernatchez 2001). This term has important legal part in e.g. the USA Endangered Species Act (ESA) (Waples 1991, 1995), or in the Australian Endangered Species Protection Act (Moritz 1994).

The connection and continuity between population genetics and systematics is self-evident today shown by many textbooks of evolution. The same molecular markers with sufficient sensitivity can be used on both fields, as mentioned in methodologies. The parallelism is clear: individuals of different species can be identified — e.g. like the different haplotypes; the phylogenetical relations can be determined — e.g. like the structure of the populations; lineage can be inferred – e.g. like the population histories.

The speed of the development of the powerful statistical methods can be applied to DNA data is comparable to that of the markers. Clearly, technical and analytical methods have facilitated each other. We could not detail the field of evaluation here, but some notes must be given. See Luikart and England (1999) for an overview and software, including relatedness/parentage and dispersal. Assignment (and related) test already mentioned (Paetkeau *et al.* 1998, Davies *et al.* 1999, Luikart and England 1999) as powerful tool studying migration. Exact tests, computer based algorithms are used frequently claiming for update our views of statistics (Rousset and Raymond 1997). On the basis of underlying theory of coalescence, maximum likelihood-based estimators are constructed to estimate population parameters using Markov chain Monte Carlo sampling. Microarray data confronted presently have raised also important issues for statistical testing (Nadon and Shoemaker 2002). The list is endless and grows further including statistical issues of database search and Bayesian approach.

Software for carrying out analyses is essential. We have a choice from plenty of possibilities. Citations and web sites are given usually in the reviews of the different methods. We mention GENEPOP, ARLEQUIN and GDA as they are used frequently. Usually, special questions need special software, and they can be often downloaded from the author's web site. We mention R (Ihaka and Getleman 1996, http://cran.r-project.org) a general software package with efficient data handling, excellent statistical and graphical capabilities, as it is free and develops very fast. Many packages are available for R, including tools for manipulate DNA data (J. Lindsey's DNA package). There are many free software available also for Linux operating system (which is very stable, fast and can be obtained also freely), including NCBI tools, Phylip, TreeView, Arlequin, R and many more (see biology software in http://www.debian.org).

The application of DNA markers should be planned carefully. Common mistake is to use inappropriate markers for a given problem (see e.g. Sunnucks 2000, Baker 2000a). Decision is often made on the availability of universal primers for

amplification irrespectively of the scale of question. This can lead to false conclusion. Sequence databases (like GenBank) are good sources of primers for the studied (or closely related) species even for species specific primers.

Finally, some cautions should be mentioned. Financial needs and technical details often override the importance of conclusions. These are methods to study problems in many fields of biology. It may be expensive relative to the classical field methods but provides high quality information. The money-information quality trade-off can be tracked in the DNA methods, too (e.g. RAPD compared to microsatellites). But the same sampling considerations must be followed (random, sufficiently large samples, etc), it is imposed by the methods of evaluation. Nevertheless, differences between populations detected by microsatellites can be resulted by plenty of processes (e.g. drift, selection, migration). Common mistake to assign differences to one of them, to migration for example (see Bossart and Prowell 1998). These are indirect methods. To decide between the alternative hypotheses, more specialised tests or direct methods are needed. Migration can be detected by field studies. Methods are good but must be used carefully.

Future prospect of applying DNA markers seems to be clear. Probably, new classes of genetic markers will be developed owing to the large scale genomic studies. The fast speed of development both in technology and related statistical methods can be illustrated by microsatellites (Jarne and Lagoda 1996, Luikart and England 1999) or the phylogeography (Burke *et al.* 1998). Interspersed nuclear elements (SINE) may provide a recent example: they are efficient markers for phylogenetic studies, as they can be handled as derived characters (e.g. Takahasi *et al.* 1998). Development of markers and screening also supposed to be simplified. Probably the best example is provided by the recent advances of microchip based technology (which is very expensive today, it is used in medicine and agriculture) for screening SNPs.

Abbreviations

AFLP: Amplification Fragment Length Polymorphism
CHD: chromo-domain-helicase DNA binding protein (gene)
cpDNA: chloroplast DNA
CTAB: cationic hexadecyltrimethyl ammonium bromide
DAF: DNA Amplification Fingerprint
DGGE: Denaturing Gradient Gel Electrophoresis
DMSO: dimethylsulphoxide
EDTA: ethylenediamine-tetraacetate
EtBr: Etidium bromide
ITS: Internal Transcribed Spacer
LINE: Long INterspersed Repeat
mtDNA: mitochondrial DNA
MHC: Major Histocompatibility Complex
PCR: Polymerase Chain Reaction
PFGE: Pulsed-Field Gel Electrophoresis
QTL: Quantitative Trait Locus
RAPD: Random(ly) Amplified Polymorphic DNA
RFLP: Restriction Fragment Length Polymorphism
scnDNA: Single Copy Nuclear DNA, it can be specific or anonymous (ascnDNA)
SDS: Sodium dodecyl sulphate
SINE: Short INterspersed Repeat
SNP: single-nucleotide polymorphisms
SSCP: Single Strand Conformation Polymorphism
SSR: Simple Sequence Repeat (microsatellite)
STR: Short Tandem Repeat, synonym of SSR (microsatellite)
STS: Sequenced-tagged-sites (including SSR, scnDNA)
TGGE: Temperature Gradient Gel Electrophoresis
VNTR: Variable Number of Tandem Repeat (minisatellite)

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TISCIA 33, 31-35

ANTS (HYMENOPTERA: FORMICIDAE) AS PRIMARY PESTS IN HUNGARY: RECENT OBSERVATIONS

G. Vörös and L. Gallé

Vörös, G., Gallé, L. (2002): Ants (Hymenoptera: Formicidae) as primary pests in Hungary: Recent observations. — *Tiscia 33, 31-35.*

Abstract. During the period between 1994 and 2001 considerable ant damages were observed in several fields, horticultural and medicinal crops in county Tolna, southern Hungary. Ants generally attacked young plants, foraging and thinning their root collars, therefore the plants fell off and then dried. They foraged skin of fruit crops and grapes and consumed fruit flesh, too. In public parks and nurseries of Budapest ants provoked lobed leaves on ornamental shrubs and trees. Majority of ants collected from damaged plants were *Tetramorium caespitum* and *Lasius* spp. In apples *Camponotus* spp. also occurred, while from grapes a Mediterranean species, *Prenolepis nitens* was identified.

Key words: ants (Formicidae), damage, crops, Hungary

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Introduction

Damages caused by ants in Hungary have been recorded already from the 19th century. Anonymous investigators (Anonym 1891, 1892, 1894, 1895) recorded *Formica rufa* Linnaeus 1758. on conifer seedlings with foraged roots, *Tetramorium caespitum* (Linnaeus 1758) feeding on greener parts of dwarf apple trees, ants of unknown species in peaches and early season grapes and attributed damages on Scotch pine and black pine to *Lasius flavus* (Fabricius 1793) and *T. caespitum*. Győrffy (1939) and Csapó (1940) reported damages on plantlets and maize, respectively without mentioning the species, but Nagy (1952) observed *T. caespitum* foraging in seeded poplars.

From the middle of the 1990s, Vörös (1995), Vályi and Vörös (1997), Vörös and Takács (1996), Vörös and Gallé (1996, 1998, 2001) and Tartally (2000) have reported on the primary damages caused by ants in various crops.

In this paper we give a brief outline of ant damages recently recorded in Hungary.

Material and methods

The waste majority of the surveys were made in county Tolna in southern Hungary (Table 1), where the predominant soil type is loess.

Losses were recorded during a thorough inspection of the fields, determining target foraging areas of ants. We sought for damages caused by ants moving or eventually feeding on the plants in every case and made sure of their primacy.

During the surveys we collected as many damaging ant individuals from the affected plants and soil as we could. Little spades, jars of different sizes (1 and 5 litres) and an aspirator were used for collection, the ants were placed in the jars together with the soil and plants.

In the laboratory the ants were sorted from the samples using a brush and placed into Eppendorf-tubes containing 75 % alcohol, with an indication of date, time, name of the host plant and other relevant data. Ants were identified under stereo microscope at the laboratory of the Department of Ecology, University of Szeged. We unified the species list based on the works of Somfai (1959) and Gallé *et al.* (1998).

Table 1. Sites and dates of the ant damage observations

Site	County	Date	Demigod plant	Note
Fadd	Tolna	May 1994	sunflower	
Felsőnána	Tolna	May 1996	maize	
Felsőnána	Tolna	May 1996	sunflower	6-19 leave stage
Szedres	Tolna	May 1996	melon	greenhouse
Szedres	Tolna	May 1996	watermelon	greenhouse
Szekszárd	Tolna	May 1996	cabbage	
Szekszárd	Tolna	May 1996	grapes	'Kékfrankos' type
Tolna	Tolna	May 1996	melon	
Fácánkert	Tolna	May 1996	melon	
Dalmand	Tolna	Oct. 1996	winter rape	
Dalmand	Tolna	May 1997	mustard	82 ha
Mözs	Tolna	May 1997	cabbage	
Fácánkert	Tolna	June 1997	watermelon	
Szedres	Tolna	May 1998	watermelon	
Szekszárd	Tolna	May 1998	strawberry	
Miszla	Tolna	May 1998	maize	100 ha
Szekszárd	Tolna	May 1998	carrot	
Kölesd	Tolna	May 1999	mustard	20 ha
Sióagárd	Tolna	May 1999	watermelon	small garden
Sióagárd	Tolna	Sept. 1999	grapes	40 ha
Siófok	Somogy	May 1999	sunflower	100 ha
Görgeteg-Lábod	Somogy	May 1999	sunflower	95 ha
Gödöllő	Pest	May 1999	melon	
Budapest	Pest	May 1999	peach plum	
Budapest	Pest	May 1999	plum	
Budapest	Pest	1999	ornamental trees & shrubs	
Tevel	Tolna	May 2000	poppy	
Tolnanémeti	Tolna	June 2000	cabbage	2 ha
Gyönk	Tolna	June 2000	maize	16 ha
Szekszárd	Tolna	June 2000	sour & sweet cherry	
Mórágy	Tolna	Sept. 2000	apple	
Szekszárd	Tolna	Oct. 2000	walnuts	
Kajdacs	Tolna	May 2001	thyme	0.15 ha
Pusztaegres	Tolna	May 2001	maize	6 ha
Jászberény	Jász-Nagykun-Szolnok	June 2001	melon	
Cserszeg	Zala	Aug. 2001	grapes & apple	

Results

The observed ant species at crops

The following ant species were found at the damaged plants (in alphabetic order): *Camponotus fallax* (Nylander, 1850); *Camponotus vagus* (Scopoli, 1763); *Formica rufibarbis* Fabricius, 1793; *Lasius alienus* (Foerster, 1850); *Lasius emarginatus* (Olivier, 1791); *Lasius neglectus* Van Loon, Boomsmsa & Andrásfalvy, 1990; *Lasius niger* (Linnaeus, 1758); *Lasius paralienus* Seifert, 1992; *Lasius plathytorax* Seifert, 1992; *Lasius psammophilus* Seifert, 1992; *Prenolepis nitens* (Mayr, 1852); *Solenopsis fugax* (Latreille, 1798) and *Tetramorium caespitum* (Linnaeus, 1758). Besides ants, the ant imitating beetle, *Formicomus pedestris* Rossi also occurred together with ants and presumably caused similar damage.

Case descriptions of ant damages on various crops

In spring 1994, instead of the "expected" wireworm damages, ant foraging was found in sunflowers. The ants concentrically overran the lower petioles of plants at 2-6 leaf stage and foraged sapful parts. In certain, most endangered sites 30 % of the

plants died. Lower leaves of the attacked but survived sunflower plants, rolling inwards dried down at their petioles. In 1995 at Felsőnána ants attacked sunflower plants of higher developmental stages, and hollowed stems below the inflorescence initiations, provoking drying of upper plant parts, which turned brown. All the ants observed on sunflower belonged to *T. caespitum* (Table 2).

In potted melons, covered with plastic flat foil we saw plants hollowed around the hypocotyl and root collar, holed and passed by foraging trails. 20-40 ants were found per plant, completely killing plantlets. In 1996, 1998 and 2001 we found similar symptoms on melons. In winter rapes we observed that the plants wilted, fell off and finally dried out due to thinning and foraging of root collars of plants at 2-4 leaf stage. In melon, besides *T. caespitum*, a beetle species, namely *Formicocomus pedestris* also caused damage (Table 2). In 1997, in transplanted watermelons ants invaded the container pots and foraged root collars. A lot of ant nests were found in the soil, the area was overrun by workers of *T. caespitum*.

In 1995, ants attacked the cabbage plantlets. Plants fell down due to the thinned root collars then dried. Losses were so significant that cabbages had to be replanted. In 1997, falling down and withering seeded cabbage of 2-leaf stage and a plenty of *T. caespitum* ant-hills were observed.

In September of 1995 ants hurt berry skin of 'Kékfrankos' grapes and consumed fruit sap. About 5-10 ants were feeding in a berry but the damage was insignificant. In 1999, intensive ant movement was observed on grapevine plants, 8-10 ants were found on each bunch at a time. Berry skin and seeds were uninjured as ants consumed only fruit flesh. In contrary to the former cases, we observed several ant species on grapes (Table 2).

In 1997 a white mustard stand showed conspicuously deficient crop stand, as the ants foraged hypocotyls of seedlings. Larger and smaller heaps indicated the entrance of high density ant nests. In 1999 ants consumed 2-4-leaf plants of white mustard in spots. At white mustard besides *T. caespitum, Lasius paralienus* and the beetle, *F. pedestris* also occurred.

In 1998, in strawberries characteristic heaps on the soil and swarming workers appeared in the centre of the plants, provoking thinning and drying of the plants. In carrots we observed tiny foraging on the upper part of the roots.

In maize, ants invaded root collars and fed on them leaving only the fibres. Damages occurred sporadically in spots. Killing of plants reached 30-40 % in certain foci, leading to an average deficiency of 5-20 % in the crop stand in 1998. In 2000, at the 2-4 leaf stage, the lower leaves began drying and caused a slightly deficient crop stand (less than 10 %). Similar damages were observed on maize in 2001, too.

In Budapest and its vicinity ants spoiled market value of peaches and plums by feeding on ripen fruits in 1999. In 2000, ants were recorded foraging ripen sweet and sour cherries on the trees. They made some very tiny or a single, bigger round hole in the fruit flesh and consumed the inner parts. More and more fruits of sound epidermis were attacked probably because of the draught and heat. Among others, large carpenter ants (*Camponotus vagus*), foraging the epidermis, hollowed fruit flesh of apples. *T. caespitum* in walnut kernels fed on sound kernel parts and covered them with gnawn fruit (without excrement!).

During the warm and arid periods in summer of 1999, similarly to leaf-cutting ants in tropic areas, they caused leaf lobes in public parks and nurseries on Japanese quince, rock cotoneaster**,** creeping cotoneaster and silver lime, and ant workers moved to their nests with the cut leaf pieces.

The only damage by *Solenopsis fugax* was observed in 2000. We found this tiny, yellowish ant in poppies, foraging holes and trails in roots and devastating the plants.

In 2001 ants foraged stem base of thyme plants; the crop died in spots, especially where a building overshadowed the surface.

Discussion

A wide range of ant-plant interactions has been published (cf. Huxley and Cutler 1991), but the documented ant damages are mainly restricted to harvester and leaf-cutting ants. In some cases, however, the role of ants as pest insects is overemphasised and although we cannot give the same answer to Robinson's (1999) question: ("What's the top pest? Ants are the answer") in Hungary, the above described cases indicate that the damages caused by the ants cannot be neglected. Whereas Cherix and Bijleveld (1994) regarded the introduced species as the main pests in Europe, in this case the majority of the damage-causing species are native in Hungary. Out of the 13 ant species observed at crops, *T. caespitum* proved to be the most dangerous pest. Some *Lasius* species were also significant. Other species occurred only sporadically and their damage, if any, was local (e.g. *Prenolepis nitens, Formica rufibarbis*) or insignificant (e.g. *Campontous fallax*), therefore they cannot be regarded as widespread agricultural pests.

Table 2. Damages by ant species in different crops in 1994-2000. *ant-imitating beetle Heteromera, Anthicidae

Plants	Ant species	Year of damage
sunflowers	*Tetramorium caespitum*	1994, 1995, 1996, 1998, 1999
maize	*T. caespitum*	1995, 1997, 1998, 1999, 2000, 2001
winter rapes	*T. caespitum* *Formica rufibarbis*	1996, 1998 1996
white mustard	*T. caespitum* *Lasius paralienus* *Formicomus pedestris**	1997 1999 1999
poppies	*Solenopsis fugax.*	2000
watermelons	*T. caespitum* *Lasius niger* *L. paralienus*	1995, 1996, 1997, 1998, 1999, 2001 1995 1998
melons	*T. caespitum* *F. pedestris**	1995, 1996, 1997 1995, 1997
cabbage	*T. caespitum*	1996, 1997, 2000
grapes	*Prenolepis nitens* *L. paralienus* *T. caespitum* *Lasius emarginatus*	1995, 1997 1999 2001 2001
apples	*Camponotus vagus* *Camponotus fallax* *T. caespitum*	2000 2001 2001
peaches	*L. psammophilus*	1998
sweet cherries	*Lasius alienus*	2000
sour-cherries	*T. caespitum* *L. alienus*	2000 2000
plums	*L. alienus,* *L. niger*	1998 1998
walnuts	*T. caespitum*	2000
Japanese quince	*L. plathytorax* *L. niger*	1999 1999
rock cotoneaster,	*L. plathytorax* *L. niger*	1999 1999
creeping cotoneaster	*L. plathytorax* *L. niger*	1999 1999
silver lime	*Lasius neglectus* *Lasius niger*	1999 1999
thyme	*T. caespitum*	2001

In the myrmecological literature there are very few recent data on similar ant damages as described here from temperate regions (cf. Cherix and Williams 1994, Williams 1994). Besides the classical Hungarian papers cited in the introduction, Hutson (1933) published the root eating ants as pests of garden plants. Myburgh *et al.* (1973) described the ants as pests of deciduous fruit, grapes and miscellaneous other horticultural crops in South Africa. Whereas this work dealt with such species as *Crematogaster* and *Iridomyrmex* spp., several papers discuss the damage caused by the carpenter ants and their control (e.g. Hansen and Akre 1994, Gooch 1999, Suiter and Bennett 1999, Drlik and Quarles 2000). There is an extensive literature on the control of other ant species or ants in general (e.g. Suiter *et al.* 1997, Tucker 1998, Katz 1999a, 1999b, Varjas *et al.* 1999).

Reimer *et al.* (1990) emphasise the significance of urban ant pests in the Hawaiian islands. In the present paper, the urbanised species were found in Budapest, damaging ornamental plants. The role of Lasius neglectus, a recently describe ant species from Budapest is crucial in this case (cf. Boomsma *et al.* 1990, Tartally 2000).

We observed heavy ant damages on maize fields in Hungary. Dejean *et al.* (2000) found interrelationships between the distribution of ants and the maize damages.

In our opinion, groundwater level, lowering due to the dry and mild winters and springs, as well as the hot summers of the past few years (especially in 1995

and 1996) have led to the drastic decrease of moisture necessary for ants, therefore they took up the necessary water from crop plants.

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TISCIA 33, 37-44

MACROZOOBENTHOS OF THREE BROOKS IN THE SOUTHERN PART OF THE PANNONIAN PLAIN: COMPARATIVE ANALYSIS OF SECONDARY PRODUCTION

I. Zivic, Z. Markovic and M. Brajkovic

Zivic, I., Markovic, Z. and Brajkovic, M. (2002): Macrozoobenthos of three brooks in the southern part of the Pannonian Plain: comparative analysis of secondary production. — *Tiscia 33, 37-44.*

Abstract. Production of macrozoobenthos was investigated at 18 localities on the Kudoski, Jelenacki, and Borkovacki brooks during April, July, and October of 2000 and in January of 2001. 16 groups of macroinvertebrates were recorded in the course of the investigation. The dominant groups in the biomass of macrozoobenthos were Hirudinea (Annelida), Mollusca, Gammaridae (Crustacea), and Trichoptera (Insecta). The greatest biomass of the bottom fauna in all months of investigation was recorded in the Kudoski brook, where it ranged from 41.3248 g/m^2 in July to 133.2384 g/m^2 in October, the next greatest one in the Jelenacki brook, and the least in the Borkovacki brook (from 23.7432 g/m^2 in July to 90.3328 g/m^2 in January).

Key words: macrozoobenthos; secondary production; Kudoski, Jelenacki, and Borkovacki brooks.

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Introduction

On the territory of the Pannonian Plain, macrozoobenthos has been investigated for the most part in the large flatland rivers (the Danube, Tisza, Sava, Begej, and Tamis) and in irrigation canals (Pujin *et al.* 1974, Djukic and Stanojevic 1979, Miljanovic and Djukic 1989, Elexova 1998, Mihaljevic *et al.* 1998, Sporka and Nagy 1998, Graf and Kovacs 2002, Nosek 2002, Oertel 2002, Schmidt-Kloiber *et al.* 2002), while considerably less attention has been paid to investigation of the bottom fauna of small streams. This is understandable, since the major part of the hydrological network of the Pannonian Plain is made up of large rivers and canals, and small streams participate more signific-antly in it only around the rim of the Plain.

Of particularly great significance in the hydrological network are small streams in the Srem region, which occupies the southern rim of the Pannonian Plain. This follows from the fact that the indicated region is completely cut off from the rest of the Plain by the Fruska Gora mountain in the north and separated by the Danube in the east. A large number of streams arise on the Fruska Gora mountain. Of these, ones that flow down the southern slope and empty into the Sava are fairly long and build a branching hydrological network. These aquatic ecosystems are exposed to human influence of varying intensity along their entire course. Except in source regions, the streams flow through agricultural fields and are used as irrigation canals, but they are also recipients of drainage waters from farm land and communal and industrial waste waters. In view of the fact that these are small streams with zoocenoses characterized by dominance of macro-zoobenthos — whose organisms on account of their way of life are good indicators of water quality (Hynes 1959) — we felt that it would be interesting to study the influence of anthropogenic pollution on abundance and biomass of their bottom fauna in comparison with mountain streams having similar hydrological characteristics.

The watershed of the Kudoski brook was selected for our investigations, the indicated watershed being composed of the Kudoski, Jelenacki, and Borkovacki brooks. These brooks flow parallel to each other from north to south and are close together (their distances are about 2-3 km). They are characterized by similar physical character-

istics (water temperature, substrate type, slope, etc.), so that their differences in regard to biomass and abundance of the bottom fauna are primarily a consequence of specific human influence.

In the present work, the biomass of the macrozoobenthos was selected as the main index for monitoring changes in quantitative composition of the bottom fauna, inasmuch as it is one of the basic parameters for quantification of the level of secondary production (Mason *et al.* 1985). This makes it very important for understanding the functioning of freshwater ecosystems, since zoobenthic organisms are an essential link in the food webs of aquatic biocenoses (Cummins 1973). In spite of this, investigations of biomass of the macrozoobenthos have been rare in aquatic habitats in the southern part of the Pannonian Plain (Mitrovic 1969, Djukic 1980, Djukic *et al.* 1991, Markovic and Mitrovic-Tutundzic 1998, 1999, 2000).

Material and methods

Secondary production of the bottom fauna in the Kudoski, Jelenacki, and Borkovacki brooks was investigated at 18 localities (Fig. 1) during April, July, and October of 2000 and in January of 2001. Sampling was conducted with a quantitative net according to Surber on sectors measuring 300 cm^2 in area. Raw mass of macroinvertebrates was measured with a precision of 0.0001 g. Biomass of organisms was expressed in g/m^2.

The Kudoski, Jelenacki, and Borkovacki brooks arise on the Fruska Gora mountain at 480, 460, and 160 m a.s.l., respectively. Fruska Gora is the greatest mountain in Vojvodina, its highest point (Crveni Cot) lying at an elevation of 534 m a.s.l. The mountain extends in an east—west direction along the southern rim of the Pannonian Plain. The 32 km long Kudoski brook is a left-hand tributary of the Sava river. Its most important left-hand tributary is the Jelenacki brook, of which the Borkovacki brook is a right-hand tributary.

Sampling of macrozoobenthos in the Kudoski brook was carried out at seven localities from rocky and pebbly substrates. Twenty-one quantitative samples were taken in the course of the investigation.

Macrozoobenthos was collected at six localities in the Jelenacki brook (which is 20 km long). Material was collected from muddy and rocky substrates. Altogether, twenty-three quantitative samples were taken in the course of the investigation.

Samples of the bottom fauna in the Borkovacki brook (which is 10 km long) were collected at five localities from rocky and muddy substrates. Eighteen quantitative samples were taken.

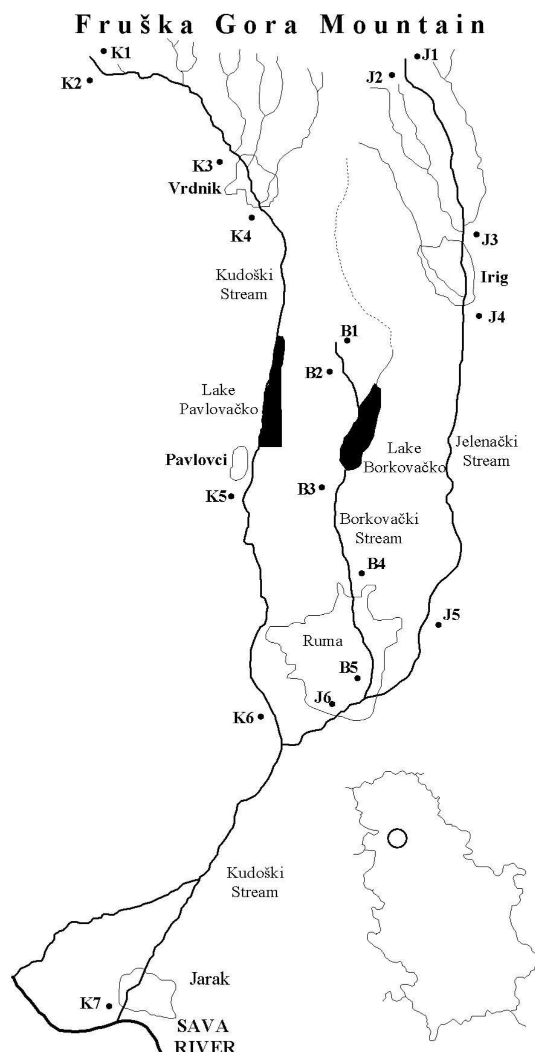

Fig. 1. Investigated localities on three brooks in the southern part of the Pannonian Plain.. Location of the three brooks in Serbia is indicated by the circle in the upper right-hand corner of the figure.

Results

Fourteen animal taxa (two phyla, three classes, nine orders, and three families) were recorded in quantitative sampling of the macrozoobenthos in the Kudoski brook. Biomass in the Kudoski brook varied annually in the interval from 41.3248 g/m^2 (in July) to 133.2384 g/m^2 (in October). At the investigated localities in this brook, no macrozoobenthos (0 g/m^2) was found at locality K7 in June and January, while the greatest biomass (518.1593 g/m^2) was recorded at locality K3 in October (Fig. 2). The greatest biomass was recorded at locality K3 in the other months of investigation as well, due to increased abundance of

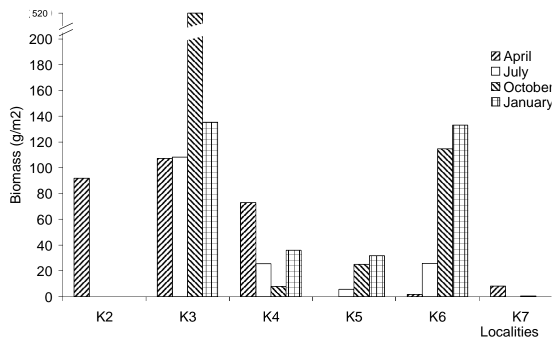

Fig. 2. Secondary production of macrozoobenthos in the Kudoski brook.

Hirudinea and Mollusca there (Figs. 3a and 3b). Not only at the third locality, but at the others also (except locality K2, where Gammaridae are dominant), the abundance of Mollusca (Fig. 3a), Hirudinea (localities K4 and K5, Fig. 3b), and Chironomidae (localities K5, K6, and K7, Fig. 3d) almost completely determined the measured biomass of macrozoobenthos. In regard to seasonal dynamics of biomass in the Kudoski brook, its high values during the winter period stand out clearly. To be specific, the greatest biomass values were recorded in January at localities K5 and K6, while the second greatest values occurred in January at localities K3 and K4.

Organisms of 14 animal taxa (two phyla, two classes, eight orders, and three families) were found in zoobenthos of the Jelenacki brook. Average biomass of macroinvertebrates varied annually in the interval from 23.6254 g/m^2 (in January) to 49.8653 g/m^2 (in April). The lowest zoobenthos biomass (4.4458 g/m^2) was recorded at locality J1 in July, the highest (113.0552 g/m^2) at locality J4, also in July (Fig. 4). In contrast to the picture observed in the Kudoski and (especially) the Borkovacki brooks, it is impossible to isolate groups whose abundance determined the biomass at the majority of localities: instead, these groups varied from locality to locality (Fig. 5). At locality J3, in addition to the indicated groups (Gammaridae, Chironomidae, Oligochaeta, and Diptera, Figs. 5a, 5b, 5c, and 5d), Trichoptera

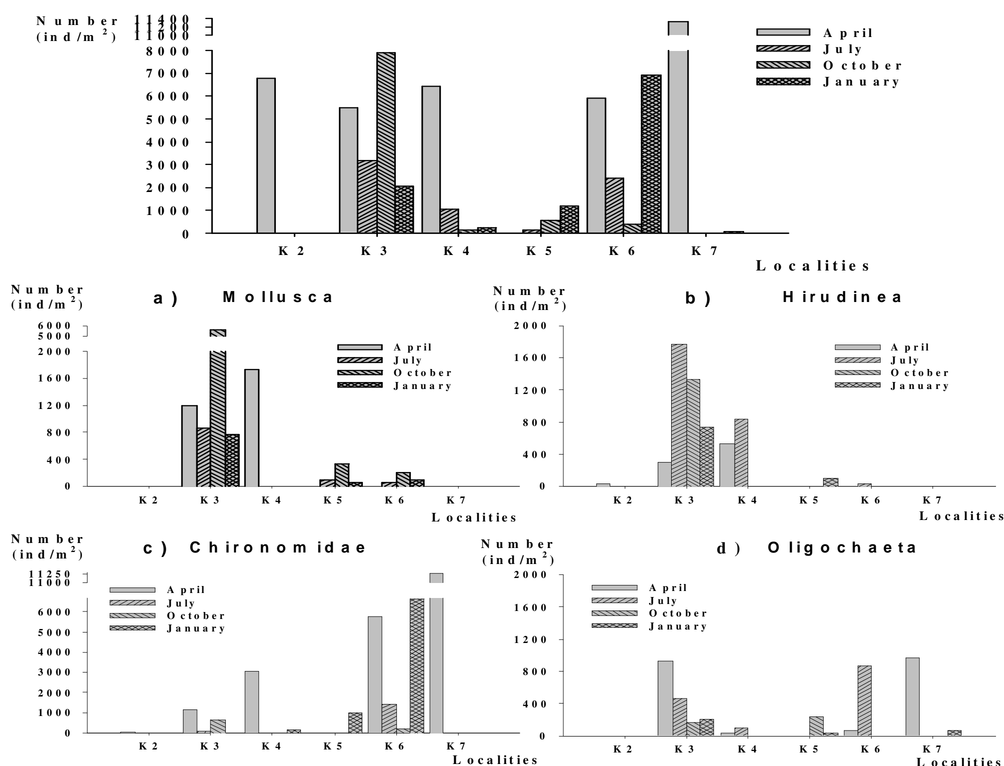

Fig. 3. Abundance of macrozoobenthos at the investigated localities in the Kudoski brook.

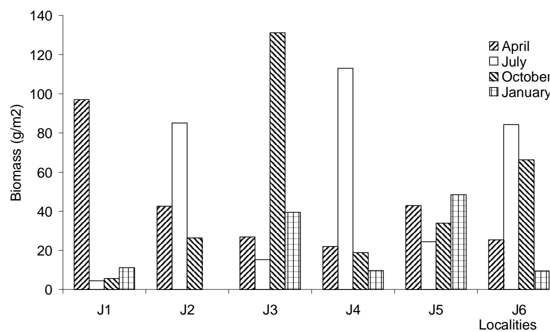

Fig. 4. Secondary production of macrozoobenthos in the Jelenacki brook.

(433 ind/m^2 in April) and Mollusca (133 ind/m^2 in October) were also numerous; at locality J4, Mollusca were abundant in April, June, and October (333, 1033, and 166 ind/m^2, respectively); and at locality J5, Hirudinea were numerous in all months of investigation, with average participation of 71.7% in abundance. Seasonal dynamics of biomass also exhibited few regularities. As in the Borkovacki brook, the greatest biomass in the source region (localities J1 and J2) occurred in spring. Localities J3 and J4 were characterized by fairly equal biomass values by seasons, with the maximum in January (Fig. 4). The other localities (especially J4 and J6) were characterized by significant seasonal oscillations of biomass (Fig. 4).

Organisms belonging to 12 animal groups (one phylum, two classes, eight orders, and three families) were found in the bottom fauna of the Borkovacki brook. The average biomass of macroinvertebrates varied annually in the interval from 23.7432 g/m^2 (in July) to 90.3328 g/m^2 (in January) (Fig. 6). The minimal biomass of zoobenthos (6.3760 g/m^2) was recorded at locality B5, while its maximal value (181.7432 g/m^2) occurred at locality B4 in January. At all localities except B5 — where Chironomidae and Oligochaeta were dominant (Figs 7b and 7d) — biomass was completely determined by the abundance of Gammaridae (Fig. 7a). Deviations from this were observed only at locality B1 in the fall and locality B2 in the spring, where Mollusca participated significantly in the biomass, with 367

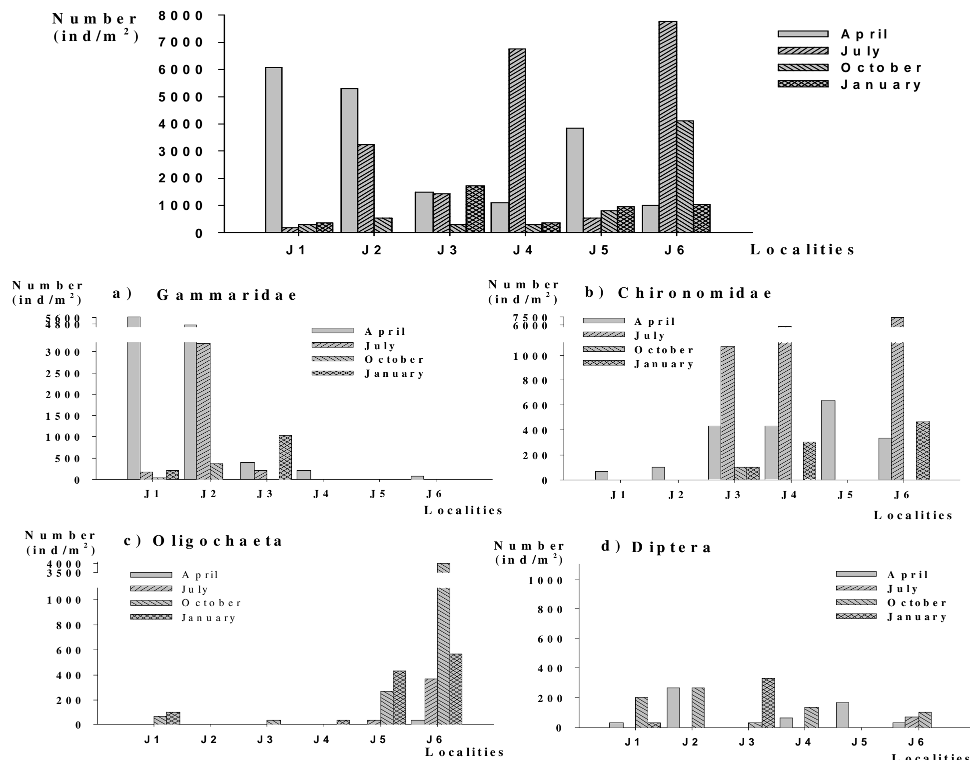

Fig. 5. Abundance of macrozoobenthos at the investigated localities in the Jelenacki brook.

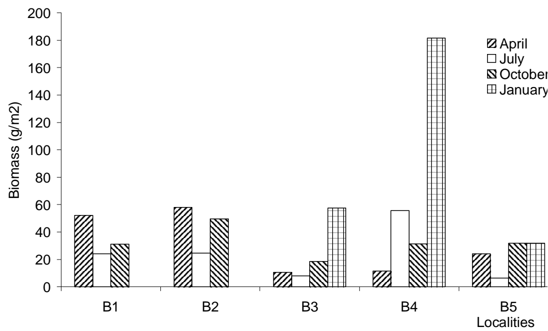

Fig. 6. Secondary production of macrozoobenthos in the Borkovacki brook

and 1666 ind/m^2, respectively (Fig. 7c). Seasonal dynamics of biomass exhibited significant regularities. In the source region (at localities B1 and B2), biomass values were the greatest in the spring, declined in the summer, and rose again in the fall (Fig. 6). At localities B3 and B4, pronounced biomass peaks were achieved in January (Fig. 6). Locality B5 was characterized by slight seasonal changes of biomass apart from its very low values in July (Fig. 6).

Discussion

Freshwater ecosystems of the temperate zone are characterized by relatively constant biomass of macrozoobenthic organisms, in which aquatic insects are dominant, together with molluscs, annelids, and crustaceans (Cummins 1973). However, it is still possible to discern certain seasonal variations in their biomass (Hynes 1970) resulting from different adaptations of the life cycle of aquatic insects (egg laying—eclosure times) to environmental factors, primarily water temperature. A large number of aquatic insects survive high summer temperatures (often accompanied by the drying up of smaller streams) in the form of eggs, pupae, or very small non-growing nymphs, the greater part of their growth and development occurring in late fall, winter, and spring (Pleskot 1961). Aquatic insects of this group are called cold water species. Apart from them, there also exists a warm water group of insects, which survive the winter period in the egg and pupa stage

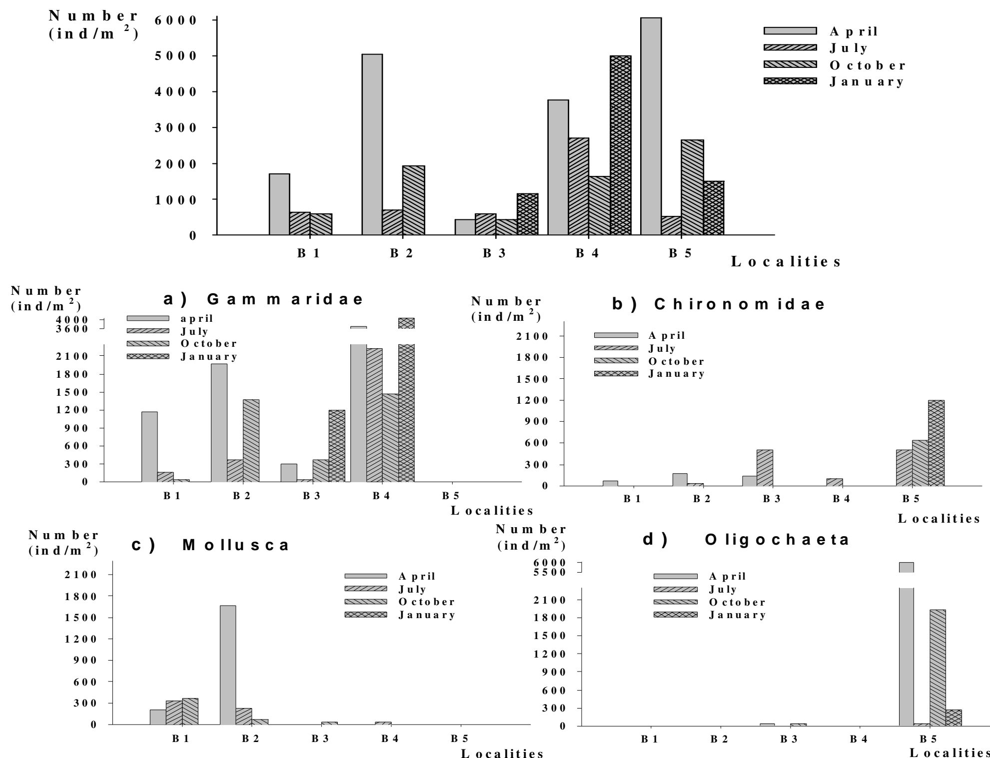

Fig.7. Abundance of macrozoobenthos at the investigated localities in the Borkovacki brook.

and attain their maximal growth and development during the summer. This group is less numerous and mostly made up of Simulidae (Anderson and Dicke 1960, Carlsson 1967), but also some Ephemeroptera (Pleskot 1961) and Trichoptera (Ulfstrand 1968). Greater representation of cold water species and the fact that some cold water species are larger than closely related warm water ones (Steffan 1963, Khoo 1964, 1968) often cause maximal biomass of the macrozoobenthos to occur in the spring, when the period of growth of cold water species is completed (Egglishaw and Mackay 1967). These considerations apply to relatively unpolluted mountain streams, in which larvae of aquatic insects constitute the dominant group of macrozoobenthos. However, it must be asked whether the same can be said about small streams of the highland type exposed to strong human influence along their entire course, such as the brooks investigated in the present study.

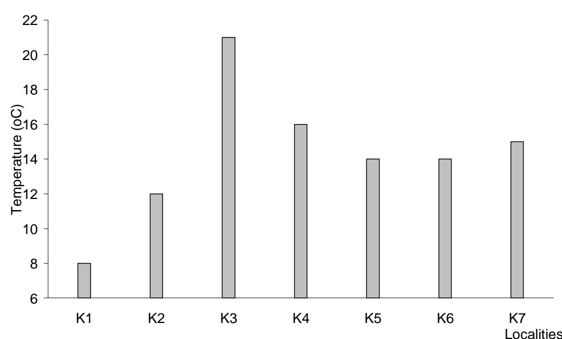

Fig. 8. Mean annual water temperatures in the Kudoski brook.

The greatest biomass of zoobenthos in all periods of investigation was recorded in the Kudoski brook at locality K3. This locality is characterized by high water temperature (Fig. 8) due to inflow of a thermal stream from the spa of Vrdnik several kilometers upstream and a high degree of organic pollution, with a biotic index value according to Plafkin *et al.* (1989) of 7.09, indicating a class of water with very significant organic pollution (unpublished data of the authors). Since Hirudinea and the determined species of the phylum Mollusca are eurythermal (Pennak 1953, Elliot and Mann 1979) and tolerant to organic pollution (Hilsenhoff 1988), and whereas it is known that temperature is positively correlated with biomass in these groups (Cummins 1973), it can be assumed that precisely the combination of these two factors brought about the high biomass at locality K3.

Occurrence of the least bottom fauna biomass (in all months) at locality K7 is a consequence of exceptionally high pollution, which is clearly indicated by the complete absence of macrozoobenthos in the summer and fall periods and finding of only two specimens of Oligochaeta in winter. Such a high degree of pollution stems from influence of the town of Ruma, whose sewage and industrial waste water (from leather, shoe, and plastic pipe factories, as well as a fruit and vegetable processing plant) flow into the Kudoski brook directly or with water of the Jelenacki and Borkovacki brooks. It is also a consequence of constant agricultural pollution, since the Kudoski brook flows through agrarian landscapes all the way to its mouth.

The lack of an animal group with dominant influence on biomass in the Jelenacki brook can be explained by the absence of dominant influence of ecological factors such as increased water temperature in the Kudoski brook and shallow depth of the stream bed in the Borkovacki brook (see parts of the discussion pertaining to these brooks) on macrozoobenthos development.

Whereas the Kudoski brook is heavily polluted throughout its entire course (with biotic index values of from 6.92 at locality K5 to 8 at locality K7) and the Borkovacki brook (if we exclude locality B5) is characterized by moderate organic pollution (with biotic index values of from 5.98 at locality B2 to 6.16 at locality B3), biotic index values in the case of the Jelenacki brook varied within a wide range (from 5.68 at locality J3 to 9.24 at locality J5) (unpublished data of the authors).

Variation of the quality of water in the Jelenacki brook results in a significant variation in composition of the bottom community from locality to locality. Thus, at locality J3 (where the water is cleanest), the more sensitive groups Trichoptera and Ephemeroptera were significantly abundant, in addition to Gammaridae and Chironomidae. On the other hand, at locality J5 (where the water is most heavily burdened with organic pollution), the tolerant group Hirudinea was dominant, together with significant participation of Oligochaeta and Chironomidae.

Such changes in the qualitative composition of zoobenthos from one locality to another result in the lack of significant regularities in biomass dynamics, since different groups of macrozoobenthos are characterized by different dynamics of growth and development.

In the Borkovacki brook, a pronounced peak of biomass was achieved in January at localities B3 and B4 (Fig. 6). This can be attributed to reduced human influence due to the absence of agrotechnological measures during the winter period, since the brook at these localities flows through agrarian ecosystems, as in the case of the Jelenacki brook (localities J3 and

J5). The great abundance of specimens of Gammaridae (which determines high biomass values at these localities) is probably a consequence of shallow water depth (0.07-0.10 m) and good aeration, factors that favor the development of species of this family (Wundsch 1922).

The slight oscillations of biomass at locality B5 probably result from consistently poor water quality, as indicated by a biotic index value of 8.04 (unpublished data of the authors), which means that the water is of a class characterized by serious organic pollution. An exception was observed during the summer period, when very low zoobenthos biomass was recorded at this locality (Fig. 6). Here it is pertinent to note that sewage water from the town of Ruma flows into the Borkovacki brook upstream from locality B5, while high summer water temperatures led to enhanced decomposition of organic matter and increased oxygen consumption, as was clearly indicated by the unpleasant odor and great muddiness of the water. Such deterioration of ecological conditions probably led to significant decrease in abundance of the tolerant groups Chironomidae and Oligochaeta, while other animal groups were not recorded.

Comparison of biomass in these three brooks reveals certain similarities. Biomass in source regions has a spring peak determined by occurrence of the greatest Gammaridae abundance in April. At localities where the brooks flow through agrarian ecosystems and intensive farming is the main source of pollution, biomass values peak in the winter, when agrotechnological activity is the lowest.

Nevertheless, in spite of their relative closeness together, parallel courses through similar terrestrial ecosystems, and similar types and levels of human influence (intensive agriculture and receipt of communal water from the settlements of Irig, Vrdnik, and Ruma), significant differences exist between the three brooks studied. These differences are primarily evident in the group of organisms which with their abundance and/or individual mass determine the total biomass. Gammaridae are that group in the Borkovacki brook; Mollusca (with significant participation of Hirudinea and Chironomidae) in greatest measure represent that group in the Kudoski brook; and several categories of organisms (varying from locality to locality and by seasons) constitute it in the Jelenacki brook.

Variation of macarozoobenthos biomass in the three investigated brooks differs in two main ways from the dynamics of seasonal biomass variation in mountain streams (Zivic *et al.* 2000).

The investigated brooks were characterized by considerable variation of biomass, which can be attributed to different levels of human influence, the factor that in greatest measure determines the qualitative and quantitative state of the macrozoobenthos in them. Thus, the share of insect groups (apart from Chironomidae) in total abundance was only 6.9%, whereas the tolerant groups Chironomidae (34.5%), Oligochaeta (12.3%), Hirudinea (7.7%), and Mollusca (10.9%) were dominant. The significance of variation of human influence on biomass is seen most distinctly in the case of crop production, whose intensity varies clearly from season to season and is lowest in winter, when biomass achieves maximum values at localities where this influence is prevalent (localities K4, K5, J3, J5, B3, and B4).

The second significant difference is that seasonal biomass dynamics (except in source regions) did not exhibit the regularities observed in mountain streams. The presence of these regularities of seasonal biomass variation in source regions of the investigated brooks is attributable to the fact that human influence is the weakest there, as is indicated by both the biotic index and the index of saprobicity. The reason why the regularities in question are absent on the rest of the stream course should be sought in the fact that they are based on the premise that insect groups are dominant or participate significantly in the quantitative composition of macrozoobenthos, which due to significant human influence is not the case in the investigated brooks.

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TISCIA 33, 45-49

GROWTH OF THE GOLDEN SPINED LOACH, *SABANEJEWIA AURATA* (FILIPPI, 1865) IN RIVER TISZA (EASTERN HUNGARY)

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Harka, Á., Györe, K and, Lengyel, P. (2002): Growth of the golden spined loach [*Sabanejewia aurata* (Filippi, 1865)] in River Tisza (Eastern Hungary). — *Tiscia 33, 45-49.*

Abstract.The paper presents data on the growth of golden spined loach, obtained on the basis of the study of 91 fish specimens. The study material was collected from an isolated lock chamber, at the same time and without any selection. Hence, its size and age distributions seem to represent those of the population well.

There were 78 first-year, 12 second-year and 1 third-year fish among the collected specimens. Their standard lengths ranged from 25 to 71 mm, their body weights, from 0.12 to 4.41. According to our results, the average standard length of the fish at age t (L_t in mm) can be expressed with the equation $L_t = 92[1\text{-}e^{-0.505(t+0.01)}]$.

There is no significant difference between the growths of males and females. Both body length and body weight increase intensely in the second year. Hence, the biomass of the second-year age group is well above that of the first-year fish, in spite of the high mortality.

The reach of the Tisza studied by us is dammed, and thus, environmental conditions are not optimal for golden spined loach. In addition, the population suffered damages also from the cyanide spill that polluted the river in February 2000. Though, the survival of the population is not in danger, as the species reaches maturity by the second-year age, and thus, there is an adequate proportion of mature specimens.

Keywords: Sabanejewia bulgarica, Bertalanffy's model, age structure, mortality, production

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Introduction

The species *Sabanejewia aurata* was first described from the territory of Hungary by Jászfalusi (1948) who classified the specimens found in the Tisza at Kőtelek into the subspecies *S. a. bulgarica*. Many authors accept the existence of the subspecies (Jászfalusi 1948, 1951, Bănărescu 1964, Bănărescu *et al.* 1977, Balon 1967, Terofal 1997) but consensus has not yet been developed in this respect. Numerous authors use only the species name *Sabanejewia aurata*, omitting the subspecific epithet (Müller 1983, Povž and Sket 1990, Györe 1995, Harka 1997, Spindler 1997), while Kottelat (1997) considers valid the species name *Sabanejewia bulgarica*.

Golden spined loach is legally protected in Hungary from 1974. At that time, its occurrence was proven only in the rivers Tisza (Jászfalusi 1948, Csizmazia *et al.* 1965) and Danube (Tóth 1971), but since, it has been detected from numerous rivers of Hungary (Harka 1986, 1997,; Sallai 1999a, 1999b). Due to the secretive habits and relative rarity of the species its biology is little known, no data on its growth rate have been available to us up to the present.

Material and methods

The study material consisted of 91 fish specimens collected between September 13 and 24, 2000, from the lock chamber of an irrigation canal branching off from the Tisza at Tiszafüred. Nets with mesh size of 3 mm were used for sampling in order to include even the smallest specimens.

Standard (L_c) and total length (L_t) measurements were done to the nearest millimetre, measurements of

weight (W) to the nearest 0.01 g. The length-weight relationship was calculated by the formula $W = a \cdot L^b$, proposed by Tesch (1968). Age was estimated using the Petersen method, on the basis of the length frequency distribution, but older specimens were aged reading the annuli of the opercular bone. Sex of the adults was determined on the basis of the lateral distension of the body present in the males. The results were corroborated by dissecting the animals and examining their gonads.

The Walford (1946) method and the Bertalanffy (1957) model, suggested by Dickie (1968), were applied for mathematical description of the growth. Condition factors (CF) were calculated following Hile (1936), biomass (B) and production (P) according to Chapman (1968). The Microsoft Excel '97 programme was used for statistical evaluation of the data.

Result

Standard lengths of the fish ranged from 25 to 71 mm. Total lengths varied between 29 and 82 mm, body weights between 0.12 and 4.41 g. The equation describing the length-weight relationship in the golden spined loach population was $W = 3 \cdot 10^{-6} Lc^{3.2753}$ in case of standard length, $W = 10^{-6} Lt^{3.3779}$ for total length (Fig. 1).

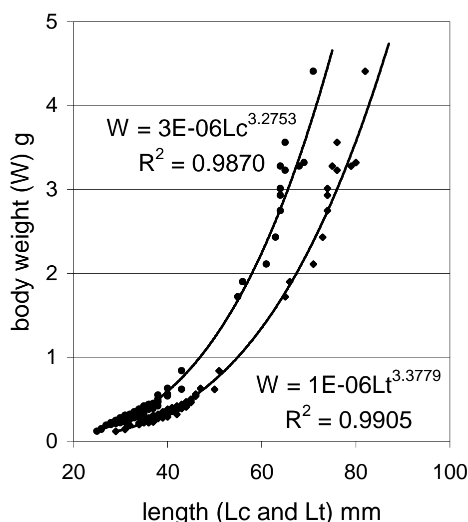

Fig. 1. The length-weight relationships

Considering that total length often figures in the results of growth studies, the relationship between the two lengths was determined in order to facilitate the conversion. The equation describing this relation is $Lt = 1.1403Lc + 1.3946$.

Length groups were formed from the standard length data of the collected specimens using 5-mm intervals. Presenting their frequency in a diagram, first-year (25 to 44 mm) and older age groups are clearly separated (Fig. 2).

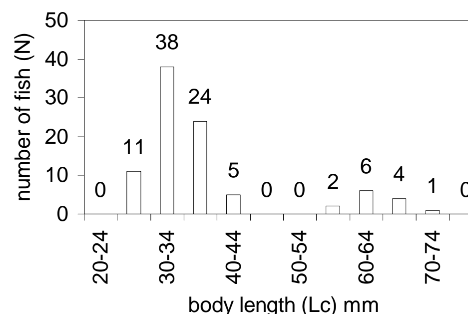

Fig. 2. Length-frequency of golden spined loach

Based on the study of the operculum, 12 of the 13 older specimens proved to be second-year (1+), and 1 to be third-year (2+). Five males were found among the second-year fish, with a mean standard length of 62.6 mm, and body weight of 2.82 g. In case of the seven females, the respective data were 63.6 mm and 2.78 g. The only third-year fish proved to be a male. The exponentially decreasing trend in the numbers of individuals in the age groups can be expressed by the equation $N = 762.99 \cdot e^{-2.1784}$ (Fig. 3).

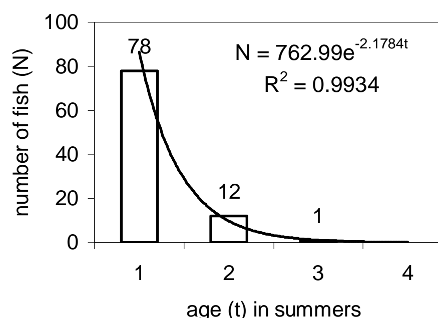

Fig. 3. Age distribution of the collected specimens

The following average values resulted from the actual measurements of standard and total lengths and body weights of the study material:

First year (0+) 33 and 40 mm, 0.34 g,

Second year (1+) 63 and 74 mm, 2.79 g,

Third year (2+) 71 and 82 mm, 4.41 g, respectively.

The Walford plot could be constructed using the average standard length data of the individual age groups, by plotting $y = Lc_{(t+1)}$ against $x = Lc_{(t)}$. The equation of the line, fitted to the data by linear regression analysis, is $Lc_{(t+1)} = 0.5958Lc_{(t)} + 37.092$, on the basis of which, the asymptotic length (L_{inf}), indicating the maximum possible size, is 91.75 ≈ 92 mm (Fig. 4).

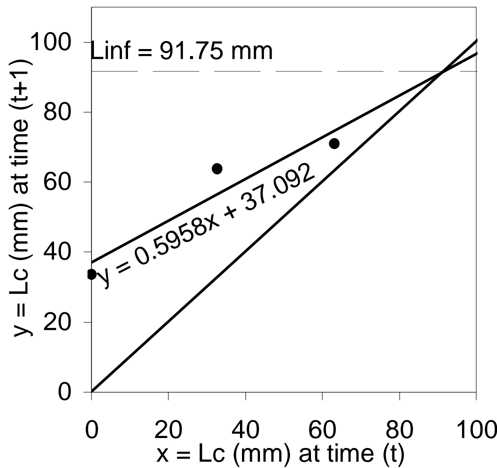

Fig. 4. Growth of golden spined loach, according to the Walford model

Standard and total lengths of the individual age groups, calculated according to the WALFORD growth model, were the following:

First year (0+) 37 mm and 44 mm,

Second year (1+) 59 mm and 69 mm,

Third year (2+) 72 mm and 83 mm, respectively.

Plotting against time the natural logarithms of the differences between the asymptotic length (L_{inf}) and standard lengths reached at different ages (L_t), with all lengths expressed in millimetres, a linear plot resulted. The equation of this was $\ln(L_{inf}-L_t) = -0.505t + 4.5133$. From this, further parameters of the Bertalanffy equation could be determined: $t_0 = -0.01$ and $K = 0.505$.

The equation of the function describing the growth of the golden spined loach population, on the basis of which the average standard length (L_t) of the t year age group can be calculated, is as follows: $L_t = L_{inf}[1-e^{-K(t-t_0)}]$, or, substituting the calculated parameters: $L_t = 92[1-e^{-0.505(t+0.01)}]$.

Standard and total lengths for the individual age groups, calculated according to the Bertalanffy equation, were the following:

First year (0+) 37 mm and 44 mm,

Second year (1+) 59 mm and 69 mm,

Third year (2+) 72 mm and 83 mm, respectively.

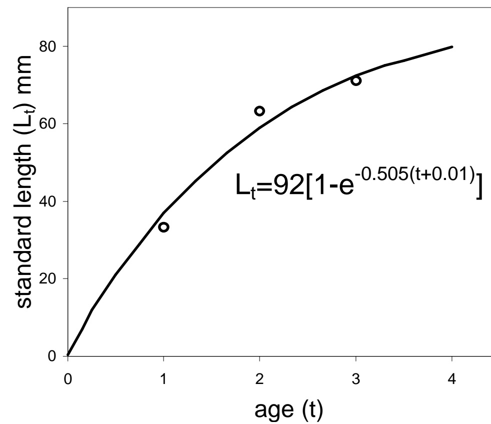

Fig. 5. Growth of golden spined loach according to the Bertalanffy model

The first two age groups were represented in the study material with a sufficient number of individuals to allow the estimation of the instantaneous mortality coefficient (Z), the survival rate (S) and the annual mortality (A). The calculated values were Z = 1.8718; S = 0.1539 and A = 0.8461.

In the studied material, biomass of the first-year fish (B_1) was 26.29 g, that of the second-year ones (B_2), 33,52 g. The instantaneous growth rate (G) of weight was 2.1148. Considering that biomass grew in the period in question, the value of G-Z, i. e. 0.2430, was used to calculate the mean biomass ($\bar{B}$) of the sample. Based on this, $\bar{B} = 29.75$ g.

Production (P) equals the multiplication product of the mean biomass and the instantaneous growth rate of weight: $P = \bar{B}\,G = 62.92$ g.

And finally, annual production (AP), expressed in percentile terms, was calculated by multiplying the $P/\bar{B}$ ratio by 100: $AP = P/\bar{B} \cdot 100 = 211.5$ %.

Discussion

Though the study material, consisting of hardly 100 specimens, cannot be regarded a big sample, it seems to represent the population adequately, considering that it was caught from one place, at the same time, and practically without any selection.

The value of the constant b of the equations describing the relation of length and weight – the so-called allometric exponent – was greater than 3, both in cases of standard and total lengths. This means that the growth rate of body weight in golden spined loach exceeds that of their length. As a consequence, condition of the fish improves with their age, as it can be seen from the increasing values of the

condition factors, calculated from standard lengths according to Hile (1936). (Table 1).

Table 1. Length, weight and condition changes in golden spined loach

Age	Standard length Lc mm	Total length Lt mm	Body weight W g	Condition 10^5 CF
0+	33	40	0.34	0.9110
1+	63	74	2.79	1.1079
2+	71	82	4.41	1.2321

According to Bănărescu (1964), males of golden spined loach are hardly smaller than females. Our experiences are in accord with this. In our sample, the length of the second-year males was only 1 mm shorter than that of the females. At the same time, males are stouter because of the lateral distension of their bodies, and thus, their body weight exceeds that of the females in spite of their shorter size. It is possible, however, that this situation can periodically change. It cannot be excluded that in spring, when eggs are fully ripened, females can take the lead in this respect, too, although at the time of spawning the distension situated behind the gill openings and that in front of the dorsal fins of males also increase in size.

Standard lengths calculated for the first- to third-year golden spined loach on the basis of the Walford and Bertalanffy models used for describing and modelling the growth, are presented in Table 2. Lengths calculated using the two methods differ only in tenths of millimetres, and thus, data rounded to millimetres are absolutely identical. However, there is a marked difference between lengths measured and calculated for the first two age groups, which requires explanation.

Table 2. Body lengths calculated on the basis of the measurements with the Walford method and the Bertalanffy equation

Age	Standard length (Lc) mm		
	According to measurements	According to Walford	According to Bertalanffy
0+	33	37	37
1+	63	59	59
2+	71	72	72

It is clearly visible in Fig. 5. that the point defined by the data pair of the second-year age group is well above the y value determined by the function $Lc_{(t)} = 0.5958Lc_{(t-1)}+37.092$, which follows from the excellent physical development of the specimens belonging to this age group. These specimens hatched in spring of 2000, when the early and long-lasting flood was accompanied by a similarly early and long-lasting warm weather. This created favourable conditions for an early spawning, while the longer growth season resulted in a more intensive development of the fry. It had been found in the fry of pike-perch in River Tisza, too, that they grew bigger than the average in the year in question (Harka, 2000).

Therefore, we assume that the outstanding size of the second-year specimens did not result from the conditions of the year of the sampling, but of the previous one. It can also be seen that the circumstances did not favour the growth of the first-year age group as much as in the previous year, and thus, their size reflects rather the conditions of worse years.

Mathematical models allow to reduce the amplitude of incidental deviations and hence, to show a better picture of the growth process. Therefore, while our measured data are valid only for the conditions of 2-3 particular years, the values calculated according to Walford or Bertalanffy show the average size conditions on a longer time span, and thus, are more suitable for making predictions.

Though the annual mortality rate, which resulted in 84.61% in our case, seems quite high, Bíró (1975) found an even higher value (89.35 %) in first-year bleak (*Alburnus alburnus*) of Lake Balaton. Taking into consideration that the youngest generation is the most vulnerable, these values can be considered realistic. Mortality probably decreases in the following year, although it can be deduced from the trend curve of Fig. 4 that the proportion of specimens surviving the fourth-year age must be negligibly small in the population. Therefore, the life span of golden spined loach in the studied reach of the Tisza can be put at 4-5 years.

In our case, estimation of biomass and production was possible only for first- and second-year fish. Lacking other data that would allow comparison, we can record only that weight gain was very rapid in the period in question (the weight gain rate was considerably higher than the values common in older generations). Hence, biomass grew in spite of the high mortality, and the annual production was above 200 %.

In conclusion, it seems – partly from our previous observations, partly on the basis of our experiences on other waters – that the environmental conditions in the dammed reach of River Tisza studied by us are not really favourable for the golden spined loach. Hence, the number of older (third- to fourth-year) specimens is little, and, according to our subjective evaluation, they do not attain the size of fish inhabiting waters that provide more favourable conditions. However, the proportion of mature

specimens still reaches the level necessary for the stability of the population, and thus, this population of golden spined loach is not immediately endangered.

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EFFECTS OF WATER POLLUTION AND GLOBAL WARMING ON THE FISH FAUNA OF THE ROMANIAN TRIBUTARIES OF THE RIVER TISZA

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Harka, Á., Sallai, Z. and Wilhelm, S. (2002): Effects of water pollution and global warming on the fish fauna of the Romanian tributaries of the River Tisza. – *Tiscia 32, 51-58.*

Abstract. In the summer of 2000, we conducted fish faunistical samplings in the Romanian reach of Upper Tisza River and its left tributaries. As a result, we found one new species (*Oncorhynchus mykiss* Walbaum, 1792) in the Szaplonca/Săpânța Brook and two new species (*Vimba vimba* Linné, 1758*, Gobio kessleri* Dybowski, 1862) in the Iza River. The fauna of the Iza is rich in natural values – 13 of its 23 fish species are legally protected in Hungary. But in the Visó/Vişeu the number of fish species (17) and their density (the number of fish samples caught in the Visó is just about 20 % of that found in the Iza) bear marks of the frequent heavy metal pollutions.
Studying the river zones, we noticed, in their fish communities, species normally inhabiting lower zones as well. This change increasingly observable in other zones of other rivers as well, which can be caused by the warming of the rivers. Numerous factors are likely to contribute to this phenomenon, but the main cause is most probably the warming that increased the surface temperature of the Northern Hemisphere by an average of 0.6 ^{0}C and that of Hungary by 0.67 ^{0}C in the 20th century.

Key words: Máramaros/Maramureş, zonation of rivers, expansion of gobiid species, water-system of Danube

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Introduction

The rivers surveyed are on the territory of Máramaros/Maramureş county, the fauna of which was first summarized by Frivaldszky (1871). He mentioned four fish species of the Iza — to which Herman (1887) added a new species —, and described six species from the Visó and its floods. Most of these species are listed by Vutskits (1904) with reference to the data of Mocsáry, and these are mentioned in the later published Fauna Regni Hungariae, in the chapter relating the fish (Vutskits 1918). These references do not mention the tributary brooks of the rivers and neither the fish fauna of the smaller Szaplonca.

The researches in the 20th century were started by Vladykov, respectively by Bănărescu. Vladykov (1931) has surveyed the right hand tributaries and the Upper Tisza, identifying 44 species of the above. Bănărescu (1964, 1969) summarized his own and the previous experiences, and pointed out the presence of 9 species in the Szaplonca, 12 in the Iza and 23 in the Visó. After that the fish fauna of the Máramaros rivers were searched by the team of the Antipa Museum, Bucharest. Bacalu (1997) has found 13 species in the Iza, 7 of which were unknown here previously. In the water system of the Visó Staicu *et al.*(1998) have found 13 species also, although they observed a very important deficiency compared to the previous very rich species-list.

Harka *et al.* (1999) described 14 species in the Tisza, between Rahó and Huszt, and 22 respectively in the Rahó–Tiszabecs reaches. The researches of Györe and collaborators on the Upper Tisza reaches have also enriched our knowledge: they have completed the fauna-list of the Szaplonca with 1, that of the Iza with 3 new species (Györe *et al.* 1999), and later that of the Visó with 1 new species (Györe

et al. 2001). Finally, we have to mention the work of Ardelean and Béres (2000), who summarize the recent researches on the vertebral fauna of the Máramaros Basin, listing 38 fish species of the Tisza, 11 of the Szaplonca, 33 of the Iza riversystem and 28 of the Visó basin.

While the water of the Szaplonca and the Iza can be declared clean, the Visó is often polluted and this represents a danger to the Tisza, which receives it. We can remember that in March 2000, 20-28 thousand m^3 of muddy sewage containing heavy metals has flown into the Visó from the industrial sewage lake of the Borsabánya (Baia Borşa) lead and zinc mine, which was followed by two more pollutions, which fortunately were of lower intensity (Szőke and Imre 2000, Hamar 2001).

Immediately after the events there were not apparent biological losses, but the damage in the living world is often shown later. Thus, during our researches we payed attention upon the changes in qualitative and quantitative distribution of the fish fauna which can be due to relatively diluted, but repetitive pollutions.

Localities and methods

The Máramaros reaches of the Tisza River present the characteristics of hilly country rivers. Its slope between the mouth of the Visó and 16 km lower at the mouth of the Iza is 2-3 m/km, but it is not smaller than 1-1.5 m/km between the mouthes of the Iza and Szaplonca on a reach of 20 km. Its current is strong, thus the bed of the river is composed by rounded rocks and rough pebbles, or gravel with different size grains, sedimental bed appearing just occasionally. The water is spread, usually not deeper than 1 m, it has lots of curves between the reefs in its way to the Lowlands.

The Szaplonca is about 20 km long with its source at 1100 m, and flows into the Tisza at 240 m above sea-level. In spite of its shortness, it is abounding in water, its average water output is 3.6 m^3/s. Its current is strong, while its drop on the upper reaches is 80-90 m/km and even at the mouth it reaches 20 m (Ujvári 1972).

The Iza has its source on Nagy Pietrosz, at 1200 m a.s.l., and it is an important tributary of the Upper Tisza. It is 83 km long and it reaches its recipient river at Máramarossziget, at 264 m height above sea-level. Its water output at the mouth is 16 m^3/s in average, but it can decrease to 0.58 at low water level, and it increases to 660 in time of great floods occuring prospectively in every 100 years. Its largest tributary is the Mára/Mara River, with the source at 1050 m a.s.l., having an average output of 9 m^3/s, and a length of 40 km (Ujvári 1972, Lászlóffy 1982).

The Visó is the first important left tributary of the Upper Tisza. Its source is in the Radna Mountains at 1693 m a.s.l., and after covering 80 km, it flows into the Tisza at 338 m a.s.l. It has a strong current with a water output of 20-50 m/km on its upper reaches, and of 2-8 m even at the mouth. Its average water output is 30 m^3/s at the lower reaches, which is just a few m^3 lower than the output of the Tisza. At low water level it carries just one tenth of this value, but its output can reach 1020 m^3 in time of a great flood expected every 100 years (Ujvári 1972, Lászlóffy 1982). Its most important tributaries are: the Vasér/Vaser and the Oroszi/Ruscova Brook. Both of them are approximately 40 km in length, with an average output of 10 m^3 (Fig. 1.).

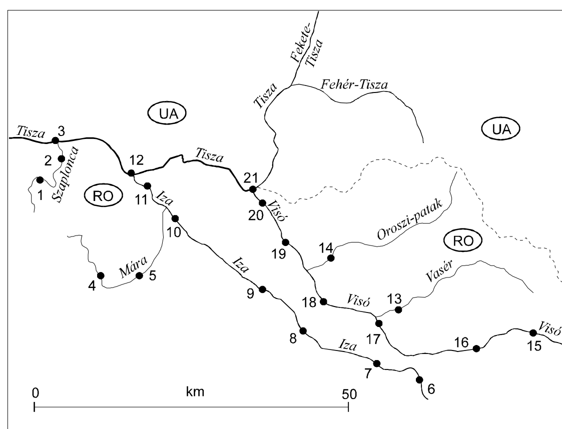

Fig. 1. A sketch map of the study area, showing the sampling sites

The fish fauna of these waters was studied between 5 and 15th of August 2001. As gathering devices we used electrical research fishing machine and — when the bed made it possible — small mesh net.

Our studies were conducted at 6-6 gathering points on the Iza and the Visó, respectively, 3 on the Tisza, 2-2 on the Szaplonca, resp. Mára and one on the Vasér and Oroszi Brook, each fishing took approximately two and a half hours. Our 21 gathering points are marked with numbers on the map of Fig. 1.

In the Szaplonca valley, including the reach of the Tisza around the Szaplonca mouth, our gathering points were: 1–Szaplonca, upper reaches, 2–Szaplonca, above Szaplonca/Săpânţa village, 3–Tisza, at the mouth of the Szaplonca.

In the Iza valley – including the Mára and the Tisza near the mouth of the Iza – our studies took part at: 4–Mára, above Karácsfalva (Mara) village, 5–Mára, at Hernécs (Hărniceşti), 6–Iza, above

Izaszacsal (Săcel), 7– Iza, under Izaszacsal (Săcel), 8–Iza, at Izakonyha (Bogdan Voda), 9–Iza, at Rozália (Rozavlea), 10–Iza, at Farkasrév (Vadu Izei), 11–Iza, at Máramarossziget (Sighetu Marmației), 12–Tisza, at Máramarossziget (Sighetu Marmației).

Our fishing points on Visó riversystem and its recipient river: 13–Vasér, above Felsővisó (Vişeu de Sus), 14–Oroszi Brook, above Visóoroszi (Ruscova), 15–Visó, near the source, 16–Visó, at Borsafüred (Stațiunea Borşa), 17–Visó, at Felsővisó (Vişeu de Sus), 18–Visó, at Alsóvisó (Vişeu de Jos), 19–Visó, at Petrova, 20–Visó, at Visóvölgy (Valea Vişeului), 21–Tisza, at Visóvölgy (Valea Vişeului).

After taxonomic identification, the collected individuals were let free. Individual number of each species was recorded exactly under 10, and approximately, rounded if their number exceeded 10. The temperature, pH and oxygen-concentration of the water were measured with a HORIBA combined water-quality assessing machine for local determinations.

Results

At our first two gathering points the temperature of the water of the Szaplonca was 17.3 and 18.1 ^{0}C respectively, the oxygen-concentration was 6.58 and 6.33 mg/l resp., and the pH values were 7.37 and 7.38 resp.

We have caught more than 400 fish samples from the Szaplonca and the Tisza around the mouth of the Szaplonca, which included 8 species regarding the Szaplonca, and 17 regarding the Tisza. Considering the species found in both, the total number is 20. These results are shown in details in Table 1.

In the period of our studies, the water temperature of the Iza varied between 12.2 and 26.6 ^{0}C. The first value was measured at the spring, and the other at the mouth. The concentration values of disolved oxygen were 6.88 and 5.65 mg/l, respectively, at the same places. The later one was the minimum value, while the maximum was measured at Bogdan Voda, 7.51 mg/l at a water temperature of 22.6 ^{0}C. The pH varied between 8.05 and 8.81 as the water becoming a little more alkaline from top to bottom. Regarding the two gathering points of the tributary Mára, our data were: 15.7 and 18.6 ^{0}C, 1.67 and 6.53 mg/l oxygen concentration, 7.62-8.32 pH.

In the Iza basin – including the Mára and the Tisza reaches around the mouth of the Iza – we could gather more than 1700 specimen. We have found 23 species in the Iza, 11 in the Mára and 13 in the Tisza, the total number of species were 25 (Table 2).

Table 1. Numbers of specimens caught in the Szaplonca/Săpânța Brook and in the recipient River Tisza at estuary of the Szaplonca/Săpânța Brook

Species	Szaplonca		Tisza
Localities	1.	2.	3.
Eudontomyzon danfordi			1
Leuciscus leuciscus			1
Leuciscus cephalus			25
Leuciscus souffia		1	4
Phoxinus phoxinus	9	70	2
Alburnoides bipunctatus		2	130
Chondrostoma nasus			4
Barbus barbus			6
Barbus petenyi		10	20
Gobio gobio			10
Gobio uranoscopus			10
Gobio kessleri			2
Barbatula barbatula	1	30	40
Cobitis taenia			1
Sabanejewia aurata			15
Oncorhynchus mykiss		10	
Cottus gobio			25
Cottus poecilopus	6	10	
Zingel streber			2

The temperature of the Visó varied between 13.5 and 22.7 degrees. Its oxygen concentration near the spring was 6.78, at the mouth 6.04 mg/l, but the minimum value was taken at Felsővisó: at a water temperature of 20.1 ^{0}C the oxygen-concentration was 4.55 mg/l. This was the point wiht the lowest pH (8.13), while on the other reaches it varied between 8.4 and 8.6.

In the Visó riversystem and in the Tisza reaches around the Visó mouth we collected about 700 fish individuals. We gathered 6 species in the Vasér, 8 in the Oroszi Brook, 17 in the Visó and 10 in the Tisza. We did not found any species in the tributaries and in the Tisza that were not present in the Visó, so the total number of species is 17. These results are shown in Table 3.

The temperature of the Tisza River, which gathers the mentioned tributaries, was 19.4 at the mouth of the Visó, while at the mouth of both the Iza and the Szaplonca was 23.5 ^{0}C, and these two last points showed almost the same pH: 8.53 and 8.59, resp. However, a considerable difference appeared in

Table 2. Numbers of specimens caught in the Iza riversystem and in the recipient River Tisza at estuary of the River Iza (+ : catch of angler)

Species	M á r a		I z a						Tisza
Localities	4.	5.	6.	7.	8.	9.	10.	11.	12.
Eudontomyzon danfordi		1	1						1
Rutlilus rutilus							1		
Leuciscus leuciscus					2	1			
Leuciscus cephalus		7			10	40	200	1	8
Leuciscus souffia		2			40	1	40		4
Phoxinus phoxinus	1	80			200	80			15
Alburnus alburnus					2		25		3
Alburnoides bipunctatus		30			20	10	40	40	50
Vimba vimba							1		1
Chondrostoma nasus					3	5	9		
Barbus barbus							3		
Barbus petenyi	8	25			40	30	80		2
Gobio gobio		1			6	15	80	3	8
Gobio uranoscopus					1	6	2		
Gobio kessleri						4	10	1	
Barbatula barbatula		30		1	30	3	20	1	2
Cobitis taenia					1	1	7	1	
Sabanejewia aurata		20			30	50	200	30	15
Salmo trutta m. fario	1			+					
Lota lota								1	
Cottus gobio	8							1	15
Cottus poecilopus			30	40					
Perca fluviatilis							4		1

Table 3. Numbers of specimens caught in the Visó/Vişeu riversystem and in the recipient River Tisza at estuary of the River Visó/Vişeu

Species	Vasér	Oroszi	V i s ó						Tisza
Localities	13.	14.	15.	16.	17.	18.	19.	20.	21.
Eudontomyzon danfordi		2				3			
Leuciscus leuciscus							1		
Leuciscus cephalus							2		3
Leuciscus souffia	1	1				2	7	1	6
Phoxinus phoxinus	200	60			15	20	30		2
Alburnus alburnus							1		
Alburnoides bipunctatus					1	2	5	6	10
Chondrostoma nasus								1	4
Barbus barbus							1		3
Barbus petenyi	2	1			1	7	7	7	15
Barbatula barbatula	10	50			40	40	50	10	20
Sabanejewia aurata								10	8
Thymallus thymallus		10				2			
Salmo trutta m. fario	1		3						
Lota lota								2	
Cottus gobio		1					1	3	30
Cottus poecilopus	2	4		15	1	1			

the oxygen-concentration, which was 4.87 mg/l at the mouth of the Iza compared to the mouthes of the the two rivers with values of 6.58 and 6.67, resp. The low value taken at the mouth of the Iza can be due to the communal pollution of Máramarossziget and its organic components, the decomposition of which needs much oxygen consumption. The fact that at the mouth of the Szaplonca the value was similar to the previous one, shows the self cleaning process of the river.

From the Tisza we could gather 10 species at the mouth of the Visó, 13 at the mouth of the Iza, while at the mouth of the Szaplonca we caught 17 species. Considering the same species in the different points, the total number is 20.

Discussion

Although in recent years others have also studied the fish fauna of these rivers, our work has brought results regarding the fauna too. We have shown the presence of new species, the Rainbow trout *(Oncorhynchus mykiss)* in the Szaplonca, and we have found that the majority of the previously described species are still living either in the stream, or in the Tisza near the mouth.

Regarding the Iza River, we have identified two species that were previously not found: the Vimba *(Vimba vimba),* and the Sand gudgeon *(Gobio kessleri)*. We have also stated that the fauna of the river represents a great natural value. From the 23 species found 13 is legally protected in Hungary (2 of them being greatly protected), regarding the European standards (Lelek 1987) the majority of them are rare or endangered. The 13 protected species also increase the natural value of the river, and most of these species are represented by a nice and large population. For example, the Blageon *(Leuciscus souffia* Risso, 1826*)*, the Minnow *(Phoxinus phoxinus* Linné, 1758*)*, the Schneider *(Alburnoides bipunctatus* Bloch, 1782*)* and the Golden spined loach *(Sabanejewia aurata* Filippi, 1865*)*, and also the greatly protected Petenyi's barbel *(Barbus petenyi* Heckel, 1847*)*. The richness of the Iza is well clearly shown by the fact, that 80 % of the 1.5 thousand samples caught was legally protected in Hungary.

We have not found any previously not identified fish in the Visó. Although we captured four species not listed in the 13 one reported by Staicu *et al.* (1998), these were mainly swimming up from the lower reaches of the Tisza, like the Dace *(Leuciscus leuciscus* Linné, 1758*)*, the Bleak *(Alburnus alburnus* Linné, 1758*)* or the Barbel *(Barbus barbus* Linné, 1758*)*. The total lack of *Gobio*-species was surprisin, because they were caught in great quantity in the Iza River conditions of which are very similar to those of Visó.

The difference is well demonstrated by the fact that compared to the 23 species of the Iza, we could only find 17 in the Visó. Besides the number of species, the number of individuals also show a great difference in the two rivers. Although we have studied the same gathering points, spending the same time with fishing, the number of fish specimens caught in the Visó were just about 20 % of that found in the Iza. We can get the same results if we make the comparison with the water system. In the two tributaries – although we surveyed just one gathering point on the Vasér and Oroszi Brook – we have caught 30 % more fish, than from the whole reach of the Visó.

In conclusion, the fish community of the Visó is greatly damaged. The geogrephical site, the size and ecological conditions of the river are similar to he Iza, but its output is much greater, so it would be able – in natural circumstances – to support a richer fish fauna than the latter one. It is absolutely sure that regular heavy metal pollution plays an important role in the fact that the river holds just a small number of fish, and we have to find a solution urgently in the favour of its recipient river, the Tisza too. The fact that there is such a small number of fish at all in this frequently polluted water, is mainly due to the tributary streams. During great pollutions a fraction of the population can get shelter in these, and the river is repopulated by them.

None of the 22 species gathered in the Tisza were new. Although we have found some, which were caught only on the lower reaches during the previous study (Harka *et al.* 1999), these were also found in the tributaries, so we will mention them relating the latter ones.

The studied waters have the same characteristics as a mountain running water source of more than 1000 m a.s.l. While the Szaplonca reaches its recipient river as a stream, the Tisza, the Iza and the Visó become smaller rivers when arriving to this region. The differences in their fish fauna are due to the differences in their size.

The Szaplonca – along almost its whole reach – shows the characteristics of the trout-zone and the composal of its fish species equivalent to this. Although the Brown trout *(Salmo trutta m. fario* Linné, 1758*)* , which is typical of this river zone, was found just in the trout-pond built near the stream, we have caught lots of samples of it settled relative, the Rainbow trout *(Oncorhynchus mykiss)*. We could also find lots of specimen of the Minnow *(Phoxinus Phoxinus),* the Petenyi's barbel *(Barbus petenyi),* the

Stone loach *(Barbatula barbatula* Linné, 1758*)*, the Siberian bullhead *(Cottus poecilopus* Heckel, 1836*)*, which make the trout-zone name obvious. The only exception is the reach around the mouth, which shows more the characteristics of the grayling-zone.

However, some species appear in the stream, which are not typical of these river zones. These are the Vimba *(Vimba vimba)* related by Ardelean and Béres (2000) and the Danubian salmon *(Hucho hucho* Linné, 1758*)*. However, the contradiction is apparent, because these fish do not live in the stream, they only swim up there occasionally. Thus they aren't the determinants, just the colouring elements of the fish population for the water, however it was surprizing that we did not find any specimen of Brown trout in the stream. On August the 9th for example – on the reach above the foresters' house – we were trying to catch it for two and a half hours, without any success, although in the previous years it was frequently caught here. Its lack should be due to the unusual heat, which characterised the weather of the Carpathian Basin at that time, where the water temperature increased up to 17.3 °C even at this height. Afterwards, we thought the possiblity that the Brown trouts withdraw till near the spring because of the heat, because in the Upper Visó we could catch them only in the uppermost reaches where the water temperature was under 14 ^{0}C. During our fishing on the Szaplonca, we haven't thought about this possibility, that's why we didn't look for proof about this idea.

The Iza — in contrast to the Szaplonca — is not a stream, but a small river, thus its fish fauna is more varied. The upper reach is a trout-zone, but the Brown trout *(Salmo trutta m. fario)* which gives the name of the zone is rare, its presence is proved only by the catch of a fisherman. However the Siberian bullhead *(Cottus poecilopus)* is frequent, a speciman of which was found in our net together with a Carpathian lamprey *(Eudontomyzon danfordi* Regan, 1911*)*, feeding from the previous.

The trout zone turns into the grayling-zone between Izaszacsal (Sacel) and Izakonyha (Bogdan Voda). We can state this, although the Grayling *(Thymallus thymallus* Linné, 1758*)* was not found, and the presence of it — to our best knowkedge — was not demonstrated. However, besides the species present in the trout-zone, like the Minnow *(Phoxinus phoxinus)*, the Petenyi's barbel *(Barbus petenyi)* and the Stone loach *(Barbatula barbatula)*, there appear numerous specimens of the Souffia chub *(Leuciscus souffia)* and the Golden spined loach *(Sabanejewia aurata)*, which are strangers in the upper zones, and disappears the Siberian bullhead *(Cottus poecilopus)* frequent in the previous zone. Under Farkasrév (Vadu Izei) the Rifle minnow *(Alburnoides bipunctatus)* appears in great quantity and other species typical to lower zones *(Rutilus rutilus* Linné, 1758*, Vimba vimba, Perca fluviatilis* Linné, 1758*)*, showing that the lower reaches of the river take part of the nase-zone.

Previously Bănărescu (1964, 1969) has shown 23 species in the Visó, but Staicu *et al.* (1998) have found the presence of 57%, while our study has shown 74% of them. However the river-zones are recognizable. Although the Brown-trout *(Salmo trutta m. fario)* was only found close to the spring, the trout-zone is extending till Felsővisó. There is the mouth of the Vasér, which – except the transitional part around the mouth – is a trout-water with a great Minnow *(Phoxinus phoxinus)* population. After the mouth of the Vasér the trout-zone turns into the grayling-zone, which extends approximately till the mouth of the Oroszi Brook. The name of this zone is given by the Grayling *(Thymallus thymallus)* of which we have found just two specimens in the river, but there were more in the Oroszi Brook, the lower reach of which is greayling-zone too. The mouth-reach of the Visó, under Petrova is a nase-zone, which is well shown by the change from the Siberian bullhead *(Cottus poecilopus)* to the Bullhead *(Cottus gobio* Linné, 1758*)* and the appearance of the Barbel *(Barbus barbus)*.

The Máramaros reach of the Tisza has recently been described as a grayling-zone (Harka *et al.* 1999), but our data suggest that it is a nase-zone. This is supported by the fact that we have found numerous species which were caught during the previous research only in the lower zones, for example the Vimba *(Vimba vimba)*, the Barbel *(Barbus barbus)*, the Kessler's gudgeon *(Gobio kessleri)*, the Pearch *(Perca fluviatilis)*. However we can also state that the partly different species-spectrum of the small and large rivers described by Bănărescu (1964) are becoming more and more similar, and the differences amongst the fish populations of the neighbouring river-zones are also decreasing.

These are supported by the presence of foreign elements in the grayling-zone of the Iza, like the Bleak *(Alburnus alburnus)*, the Nase *(Chondrostoma nasus)* and the Kessler's gudgeon *(Gobio kessleri)*. The Pearch *(Perca fluviatilis)* is also a stranger in the mentioned zone of both the Iza and the Tisza.

The occurrence of one or two species in a foreign environment can be occasional, but we have found several individuals of several species. Thus this is a marked tendency which needs to be explained.

It is well known that the different fish-zones of a river, following each-other are distinguished by the fish populations composed by characteristic species to that river-zone, which are determined by the dominating ecological relations. The most important environmental factors are: the speed, the temperature and the oxygen-concentration of the water and the material and the quality of the bed. In our case one of these differed from the usual grayling-zone: the temperature of the water. Thus the explanation is obvious: the phenomenon was caused by the fact that the temperature of the water was higher than usual – due to the hot weather at that time.

At first this seems to be a satisfactory explanation, but in our opinion it needs a detailed survey, as it is not a single case. Bacalu (1997) and Györe *et al.* (1999) have searched the Iza at different times and they have also remarked the presence of the Bleach *(Alburnus alburnus)* and the Barbel *(Barbus barbus)*. Ardelean *et al.* (2000) as well as Györe *et al.* (2001) have observed the expansion towards the upper reaches of the Carp *(Cyprinus carpio* Linné, 1758*)*, the Crucian carp *(Carassius carassius* Linné, 1758*)*, the Pike *(Esox lucius* Linné, 1758*)*, the Perch *(Perca fluviatilis)*, the Bream *(Abramis brama* Linné, 1758*)* and the Brown bullhead *(Ictalurus nebulosus* Le Sueur, 1819*)*, while the Chub *(Leuciscus cephalus* Linné, 1758*)*, the Nase *(Chondrostoma nasus* Linné, 1758*)* and the German carp *(Carassius auratus* Linné, 1758*)* were found right up to the mountain streams. The same results were shown by some Slovakian researches (Harka *et al.* 2000) which noted the presence of the Nase *(Chondrostoma nasus)* and the Barbel *(Barbus barbus)* in the grayling-zone of the Laborc (Laborec) River. The upwards-expansion – as a phenomenon – is not limited just to the nase- and grayling-zones, it can be observed on the middle and lower reaches of our rivers. At the end of the 19th century the Tubenose goby *(Proterorhinus marmoratus* Pallas, 1811*)* reached only up to Bratislava on the Danube, by now it has reached Germany and it gets upper and upper in the Dráva, Tisza and Körös too (Harka 1990). A similar phenomenon is observable concerning the Monkey goby *(Neogobius fluviatilis* Pallas, 1811*)* which has conquered several new waters in the Carpathian Basin (Harka 1993, 1997, Ahnelt *et al.* 1998, Sallai 2000). The Bighead goby *(Neogobius kessleri* Günther, 1861*)*, the Syrman's goby *(Neogobius syrman* Nordmann 1840*)*, the Round goby *(Neogobius melanostomus* Pallas, 1811*)* and the Racer goby *(Neogobius gymnotrachelus* Kessler, 1857*)*, previously found only around the mouth of the Danube, have appeared in the last ten years in the middle, Hungarian-Slovakian-Austrian reaches of the river (Zweimüller *et al.* 1996, Erős and Guti 1997, Guti, 1999, Wiesner *et al.* 2000, Ahnelt *et al.* 2001).

Regarding the way of this expansion there are only speculations. There is a possibility of illegal introduction of these species by aquarists, and also the importation of these by ballast water of the ships. But neither of these speculations give a reassuring explanation why these changes have just occurred recently, although both aquaristics and shipping look back on a long past. Certainly there is the possibility of active migration, but the „Why exactly now?" question is still not answered.

If we consider that the appearance and expansion of the Black Sea-origin gobiid species towards our rivers is the same phenomenon of striving of the fishes from lower to upper reaches, has probably the same reason as in the Máramaros rivers: warming. Considering that this is not an oscillating phenomenon, but it has a strict direction, we have to look for a tendency-like changing of the weather. And this is not a change of the weather, but of the climate. The so-called global warming means that the temperature of the surface of the Earth has increased with 0.6 ^{0}C, while that of Hungary with 0.67 ^{0}C in the 20th century (Szalai and Szentimrey 2001). This has become more evident in the last ten years, which was the warmest decade of not just the century but also the millennium. Corresponding to this, we have observed the expansion of the Tubenose goby (*Proterorhinus marmoratus*) in the last 100 years, the Monkey goby (*Neogobius fluviatilis*) has conquered our waters in the last 20-30 years, while the appearance of the other gobiid species has occurred in the last decade.

Thus we consider that different species of the fish communities of the river-zones and the immigration of the Ponto-Caspian species into Central Europe are both due to the warming of the waters. This may be due to several reasons – like the building of water-reservoirs, the communal pollution, the warm coolants of the power stations, etc. – but the main factor is the global warming, the accelerated warming of the surface temperature of the Earth. This means that there will be more changes in the composal and dominancy relations of the species of the fish community in the different river-zones and new species will appear from South to the Carpathian Basin in the future too.

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TISCIA 33, 59-66

THE DIET OF DIPPERS (*CINCLUS CINCLUS*) IN THE AGGTELEK KARST

R. Horváth

Horváth, R. (2002): The diet of dippers (*Cinclus cinclus*) in the aggtelek karst. *— Tiscia 33, 59-66.*

Abstract. The study highlights the changes in the food-composition of dippers (*Cinclus cinclus*) based on the method of pellet and faecal analyses. The collected reguriated pellets and faeces were analysed together with the evaluation of the potential food supply for dippers. By applying the two methods simultaneously, I managed to prove the change in the feeding tactics of dippers as well as the efficiency of the two methods.

Keywords: Aggtelek National Park, Cinclus cinclus, diet of dippers, faecal analysis, pellet analysis

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Introduction

The dipper (*Cinclus cinclus*) is a rare, endangered and a highly protected singing bird in Hungary. Individuals nestling on a regular basis can only be found in the clear and swift-running mountain streams abounding in water in the Bükk and Zemplén mountains and the Aggtelek Karst. Due to the natural endowments of the middle-ranged mountains of Hungary this species could not spread all over, in fact it could reach only marginal areas of Hungary. Therefore, Hungary has never had large populations of dippers.

Since our rivers became polluted their water output has decreased to a great extent due to the catchment and the inaccurate practices of forestry management. Thus the invertebrate wildlife has become extremely poor and has changed in many aspects furthermore, the nestling sites of our birds have become treated with concrete leading to a decreased number of birds. In the 1950s the estimated number of dipper pairs reached as far as 50, in 1983 only 23 pairs were recorded in Hungary (14 pairs in the Bükk mountains alone). By 1989 and then by 1992 this number fell to 9 and 3 pairs. In the mid 1990s there was only one pair nestling on the Aggtelek Karst (Horváth 1993, Horváth and Szép 1998, Horváth, Boldogh and Varga 1999)

So far 3 studies on the diet of dippers have been published in Hungary. The first one studied the stomach content of 482 individuals (Vollnhofer 1906) out of which only three had come from the present area of Hungary. The second research involved the analysis of only 39 reguriated pellets (Rékási 1985), therefore it provided rather poor data on this subject. Although the third paper focused exclusively on the winter diet of this species (Horváth and Andrikovics 1991) it involved the analysis of an extraordinary number of dipper pellets reaching as far as 1362.

Reguriated pellet sampling (Jost 1975a) and analysis (Jost 1975b, 1975c, Spitznagel 1985, 1988) are known to be quite wide-spread abroad with an extensive literature. Another method of the analysis, the faecal analysis has also been applied several times (Ormerod, Tyler and Lewis 1985, Ormerod, Boilstone and Tyler 1985, Ormerod 1985a, 1985b, Ormerod and Tyler 1986, Smith and Ormerod 1986, Ormerod, Efteland and Gabrielsen 1987). There has only been one attempt involving only a low number of samples, that is 15 pellets and 20 faecal pellets to compare the two different methods (Spitznagel 1985). Thus, up to this point there was not one thorough study to compare the two methods.

Almost all of the studies dealing with the diet and food-composition of the dippers focused on the indicative characteristics as well as the protection of this species (Andrikovics and Horváth 1997). Classical examples of this include the investigations that give account of the fact that the number of birds were decreasing and the diet, food-composition and food supplies of the dippers were also changing in

parallel with the acidification of our rivers (O'Halloran, Gribbin, Tyler and Ormerod 1990, Ormerod and Tyler 1991, Tyler and Ormerod 1994).

My research was aimed at getting information about the food-content of the dippers nestling on the area of the Aggtelek Karst. I have observed that in spring dippers tend to feed on and show a preference for caddisfly larvae. During the study I was to support these observations with precise data. I wanted to learn whether and how the diet of the dippers changes with the breeding season. Furthermore, I wanted to draw a comparison between the results of the reguriated pellet and the faecal analysis to see whether the two methods could be successfully applied together. The two applied methods could be a great help in assessing and studying dipper populations as well as protecting their habitats, which could eventually lead to bringing the decrease in the numbers of dippers to an end.

The study area

The study area coincided with the distribution of dippers in Hungary (Horváth 1988, Horváth and Szép 1998). We collected samples from the dippers nestling on the area of the Aggtelek Karst, around the river-head of the Jósva stream and lake Tengerszem. The Jósva stream forms several branches with artificial or partially natural streambeds, all heading towards Tengerszem lake. The environment of the current leaving behind the storage lake is completely natural.

Methods

We collected samples from the above mentioned regions both during the winter period (November-December) in 1991 and during the following breeding season (February-March) in 1992 (Table 1). On the artificially constructed bank where the dippers foraged for food on a regular basis we managed to collect the characteristic faecal and reguriated pellets of the adult dippers. The conspicuous empty shells of the Trichoptera larvae (Jost 1972) that were eaten by the dippers were also collected at this site. We tried to identify the caddisflies consumed by dippers on the basis of these, as well.

The benthic fauna of the riverbed serving as a potential food resource was collected with the aid of a special net, at the feeding sites of the birds. During sampling we made an attempt to collect samples the same way each time by focusing on the fact that both the number and the length of each drawing should be the same.

The reguriated pellets and the faeces were collected simultaneously in the winter of 1991 and in

Table 1. Data obtained from dipper faeces, reguriated pellets, empty caddisfly shells and potential prey items collected near the river-head of the Jósva stream and Tengerszem lake in relation to the breeding period of dippers.

Data	Pellet Gammarus/Trichoptera %	Faecal Gammarus/Trichoptera %	Caddisfly shells	Breeding of Dipper
04.09.1991			none	hatch
16. 04.			10/day	hatch out
20.04.- 01. 05.			203-489	
03. 05.			115	nestling fly away
06-0805.			244-344	
09. 05.			59	
10-13. 05.			10	nestling independent
end of November	89 / 6,5	69 / 5,6		
first part of December	82 / 17	78 / 9,3		
18-20. 02.1992	76 / 18	72 / 12		
12. 03.	67 / 31	60 / 18	none	
18-20. 03.	38 / 60	42 / 35	1-6	
22. 03.			140	
24. 03.	52 / 47		71	
25. 03.			32	
27. 03.	37 / 62		103	
28. 03.			29	
30. 03.			24	hatch out
06. 04.			98	nestling destroyed

1992. The reguriated pellets were preserved in 70 % alcohol, hand-sorted and then they were examined under a stereobinocular microscope at magnifications X40-X100 (Spitznagel 1985, 1988). The dipper faeces were fixed in M/2 sodium hydroxide solution for four hours (Ormerod 1985a). Only after this were the remains of the prey items identified. The benthic fauna (as a possible food supply) of the riverbed was preserved in 70% alcohol and was identified using a microscope. The individuals of *Salamandra salamandra* were not collected since they are protected species. These individuals were pooled and soon after sampling they were released. Only after identification did we dry and weigh the prey items. In the results the dry weights are given and the values are percentage contributions by dry weight.

The empty caddisfly shells were collected and counted in the breeding season of 1991 and 1992. The empty shells collected in 1991 were identified under a microscope whereas those sampled in 1992 were not identified. Nevertheless, the proportion of empty caddisfly shells of 1991 and 1992 were more or less the same (Table 1).

The remains of the prey items were identified to the lowest taxon possible. During the study we identified a total of 5 samples of potential food resources (with 4.985 individuals) including 210 reguriated pellets (90 out of which were collected in winter and 120 in the breeding period), 209 pieces of faeces (80 collected in winter and 129 in the breeding season) and 2900 empty caddisfly shells.

Results

The results of the analyses of the samples (i.e. the potential food resources)

2077 individuals (as potential prey items for dippers) belonging to 6 species and one higher taxon were identified in the winter sample of 1991 (Table 2). *Gammarus fossarum, Sadleriana pannonica* and Coleoptera larvae were the most abundant in the sample. Interestingly enough, only 11 individuals of Trichoptera larvae were found in it.

A total of 2908 individuals (as a potential food resource) belonging to one vertebrate and 10 invertebrate taxa were identified in the three samples collected during the breeding season (Table 2). *Gammarus fossarum* and *Sadleriana pannonica* were present here with the largest numbers. The ratio of *Gammarus fossarum* and *Sadleriana pannonica* in the four samples did not change significantly. However, there was an increase in the proportion of Trichoptera larvae. Although it increased more than tenfold, it still did not reach 10 %.

Table 2. The range of potential prey items for dippers near Jósva stream during the winter of 1991 and the spring of 1992 (number and percentage)

<table>
<tr><th rowspan="2"></th><th rowspan="2">Glossiphonia complana (Annelida)</th><th rowspan="2">Gammarus fossarum</th><th rowspan="2">Ephemera vulgata</th><th rowspan="2">Coleoptera larva</th><th>Trichoptera sp.</th><th>Parachiona picicornis</th><th>Sericostoma personatus</th><th>Sericostoma turbatum</th><th>Chaetopteryx sp.</th><th>Limnephilidae</th><th rowspan="2">Sadleriana pannonica</th><th rowspan="2">Succinea putris</th><th rowspan="2">Laciniaria turgida</th><th rowspan="2">Arionidae</th><th rowspan="2">Salamandra salamandra (Amphibia)</th><th rowspan="2">Total number</th></tr>
<tr><th colspan="6">TRICHOPTERA</th></tr>
<tr><td rowspan="2">01.12.199</td><td rowspan="2"></td><td rowspan="2">1390 ind 66,9 %</td><td rowspan="2">20 ind 1,0 %</td><td rowspan="2">32 ind 1,5 %</td><td rowspan="2"></td><td>1 ind</td><td>7 ind</td><td>3 ind</td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2">624 ind 30 %</td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2">2.077</td></tr>
<tr><td colspan="3">0,5 %</td></tr>
<tr><td>21.02.1992</td><td>7 ind</td><td>437 ind 61 %</td><td></td><td>1 ind</td><td></td><td></td><td></td><td></td><td>9 ind 1,2 %</td><td></td><td>248 ind 35 %</td><td>9 ind</td><td>1 ind</td><td></td><td></td><td>712</td></tr>
<tr><td rowspan="2">22.03.1992</td><td rowspan="2"></td><td rowspan="2">553 ind 46 %</td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2"></td><td>47 ind</td><td>16 ind</td><td rowspan="2">576 ind 48 %</td><td rowspan="2">9 ind</td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2">1.201</td></tr>
<tr><td colspan="2">5,2 %</td></tr>
<tr><td>30.03.1992</td><td></td><td>347 ind 52 %</td><td></td><td>1 ind</td><td>56 ind 8,4 %</td><td></td><td></td><td></td><td></td><td></td><td>258 ind 39 %</td><td></td><td></td><td></td><td>1 ind</td><td>663</td></tr>
<tr><td>06.04.1992</td><td></td><td>134 ind 40 %</td><td></td><td></td><td>17 ind 5,1 %</td><td></td><td></td><td></td><td></td><td></td><td>177 ind 53 %</td><td>2 ind</td><td></td><td>2 ind</td><td></td><td>332</td></tr>
</table>

Table 3. The results of reguriated pellet for dippers during the winter of 1991 and the spring of 1992 (number and dry weight)

	Gammarus fossarum	*Anacaena globulus*	*Lymnus volcmari*	Trichoptera indet.	*Hydropsyche* sp.	*Rhyacophila* sp.	Lepidoptera caterpillar	*Sadleriana pannonica*	Part of plants	Stone	Total weight Number of pellet Average weight
				TRICHOPTERA							
10.1991	2,9499 g 89,29 %			0,1100 g 3,33 %	0,0420 g 1,27 %	0,0636 g 1,93 %	0,0620 g 1,87 %	5 ind	0,0120 g 0,36 %	0,0642 g 1,94 %	3,3037 g 30 pcs 0,1101 g
				6,53 %							
12.1991	5,2056 g 82,29 %	0,0119 g 0,18 %			0,4391 g 6,94 %	0,6548 g 10,35 %		3 ind	0,0012 g 0,01 %	0,0132 g 0,2 %	6,3258 g 60 pcs 0,1054 g
				17,29 %							
18-20. 02.1992	6,3846 g 76,73 %	0,0102 g 0,12 %	0,0016 g 0,02 %	0,3666 g 4,41 %	0,6896 g 8,28 %	0,4896 g 5,88 %		7 ind	0,0820 g 0,99 %	0,2962 g 3,56 %	8,3204 g 65 pcs 0,1280 g
				18,58 %							
12.03. 1992	1,6140 g 67,92 %		0,0011 g 0,04 %	0,3706 g 15,59 %	0,1712 g 7,20 %	0,2072 g 8,72 %		42 ind	0,0020 g 0,08 %	0,0104 g 0,44 %	2,3765 g 20 pcs 0,1188 g
				31,52 %							
18-22. 03.1992	1,0190 g 38,14 %		0,0019 g 0,07 %	0,8422 g 31,52 %	0,4211 g 15,76 %	0,3428 g 12,80 %		12 ind	0,0021 g 0,08 %	0,0427 g 1,60 %	2,6718 g 21 pcs 0,1272 g
				60,11 %							
24-25. 03.1992	0,2105 g 52,59 %		0,0011 g 0,27 %	0,0813 g 20,31 %	0,0668 g 16,69 %	0,0406 g 10,14 %		1 ind			0,4003 g 4 pcs 0,1001 g
				47,14 %							
27.03. 1992	0,4314 g 37,70 %		0,0016 g 0,14 %	0,4212 g 36,81 %	0,1032 g 9,02 %	0,1868 g 16,33 %					1,1442 g 10 pcs 0,1144 g
				62,16 %							

The results of the pellet analysis

The 90 reguriated pellets collected on two occasions in the winter period of 1991 have much in common (Table 3). The majority of their dry weigh was provided by *Gammarus fossarum* in both samples (89% and 82 %). The number and proportion of Trichoptera larvae, however, showed a slight increase: from 6,5% over 17%.

During the spring of 1992 we collected samples on six different occasions. In the breeding season 120 reguriated pellets with individuals belonging to 6 invertebrate species as well as plant matters and smaller stones were found. Almost all the samples contained large numbers of *Gammarus fossarum* and various Trichoptera species (Table 3). Taking a closer look at the tables, it becomes obvious that while the proportion (77%) of *Gammarus fossarum* in the 65 pellets of February is similar to the values of the November and December samples of 1991 (89 % and 82 %), it keeps decreasing gradually in the successive pellets until it reaches 37 %. In the meantime the ratio of caddisflies seemed to be increasing in the samples (from 19 % to 62 %).

The results of the faecal analysis

At the end of 1991 we did not collect only reguriated pellets but faeces, as well. In the winter period 80 pieces of dipper faeces were gathered on two occasions. The faecal analysis revealed similar results as the reguriated pellet analysis (Table 4). In the second sample the proportion of Trichoptera larvae also rose from 6 % over 9 % in the faeces. Moreover the increased number of small stones in the November sample is also of high significance.

During the spring of 1992 we collected 129 pieces of faeces simultaneously with pellets. The results of the faecal analysis are close to that of the reguriated pellet analysis. The ratio of caddisflies increased from 12 % to 35 % at the expense of that of the Amphipods. Simultaneously, the portion of *Gammarus* decreased to 42 %.

The results of the identification of the caddisfly shells

98.9 % (i.e. 2868 pieces) of the 2900 empty caddisfly shells, which were collected in 1991, were

Table 4. The results of faecal analysis for dippers during the winter of 1991 and the spring of 1992 (dry weight)

	Gammarus fossarum	*Gammarus* – Trichoptera	Coleoptera indet.	*Lymnus volcmari*	Trichoptera indet.	*Rhyacophila* sp.	*Hydropsyche* sp.	*Sadleriana pannonica*	Secondory product of digestion	Stone	Total weight Number of faecal Average weight
11.1991	0,8831 g 69,81 %	0,1124 g 8,89 %		0,0340 g 2,69 %	0,0327 g 2,58 %	0,0244 g 1,93 %	0,0138 g 1,09 %		0,0668 g 5,28 %	0,0978 g 7,73 %	1,2650 g 20 pcs 0,0632 g
						0,0709 g 5,60 %					
12.1991	2,3082 g 78,47 %	0,2606 g 8,86 %		0,0016 g 0,05 %		0,0978 g 3,32 %	0,1772 g 6,02 %		0,0966 g 3,28 %		2,9420 g 60 pcs 0,0490 g
						0,2750 g 9,35 %					
18.02.1992	2,8681 g 72,20 %	0,4482 g 11,28 %	0,0012 g 0,03 %			0,2107 g 5,30 %	0,2812 g 7,08 %		0,0826 g 2,08 %	0,0800 g 2,02 %	3,9720g 72 pcs 0,0552 g
						0,4919 g 12,39 %					
12.03.1992	0,8012 g 60,47 %	0,1341 g 10,12 %				0,1279 g 9,65 %	0,1218 g 9,19 %	2 ind	0,1213 g 9,16 %	0,0188 g 1,41 %	1,3251 g 20 pcs 0,0663 g
						0,2497 g 18,84 %					
18.03.1992	1,0066 g 42,39 %	0,4042 g 17,02 %			0,3640 g 15,32 %	0,1981 g 8,34 %	0,2842 g 11,96 %		0,0846 g 3,56 %	0,0329 g 1,39 %	2,3746 g 37 pcs 0,0642 g
						0,8463 g 35,64 %					

successfully identified to subgenus or family. 65 % of the identified caddisflies belongs to the *Anabolia* and *Halesus* subgenus. Furthermore, individuals belonging to the following two taxa: the *Allogamus* subgenus (12 %- 349 pieces) and the Limnephilidae family (11% - 321 pieces) were present in large numbers. In Table 1 it is evident that there is a correlation between the feeding period of the nestling and the mass appearance and disappearance of the empty shells of Trichoptera larvae (larvae eaten by dippers).

Table 1 well demonstrates that despite the early nestling, the mass appearance of the empty shells of the Tricheroptera larvae, which are consumed by dippers, also correlated with the nestling period of these birds in 1992. Unfortunately the diurnal fluctuations in the number of caddisflies could not have been accurately observed because of the heavy tourist industry and the subsequent disturbances.

Discussion

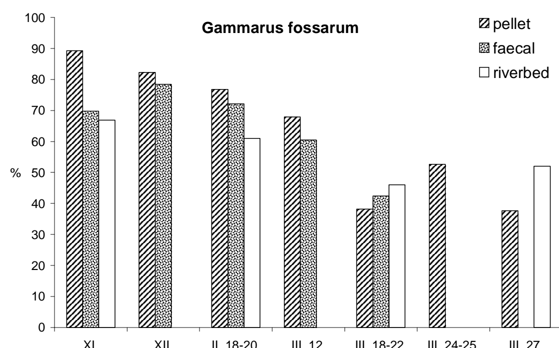
The results of the pellet and the faecal analysis

The results of the faecal analysis can be used at least on the area of the Aggtelek National Park. We could now compare it with the results of the modified (Spitznagel 1985) reguriated pellet analysis (Figs 1. and 2.). The results of the faecal and pellet analyses, which have been carried out simultaneously, seem to be very similar. They show slight differences in only few cases and these minor differences are far from showing any real tendencies. This method thus makes it possible to apply the faecal analysis alone when assessing the diet of dippers and to compare the results of the reguriated pellet and faecal analysis within smaller regions.

Diet of dipper

The reguriated pellet an faecal analyses proved the presence of 9 invertebrate animals (taxa), as well as small stones, plant matters and feathers. The feathers and the various plant tissues (mainly taken from mosses) are likely to have been mixed with the samples while collecting them. The small stones (perhaps stones present in the gizzard of the birds) may also have got mingled with the samples the same way.

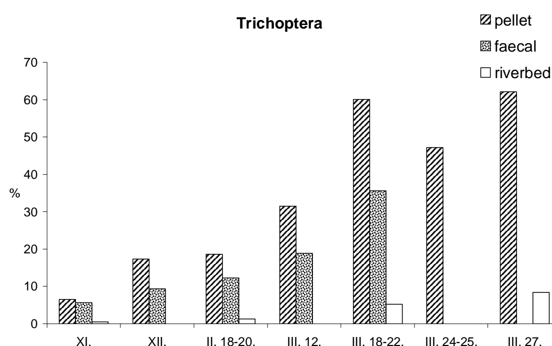
The data of the reguriated pellet analysis supports our earlier observations (Rékási 1985, Horváth and Andrikovics 1991), namely that *Gammarus* is predominant (over 80 %) in the winter diet of dippers in Hungary. Such high values have only been reported from the area of Angara (Pastuchov 1961). Having examined more than 6000 items of food, the author reported a proportion of 93,8 %. Although in Germany the ratio was alleged to be below 40 % (Jost 1975/B, Spitznagel 1985), there were some pellets with a dominance of more than 90 % (Spitznagel 1985). In Wales 5,4 % of the number prey and 3,2 % of the mass pray *Gammarus* made up. Nevertheless, it was not found in the dipper pellets in Spain (Santamarina 1990) and Morocco (Tyler and Ormerod 1991).



1. Fig. The ratio of *Gammarus* on the diet of Dippers.

In Hungary the proportion of *Gammarus* in the dippers' diets sometimes starts dropping even in February, at the beginning of the nestling period. By the time the nestlings have hatched, it does not even reach 50 %. In the meantime, the ratio of Trichoptera larvae is gradually increasing, sometimes it may even exceed 60 %. Similar data was observed and obtained in Germany (Jost 1975b, Spitznagel 1985), in Spain (Santamarina 1990) and Scotland (Vickery 1988). In Wales (Ormerod and Tyler 1986), Ireland (Tyler and Ormerod 1991) and Norway (Ormerod, Efteland and Gabrielsen 1987) the ratio of Trichoptera larvae in the reguriated pellets of dippers reached as far as 30 %.

Still, it is noteworthy that on the area of the Carpathian Basin (not on the present territory of Hungary) a proportion of 10 % of *Gammarus* and about 50 % of *Trichoptera* was recorded in 1906 (Vollnhofer 1906). We should also emphasise the fact that the data was obtained by analysing the stomach-content of shot and dissected individuals from more than 1000 metres above sea level.



2. Fig. The ratio of caddisflies on the diet of Dippers.

Studying the benthic fauna of the stream it became evident that the reason for the previously recorded predominance of *Gammarus* (Rékási 1985, Horváth and Andrikovics 1991) during the winter period lies in the composition of available food resources. In winter mostly *Gammarus* and *Sadleriana pannonica* were abundant in the stream as well as smaller numbers of caddisfly larvae without shells. That is the reason why dippers tended to feed on *Gammarius*.

At the end of winter, however, the ratio of caddisflies (as a potential food resource) rose and exceeded 8 % (Table 2). The increase is even more significant if we exclude *Sadleriana pannonica*, towards which dippers do not show much preference and which they rarely feed on. In this case the proportion of caddisflies (as potential food resources) reaches up to 10 % (30th March: 14 %, 6th April: 11 %). Dippers seem to exploit it since the ratio of Trichoptera larvae was gradually increasing in its food. These are primarily the so called caddisflies without shells (*Hydropsyche*, *Rhyacophila*), which are abundant here (Nógrádi – Uherkovics, 1988). Just before the nestlings hatch, the proportion of caddisflies in the dippers' diet swells even exceeding that of the Trichoptera available in the benthic fauna (Table 1 and 2). This even supports the idea that dippers tend to show preferences for certain foods within the available

prey, namely they seem to prefer caddisfly larvae. An evident explanation for this is the difference in the energy content between the two prey types. While *Gammarus* provides the birds with 3900 cal/g energy, Trichoptera larvae mean 6000 cal/g. This might even be supported by the observation that when the nestlings hatch, the number of caddisflies (mainly *Halesus*) start to swell (Table 1).

It is conspicuous how little role *Sadleriana pannonica* plays in the diet of the dippers (Tables 3 and 4), though they are present in great numbers all over the year (Table 2). It is the size of the shell and the great percent of indigestible parts (more than 46 %) that accounts for the dippers' lack of preference for these prey (Horváth and Andrikovics 1991).

On the basis of the above mentioned data (and our observations using binoculars) we could say that like in Wales and Norway (Omerod 1985a, Ormerod, Efteland and Gabrielsen 1987) dippers also feed their nestlings with caddisflies in Hungary. Owing to some failed experiments, however, we did not succeed in proving this directly either by the method of ligature nor by an artificial nestling. My theory can also be supported by the fact that *Gammarus* was not proved from the faeces of the nestlings.

The changes in the feeding strategies of the dippers have already been mentioned by researchers (Ormerod, Efteland and Gabrielsen 1987). Still the details of the process and the transition have not been thoroughly examined. Therefore studies involving such questions may be relevant for further research. They could help us reveal the correlation between water pollution, the transformations and changes in the invertebrate aquatic wildlife and the disappearance of dippers.

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TISCIA 33, 67-77

INITIAL STEPS IN THE REGENERATION OF A FLOODPLAIN MEADOW AFTER A DECADE OF DOMINANCE OF AN INVASIVE TRANSFORMER SHRUB, *AMORPHA FRUTICOSA* L.

Cs. Szigetvári

Szigetvári, Cs. (2002): Initial steps in the regeneration of a floodplain meadow after a decade of dominance of an invasive transformer shrub, *Amorpha fruticosa L. — Tiscia 33, 67-77.*

Abstract: The flora and vegetation of two floodplain meadow sites invaded by the nonindigenous shrub, *Amorpha fruticosa* L. were compared. One of the sites (site [A]) was previously completely invaded by the species, which had transformed the habitat into a monodominant thicket for more than ten years before it was cleared in 2000. The other site (site [B]) was more or less continuously mowed therefore *Amorpha* only reached here 50 % cover on average and large patches of the grassland community remained intact. The two sites are managed similarly, by mowing or stalk-cutting twice a year, which prevents seed set and sprouting of *Amorpha*. The flora of the sites was censused and vegetation was sampled by estimating species cover in 4×4 m quadrates, 30 sampling units in each site. Our floral investigations detected 107 species in site [A] and 115 in site [B]. The species pool of the two sites was similar, and almost all species characteristic to the target vegetation (represented by site [B]) were present in the recovering area, site [A]. The vegetation investigations revealed that species richness per quadrates was significantly lower in site [A], which was mainly due to the rareness of subordinate species. The cover of *Amorpha* did not correlate with species richness within the sites. Fifteen species had significantly higher cover in site [B] while only two species, including *Amorpha* had higher cover in site [A]. PCoA ordination of the quadrates based on species presence information markedly discriminated the two sites, while NMDS ordination based on species cover data did not segregate them to much extent. The points representing site [A] were more scattered in the ordination space. The orientation of the quadrates in the ordination scattergram was not associated with the cover of *Amorpha* when within site variability was investigated. The variation in species combinations and dominance relationships was associated mainly with the opposite behaviour of two dominants: *Alopecurus pratensis* and *Bidens tripartita*. Interspecific correlations showed that the invasive species has almost as mani positive as negative correlations in both sites at the scale of investigations. The most remarkable is the strong negative correlation between *Amorpha* and the dominant *Alopecurus pratensis* in site [A].
We concluded that although only weak evidence supports that *Amorpha* has considerable present impact on the within-site vegetation variation, the lower species richness, the absence or low cover of subordinate meadow species and the patchiness of vegetation in site [A] can be attributed to the past dominance of the invasive species. The species pool of the site [A] area is promisingly rich for future rehabilitation. The dominants of the target vegetation are already quite frequent which assures regeneration, while most of the subordinate species are rare and scattered. The vegetation seems to have low resistance to colonization which might enforce recolonization of both natural species and invasion of aliens. Only systematic management of the area assures successful regeneration.

Keywords: plant invasion, community rehabilitation, Tisza river, inundation area, Alopecurus pratensis, Bidens tripartita

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Introduction

False indigo, *Amorpha fruticosa*, a leguminous shrub of North-American origin is considered as one of the most problematic invasive plant species in the inundation area of the Tisza river. The species has effectively utilised the ecological corridor properties of the river and by this timeit is abundant all over the Tisza flood area (Bartha and Mátyás 1995). Although *Amorpha* is able to invade different seminatural riverside communities with more or less success (Zavagno and D'Auria 2001), the most endangered target communities are probably the floodplain pastures and meadows. These species rich seminatural communities evolved under the traditional human disturbance regime. As the traditional extensive management practices, grazing and mowing are abandoned (as has been happening near the Tisza in the recent decades, cf. Tóth 2001) they are reoccupied by gallery forests as the course of a natural succession. The presence of *Amorpha* significantly alters the above outlined dynamics: in the absence of grazing or mowing the invasive species is able to immediately colonise the meadow community, and with its rapid growth it transforms the habitat into a homogeneous thicket within 5-6 years (Kóra 2002). Under the closed canopy of *Amorpha* most of the meadow species cannot survive, and a very poor community of opportunistic species develops (Zavagno and D'Auria 2001, Kóra 2002). The homogeneous *Amorpha*-thicket represents a long-lasting successional state in the meadow-forest sere. The development of the gallery forest state is supposed to eventually happen (Zavagno and D'Auria 2001), but it is only supported by anecdotal information, and might only happen after 25-30 years when the *Amorpha* stand grows old and collapses probably due to self-generating processes (Tóth 2001, Kóra 2002).

The floodplain meadows have high natural conservation value, therefore it is desirable to prevent their invasion by *Amorpha fruticosa* and reverse the process if it is possible. The aim of the present study is to evaluate the first observations of a recent rehabilitation experiment, in which a cca. 10-years-old homogeneous *Amorpha*-thicket developed in an abandoned floodplain meadow was cleared one and a half years before the observations. As a control area, we used a neighbouring meadow which has been mown more or less regularly, and abundance of the invasive species has been kept at a relatively low level.

The questions of the study are the following: 1) How similar is the species pool of the two sites; are the species characteristic of the floodplain meadow present in the recovering area? 2) What are the characteristics of the dominance relations of the vegetation in the two areas; which species' abundance values are different between the two sites? 3) Are plant community characteristics different in the two sites? 4) Is the dominance of *Amorpha* related to the variation of the vegetation between and within the two sites?

Materials and methods

Study species

Amorpha fruticosa L. (Fabaceae) is a shrub of 3 to 4 m maximum height (Gencsi and Vancsura 1992). It originates from the South-eastern parts of North-America (Wilbur 1975, Zavagno and D'Auria 2001), where it is a pioneer of riverbanks and wetlands. The species was introduced into Hungary before 1907 (Priszter 1997). *Amorpha* was spread by the forestry mainly after the 1st World War all over the country (Szentesi 1999). Its spontaneous invasion has been most intensive in the floodplains of the Great Hungarian Plain (Bartha and Mátyás 1995).

Amorpha fruticosa propagates sexually by one-seeded indehiscent pods. Plants in Hungary usually blossom from June to July. Pod production of an average individual in a homogeneous stand is estimated ca. 12,000 a year (Kóra 2002). Seed loss due to seed predation is low (Szentesi 1999). Most effective germination is from shallow (> 1 cm) soil depths and at high (20/30°C) temperatures (Zasada and Martineau n.d.). Anecdotal evidence suggests that dry soil conditions prevent successful germination. Seeds have a limited viability of 3-5 years under laboratory conditions (Zasada and Martineau n.d.). The buoyant pods are spread by water, but birds and small mammals might also play some role in the seed dispersal.

Vegetative reproduction is possible by sprouting, which can be very intensive when the stems are frozen or cut (Gencsi and Vancsura 1992, Kóra 2002). After cutting or mowing *Amorpha* quickly regenerates by sprouting. Stems can also root at the nodes (Kóra 2002).

Amorpha is a week competitor in forests and is usually excluded by tree species (Magyar 1960), but due to its fast growth, shading, and probably its allelopathyc effects (Elakowich and Wooten 1995, Csiszár, unpublished data) and nitrogen-fixing ability (Wang et al. 1999) it is a superior transformer species in meadows and pastures. *Amorpha fruticosa* is unpalatable for most invertebrates except an introduced seed predator (Szentesi 1999), but ruminants readily eat its leaves and young shoots (Papachristou et al 1999, Tóth and Gadó 2000).

Study site

The study site is situated at 46º50'40" north 19º59'30" east, near the village Tiszaalpár in the Great Hungarian Plain. The site is the part of a cca. 0.5 km^2 meadow-marsh complex about 3 km from the recent riverbed of Tisza. The original riverbed (the Lakitelek-backwater), that was artificially separated in the 1870' s is about 1 km away. Although recently the study area was inundated quite frequently (in the winter of 1999/2000, and in the spring of 2000 and 2001), the period from 1970 to 1999 was flood-free. The area is bordered by *Alnus glutinosa* forests from the East and North, by a marsh (Sulymos-Lake) from the West, and by a cca. 50 m wide marshy channel from the South. The area contains three almost equal-sized meadows that are completely separated by two marshy channels (50 and 200 m wide, respectively) (Fig. 1). The three meadow patches have similar abiotic conditions, but the invasion of *Amorpha* reached different levels. The eastern patch (patch [A] Fig. 1) is not easily accessible and its mowing was stopped in the second half of the 1980's. Than, a homogeneous *Amorpha*-thicket developed there, which reached more than 3 m height by 2000 when it was cleared by stalk-cutter. Since then, the area has been managed by stalk-cutter twice a year to prevent reestablishment of *Amorpha* by sprouts and to mimic the traditional mowing regime (mowing is only possible after some years, after new *Amorpha* sprouts become week enough). In the year of the study *Amorpha* reached here 2 m height and 60-70 % cover (maximal data, i.e. before cutting). The mowing of the central patch (patch [B] Fig 1) was not stopped, but it was not regular except for the last two years, therefore *Amorpha* was able to permanently establish with 30-40 % cover and 1m height (maximal data, i.e. before mowing). Both [A] and [B] meadows are bordered by a thick and continuous line of adult *Amorpha* shrubs at the edge of the marshy channels, where management is not effective. This shrubby border is a source of a continuous and heavy propagule pressure. The western site, which is the most easily accessible was managed continuously and *Amorpha* forms only small patches. Our investigations were conducted in the eastern [A] and the central [B] meadows. In the present paper site [B] serves as the control area since it represents a reasonable target state of the vegetation that might be reached by systematic management.

The vegetation of the meadows is not homogeneous. Although the variation of the surface is less then 1 m (81.0-81.5 m above sea level), the area slightly slopes towards the east and the marshy channels. As far as it is discernible in the presence of the invader, the following vegetation types can be distinguished *a priori* on the basis of the dominant species: most of the two sites is covered with *Alopecurus pratensis* dominated grassland community. Abundance of *Amorpha* is high in this vegetation type. A considerable part of the *Alopecurus* grassland was burnt in 2001 in site [A]. This patch is abundant in opportunistic coloniser species. In the lower elevations *Carex acuta* dominated dense sedge communities prevail, which seem to be quite resistant to *Amorpha* invasion. In a single invader-free wet patch in site [A] *Scirpus lacustris* ssp. *lacustris* prevails. In the edges of the sites near the marshy channels *Amorpha* predominates with some marsh species and alluvial climbers like *Calystegia sepium, Solanum dulcamara*, and the nonindigenous *Echinocystis lobata*.

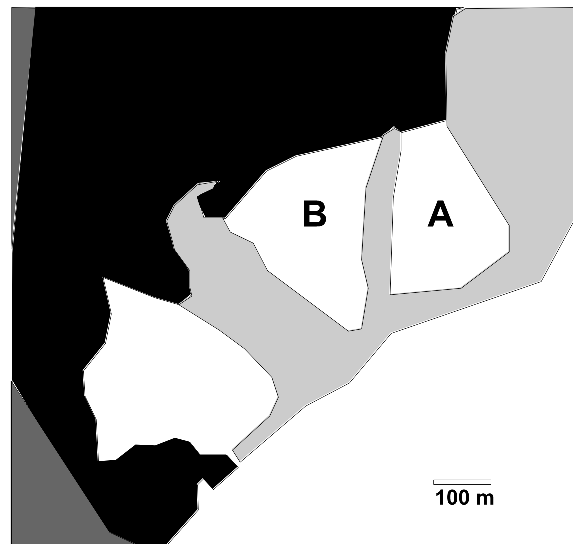

Fig. 1. The landscape location and the surrounding habitats of the two investigated sites [A] and [B]. Black: forest vegetation, dark grey: anthropogenic habitats, light grey: marsh vegetation, white: meadow vegetation.

Sampling

The flora of the two sites was investigated in 2002 from early spring to late autumn. It is more than probable that some of the rare species were not found (especially in site [A] where the dense and high *Amorpha* shrub made it more difficult to find all species), but the intensity of the field work assures reasonable representativity.

The vegetation of the sites was sampled in 30-30 quadrates of 4×4 m area each. Percent cover of each plant species was estimated in the quadrates. In the arrangement of the sampling units our aim was to

representatively sample the meadow vegetation types which are affected by *Amorpha* invasion and are common in both sites. Therefore the *Carex acuta* dominated patches were not sampled in neither sites, nor the burnt areas or the *Scirpus lacustris* dominated patches in site [A]. Except for the omitted types the samples were blocked in 6 lines uniformly arranged in the site. Within the lines the 5-5 quadrates were placed randomly.

Data analysis

The species richness of the two sites was compared first on the basis of the total flora and second on the basis of the species pools of the quadrates. Species richness per quadrate was compared with the nonparametric Wilcoxon-Mann-Whitney test. The abundance of each population was also compared with the above mentioned test (because of the great number of simultaneous tests we used a significance level of $\alpha = 0.01$ in this case). We also computed the correlation between *Amorpha* cover and plant species richness, separately for the two sites (N = 30). The applied statistics was the non-parametric Goodman-Kruskal's gamma correlation.

For the evaluation of the variance in the vegetation we performed two kinds of ordination on the basis of the data of the 4×4 m quadrates. In the first type of ordination we ignored cover data and only the similarity of species combinations was taken into account. The similarity matrix was computed using Jaccard's index (JAC = a/[a+b+c], the notation refers to the cells of the 2×2 contingency table), the ordination method was the principal coordinates analysis (PCoA). In the second type species dominance relations were also taken into account. The similarity matrix in this case was the correlation matrix based on the species' percent cover values, the ordination method was the non-metric multidimensional scaling (NMDS), because this method is quite robust to nonnormality and nonlinearity in data (Podani 1997). The ordinations were performed for the whole sample. Only those species were taken into account in the ordination that were present at least in 3 quadrates. Because the aim of the study was to assess the relation of the invasive species on the variation of the vegetation, the cover of *Amorpha* was not taken into account in computing the NMDS ordination in order to avoid circularity (the results of the PCoA are not affected, because *Amorpha* was present in all quadrates). The performance of the PCoA was evaluated on the basis of the explained percent variance of the first three axes. This value was rather low (less than 30%), but preliminary analysis showed that sequential omission of the rarest species results in gradual increase of explained variance without considerably affecting the final configuration of the quadrates in the ordination space. Therefore we consider the results of the PCoA acceptable. The performance of the NMDS analysis was evaluated on the basis of the Goodman-s stress value, which was sufficiently low (0.13) in our case. The ordination analyses were performed by the Syn-Tax 5.0 program packet (Podani 1993).

The coordinates of the quadrates on the first two axes of the ordination scattergrams were correlated with the cover of the species that were present at least in 1/5 of the sampling units (e.g. 12 quadrates). The applied statistics was Goodman-Kruskal's gamma, because this type of nonparametric correlation performs well in cases when a lot of identical rank values are present in the sample (Siegel and Castellan 1988). Because of the great number of simultaneous test we used a significance level of $\alpha = 0.005$ in this case.

Results

Altogether 131 species were found on the two sites during the year: 107 species in site [A] and 115 in site [B] (Appendix 1). The difference of the species pools results from mainly occasional species represented by one or a few individuals, with the only exception of *Cichorium intybus* and *Cynodon dactylon* that was quite common in site [B] but absent from site [A]. In the sixty 4×4 m quadrates taken in June 92 species were detected: 67 species in site [A] and 78 species in site [B]. This means an approximate 6:7 ratio. The most common species (*Amorpha fruticosa, Alopecurus pratensis, Bidens tipartita, Potentilla reptans*) are the same in both places, but many of them appear in considerably different frequencies (Table 1). The species number per quadrate was significantly ($p << 0.001$) higher in site [B] than in site [A] (medians 24 and 15, interquartile range 6 and 3, respectively). When only species with frequency of 20% or higher are considered, the species pool ratio is about 3:4 indicating that subordinate species are more common in site [A]. The most valuable species from a nature conservation view, *Leucanthemella serotina* and *Ophioglossum vulgatum*, are present in both places. The former is somewhat more abundant in site [A] and the latter is much more common in site [B] (the difference was not detectable by the 4×4 m quadrates because by the time of sampling the aboveground parts of *Ophioglossum* had mainly disappeared while *Leucanthemella* was just beginning to sprout).

The cover of *Amorpha* did not correlate significantly with the species richness of the

Table 1. The most frequent (fr > 4) species in the 4×4 m quadrates in the two sites [A] and [B]. Species are sorted by descending frequency.

A

Code	Species	Frequency
AMOFRU	*Amorpha fruticosa*	30
ALOPRA	*Alopecurus pratensis*	28
POTREP	*Potentilla reptans*	28
BIDTRI	*Bidens tripartita*	25
LYSVUL	*Lysimachia vulgaris*	21
CARPRA	*Carex praecox*	20
FRAPEN	*Fraxinus pennsylvanica (juv.)*	20
CARHIR	*Carex hirta*	19
LACSER	*Lactuca serriola*	18
GLEHED	*Glechoma hederacea*	17
VALLOC	*Valerianella locusta*	16
CARMEL	*Carex melanostachya*	15
VICANG	*Vicia angustifolia*	13
IRIPSE	*Iris pseudacorus*	11
POATRI	*Poa trivialis*	11
RANREP	*Ranunculus repens*	11
LYTVIR	*Lythrum virgatum*	10
GALAPA	*Galium aparine*	9
VICCRA	*Vicia cracca*	9
ELYREP	*Elymus repens*	8
VICHIR	*Vicia hirsuta*	8
CALSEP	*Calystegia sepium*	7
VIOPUM	*Viola pumila*	7
ACENEG	*Acer negundo (juv.)*	6
ATRPRO	*Atriplex prostrata*	6
CIRARV	*Cirsium arvense*	6
SOLDUL	*Solanum dulcamara*	6
XANITA	*Xanthium italicum*	6
LYCEXT	*Lycopus sp.*	5
SONASP	*Sonchus asper*	5
VICTET	*Vicia tetrasperma*	5

B

Code	Species	Frequency
ALOPRA	*Alopecurus pratensis*	30
AMOFRU	*Amorpha fruticosa*	30
BIDTRI	*Bidens tripartita*	30
POTREP	*Potentilla reptans*	30
CARHIR	*Carex hirta*	29
CARPRA	*Carex praecox*	29
GLEHED	*Glechoma hederacea*	25
LYSVUL	*Lysimachia vulgaris*	24
CONCAN	*Conyza canadensis*	23
POTANS	*Potentilla anserina*	23
CARMEL	*Carex melanostachya*	22
PLALAN	*Plantago lanceolata*	22
DAUCAR	*Daucus carota*	21
TRIPRA	*Trifolium pratense*	21
VICANG	*Vicia angustifolia*	21
ELYREP	*Elymus repens*	18
VICCRA	*Vicia cracca*	16
JUNCOM	*Juncus compressus*	15
TRIDUB	*Trifolium dubium*	14
CERDUB	*Cerastium dubium*	13
RANREP	*Ranunculus repens*	13
LACSER	*Lactuca serriola*	12
IRIPSE	*Iris pseudacorus*	10
LYCFLO	*Lychnis flos-cuculi*	10
LYCEXT	*Lycopus sp.*	10
SYMOFF	*Symphytum officinale*	10
VICHIR	*Vicia hirsuta*	10
AMBART	*Ambrosia artemisiifolia*	9
ASCSYR	*Asclepias syriaca*	9
CREBIE	*Crepis biennis*	9
PLAMAJ	*Plantago major*	9
SONASP	*Sonchus asper*	9
VALLOC	*Valerianella locusta*	9
CERFON	*Cerastium fontanum*	8
FRAPEN	*Fraxinus pennsylvanica (juv.)*	8
INUBRI	*Inula britannica*	8
POATRI	*Poa trivialis*	8
CENJAC	*Centaurea jacea*	7
CICINT	*Cichorium intybus*	6
CIRARV	*Cirsium arvense*	5
CIRVUL	*Cirsium vulgare*	5
GALAPA	*Galium aparine*	5
LYTVIR	*Lythrum virgatum*	5
PRUVUL	*Prunella vulgaris*	5
VERCHA	*Veronica chamaedrys*	5

quadrates in neither sites. It should be mentioned, however, that the correlation was positive (gamma = 0.277) and marginally insignificant ($p = 0.052$) in site [A] (In site [B]: gamma = -0.114, $p = 0.405$).

When comparing the cover values of the species in the quadrates the difference is significant ($p < 0.01$) in 17 cases (Table 2). Only two species have higher dominance in site [A]: *Amorpha fruticosa* itself and another alien species *Fraxinus pennsylvanica*, which is represented only by juvenile individuals. On the contrary, 15 species have significantly higher cover in site [B] (Table 2). Most of them are subordinate generalists of mesophylous meadows but some (*Carex hirta, C. praecox, C.*

melanostachya, Potentilla reptans, Elymus repens) are codominants of the community with considerable dominance in site [B]. It is also worth mentioning that there is no significant difference in the cover of opportunistic species of disturbed inundation areas, like *Xanthium italicum, Calystegia sepium, Echinocystis lobata, Bidens tripartita* (although the latter has much higher maximal cover percentages in site [B], and a wider range of values, which unfavourably affects the power of the Wilcoxon-Mann-Whitney test). The cover of the dominant grass species *Alopecurus pratensis* tends to be somewhat higher in site [B], but the difference is not significant ($p = 0.032$).

The PCoA ordination of the quadrates based on the Jaccard similarity index markedly separated the two sites along the 1st axis (Fig. 1). It is also noteworthy that the points belonging to site [A] are more scattered, which indicates that the variation of the species combinations (despite the lower species number per quadrate) is greater here than in site [B], e.g. the vegetation is less coordinated.

Table 2. The significant ($p < 0.01$) results of the Wilcoxon-Mann-Whitney test on the cover of the species in the two sites. For the abbrevations of the species see Table 1.

Species	rank sum		U	p-value
	(A)	(B)		
POTANS	576	1440	111	1.268E-07
POTREP	584	1432	119	2.299E-07
CONCAN	617	1399	152	2.364E-06
PLALAN	630	1386	165	5.607E-06
AMOFRU	1288	728	167	6.386E-06
CARPRA	632.5	1383.5	167.5	6.596E-06
DAUCAR	645	1371	180	1.462E-05
TRIPRA	645	1371	180	1.462E-05
CARHIR	666	1350	201	5.221E-05
JUNCOM	702	1314	237	0.0003849
TRIDUB	731	1285	266	0.0016258
VICANG	743.5	1272.5	278.5	0.0028895
CARMEL	747.5	1268.5	282.5	0.0034532
CERDUB	765	1251	300	0.0072872
VICCRA	768	1248	303	0.0082382
FRAPEN	1149.5	866.5	305.5	0.009114
ELYREP	771.5	1244.5	306.5	0.0094869

The results of correlation analysis between the PCoA coordinates and the species' cover (Table 3) are qualitatively the same in the case of the 1st axis as that of the Wilcoxon-Mann-Whitney test on the species cover values (c.f. Table 2). This means that the discrimination of the quadrates of the two sites is mainly based on the numerous species common in site [B] but rare or absent in site [A]. The only species correlating with the 1st axis that are common in both places are *Amorpha fruticosa, Potentilla reptans, Carex hirta* and *C. melanostachya*. The species correlating with the 2nd axis (Table 3) represent the variability within the sites. Of these the two most common and dominant ones, *Alopecurus*

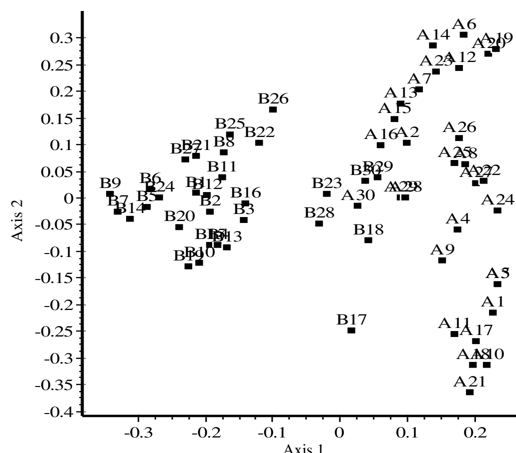

Fig. 2. The scattergram of the PCoA ordination of the 4×4 m quadrats based on Jaccard's similarity index.

Table 3. Goodman-Kruskal's gamma correlations ($p < 0.005$) between the species cover values and the PCoA axes (see Fig. 2). For the abbrevations of the species see Table 1.

PCoA axis coordinates and species	gamma	p-value
axis1 & AMOFRU	0.469	0.00000
axis1 & FRAPEN	0.503	0.00000
axis1 & ACENEG	0.505	0.00290
axis1 & POTANS	-0.785	0.00000
axis1 & TRIPRA	-0.832	0.00000
axis1 & DAUCAR	-0.764	0.00000
axis1 & PLALAN	-0.696	0.00000
axis1 & CERDUB	-0.801	0.00000
axis1 & JUNCOM	-0.666	0.00000
axis1 & POTREP	-0.472	0.00000
axis1 & CONCAN	-0.514	0.00000
axis1 & TRIDUB	-0.581	0.00000
axis1 & CARPRA	-0.409	0.00001
axis1 & SYMOFF	-0.508	0.00017
axis1 & CARMEL	-0.342	0.00028
axis1 & ELYREP	-0.362	0.00066
axis1 & LYCEXT	-0.392	0.00175
axis1 & CARHIR	-0.254	0.00485
axis2 & POATRI	0.624	0.00000
axis2 & BIDTRI	0.471	0.00000
axis2 & GALAPA	0.624	0.00000
axis2 & GLEHED	0.381	0.00007
axis2 & VALLOC	0.407	0.00014
axis2 & LACSER	0.393	0.00017
axis2 & CARMEL	0.283	0.00269
axis2 & ALOPRA	-0.516	0.00000
axis2 & PLALAN	-0.396	0.00060

pratensis and *Bidens tripartita* together with *Carex melanostachya* show correlation in opposite directions (Table 3). As the variation of cover of

both *Alopecurus* and *Bidens* is less is site [B] than in site [A] this result is consistent with the fact that the quadrates of site [B] are less scattered among the 2nd axis of the PCoA scattergram (cf. Fig. 2).

The NMDS ordination of the quadrates (Fig. 3) based on the correlation matrix did not as markedly separate the quadrates of the two sites as the PCoA analysis (Fig. 2). Points belonging to site [B] are mainly concentrated along the diagonal in the NMDS scattergram (Fig. 3). Those belonging to site [A] are more scattered, with a more compact subset in the bottom left quarter.

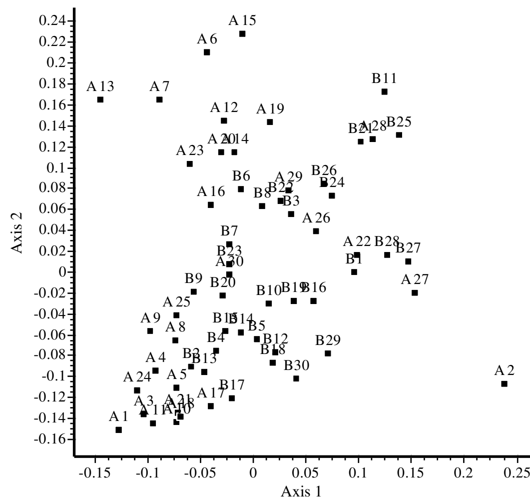

Fig. 3. The scattergram of the NMDS ordination of the 4×4 m quadrates based on the correlation matrix.

Table 4. Goodman-Kruskal's gamma correlations ($p < 0.005$) between the species cover values and the NMDS axes (see Fig. 3.). For the abbreviations of the species see Table 1.

NMDS axis coordinates and species		gamma	p-value
axis1	& POTREP	0.465	0.00000
axis1	& PLALAN	0.429	0.00020
axis1	& DAUCAR	0.405	0.00086
axis1	& POATRI	-0.375	0.00105
axis1	& LYSVUL	-0.282	0.00175
axis2	& BIDTRI	0.513	0.00000
axis2	& POATRI	0.527	0.00000
axis2	& LYSVUL	0.372	0.00004
axis2	& GALAPA	0.427	0.00085
axis2	& GLEHED	0.313	0.00101
axis2	& CARMEL	0.304	0.00124
axis2	& ALOPRA	-0.636	0.00000
axis2	& FRAPEN	-0.325	0.00151

The correlation analysis with the NMDS axes (Table 4) has different results for the 1st axis than in the case of the PCoA, but the results for the 2nd axis are similar (c.f. Table 3). Three of the species correlating with the 1st axis probably represent between-site variability (*Potentilla reptans, Plantago lanceolata, Daucus carota*) but the other two (*Lysimachia vulgaris, Poa trivialis*) do not. The coordinates of the quadrats along the 2nd axis are associated with the opposite behaviour of some dominant species: *Alopecurus pratensis* represents one direction while *Bidens tripartita* and some others, like *Lysimachia vulgaris* and *Carex melanostachya* the other (Table 4), like in the case of the PCoA (c.f. Table 3). The cover of *Amorpha fruticosa* does not correlate significantly with any of the axes (note that the cover of this species was not used in the calculation of the NMDS).

Table 5. Goodman-Kruskal's gamma correlations ($p < 0.05$) of Amorpha fruticosa with the other species in the two sites. For the abbreviations of the species see Table 1.

		gamma	p-value
site (A)			
AMOFRU	& LACSER	0.5404531	0.0003993
AMOFRU	& GALAPA	0.65	0.0005640
AMOFRU	& POTREP	0.4670185	0.0007149
AMOFRU	& VALLOC	0.3648649	0.0198986
AMOFRU	& VICHIR	0.4385965	0.0342645
AMOFRU	& ALOPRA	-0.480315	0.0004754
AMOFRU	& VIOPUM	-0.6219512	0.0027716
AMOFRU	& CIRARV	-0.6231884	0.0062587
AMOFRU	& CALSEP	-0.4819277	0.0203251
AMOFRU	& ACENEG	-0.4893617	0.0298758
AMOFRU	& CIRVUL	-0.6410257	0.0331997
site (B)			
AMOFRU	& VALLOC	0.5532995	0.0039416
AMOFRU	& LYTVIR	0.5593221	0.0240022
AMOFRU	& GALAPA	0.5254237	0.0339808
AMOFRU	& SONASP	0.4010152	0.0344457
AMOFRU	& CENJAC	-0.6923077	0.0007993
AMOFRU	& TRIPRA	-0.4715909	0.0009681
AMOFRU	& PRUVUL	-0.7	0.0040703
AMOFRU	& PLAMAJ	-0.4653465	0.0137923
AMOFRU	& LYSVUL	-0.3090909	0.0242866

Amorpha fruticosa has approximately as many positive as negative correlations with other species (Table 5). There are only two species (*Galium aparine* and *Valerianella locusta*) that correlate with the invasive species in the same way (positively) in both sites. The dominants are not involved in the correlations except the strong negative association with *Alopecurus pratensis* in site [A] (Table 5).

Discussion

Species pool of the two sites

The species pool of the two sites can be considered essentially identical. The difference is partly due to the fact that in a small fraction of site

[A] the abiotic conditions are different: in a wet patch species like *Scirpus lacustris* ssp. *lacustris*, *Bolboschoenus maritimus, Glyceria maxima* prevail, and in the burnt area some early coloniser species like *Myagrum perfoliatum, Chenopodium strictum, Echinochloa crus-galli* or *Fumaria vaillanti* appear. On the other hand, some generalist meadow species, like *Cichorium intybus, Leontodon autumnalis, Achillea millefolium, Trifolium repens or Cynodon dactylon* were not found in site [A]. In addition some opportunistic species of drier grasslands, like *Arenaria serpyllifolia, Holosteum umbellatum, Thlaspi arvense* or *Bromus tectorum* were detected only in site [B]. Probably some of the above species might have been found with a more thorough survey, but still their absence in site [A] is indicative. It is reasonable to attribute it to the preceding monodominance of *Amorpha fruticosa*. Nevertheless, the species richness of site [A] almost equals that of the control area, and most species characteristic to the potential (target) vegetation are already present, including those of high conservation value. The cause of the relative richness of site [A] is not clear: some species might survive under the closed canopy of the invader (e.g. *Ophyoglossum vulgatum*, which develops in the soil for years must have been present before *Amorpha* was cleared from the area). The propagules of other species might have been transported by the recent years' frequent floods, but other dispersal mechanisms might have also been important (e.g. *Cirsium brachycephalum*, an anemochorous species, which was represented by one flowering specimen in both areas has large stands southwards some hundreds of metres away, and was probably transported by wind). It is not clear which was the dominant mechanism. Previous studies by Kóra reported only a few opportunistic species living under complete *Amorpha* cover (e.g.: *Galium aparine, Urtica dioica, Calystegia sepium, Humulus lupulus*) (Kóra 2002). However, gaps of a few square metres where the canopy of the invader was not closed could serve as refuges for many species. Similarly, the coenotone zones between the *Alopecurus* grassland and the invasion-resistant *Carex acuta* sedge meadow could also preserve some species (e.g. *Leucanthemella serotina* was found mainly in such situations in site [A]).

Vegetation of the two sites

The vegetation of the two sites is rather different, when only compositional data are assumed (PCoA results, Fig. 2), but they are much more similar when species dominance data are taken into account (NMDS results, Fig. 3). Apart from the invasive species, the characteristics of the *Alopecurus pratensis*-meadow typical of floodplains can be identified in both sites, although the vegetation of site [A] is more patchy and inhomogeneous (see Figs 2 and 3) and species-poor on the local (quadrate) scale. The *Alopecurus pratensis* dominated meadow community shows transition either towards the more humid sedge meadows or towards the drier grassland types, forming a zonation complex, as detailed by Bodrogközy (1962, 1982, 1990). In our case, distinct appearance of zones was not detected: in site [A] the vegetation is still not well developed, while in site [B] species of humid (e.g. *Carex melanostachya, Eleocharis palustris*) or dry (e.g. *Carex praecox, Convolvulus arvensis*) habitats coexist from the scales of a few centimeters to several meters (Szigetvári, unpublished data). In our case another transition seems to be more important in the variation of the vegetation, which most characteristically manifests in the opposite behaviour of three dominant species: *Alopecurus pratensis, Bidens tripartita* and *Carex melanostachya*. The variation of cover of these species can be related to the variation of the species combinations (see the PCoA results: Fig. 2 and Table 3) and to the species dominance relations (see the NMDS results: Fig. 3 and Table 4). Their role is probably different: *Alopecurus* and *Carex melanostachya* are the dominant competitor species of the community, while *Bidens* is a colonizer of open surfaces with good competitive abilities. In earlier long-term studies Bodrogközy (1979) has shown that there is a peculiar relationship between these species: after years of permanent floods the cover of *Carex melanostachya* increases to the expense of *Alopecurus*. In flood-free years *Alopecurus* regenerates, but in the transitional period opportunistic species the propagules of which was previously spread by the flood increase their dominance (Bodrogközy 1979). This scenario is probably applicable to our case, with some remarks (see below).

Probable impact of the invader on the vegetation

The impact of the invader can be evaluated in two aspects: first, the relationships of the invader on the within-site variation can be investigated. Formally, we can draw conclusions from a vegetation "snapshot", but such result gained from simple observations are usually doubtful (Lepš 1990). Satisfactory conclusions can only be drawn from long-term studies (Blossey 1999) or planned experiments (Morrison 1997). Second, the relationships of the invader with the between-site

variation can be used. The costs of the latter approach are that we should a priori hypothesise that (1) the characteristics of the two sites are essentially identical, and their difference is (2) due to the impact of *Amorpha fruticosa* in site [A] in the preceding period. The first hypothesis can be supported by the similar species pools of the sites. The second hypothesis cannot be directly supported by the present study, we should rely on anecdotal information and literature data concerning the properties of the species.

The present study provides only week support to the hypothesis that *Amorpha* has any impact on the within site vegetation variation. The cover of the species does not correlate with the species richness per quadrate in either sites (or the "nearly significant" correlation is positive, like in site [A]). Also in the ordination analyses, the cover of *Amorpha* does not show any relationships with the variation of the species combinations or the dominance relationships within the sites. Some conclusions can be drawn from the analysis of interspecific correlations, which show that some species are positively, some are negatively associated with the invader. Only one of them, the negative correlation in site [A] between *Amorpha* and *Alopecurus pratensis* (the dominant of the target vegetation) suggests that the invasive species has adverse effects on the vegetation.

If we compare the two sites, the differences: lower species richness, absence or low abundance of many subordinate meadow species, greater patchiness (as manifested in the more scattered points in the ordination scattergram), can be interpreted as the after-effect of the permanent dominance of *Amorpha fruticosa*. Aware of the relative competitive abilities and allelopathyc capacity of the invader it is reasonable to hypothesise that these assumptions are correct. Two years since the *Amorpha* thicket was cleared and the systematic management has started most of the species have not been able to regenerate and spread, and there are still open surfaces to colonise in site [A]. Perhaps this state is similar in some aspect to that after the permanent floods. The lasting inundation weakens the meadow vegetation, and before the regeneration of *Alopecurus pratensis* is completed opportunistic coloniser species, like *Bidens tripartita* increase their cover (Bodrogközy 1979). It is also indicative, that the abundance of *Fraxinus pennsylvanica* seedlings is significantly higher in site [A] than in site [B] which — aware of the fact that there are a lot of ash trees near both sites, therefore propagule limitation perhaps does not play any role — is probably due to the many gaps ready to colonisation. The success of *Fraxinus pennsylvanica* in site [A] refers to reduced invasion resistance, which can also be interpreted as the after-effect of *Amorpha* invasion.

Prospects of regeneration

As the present investigations revealed, the species pool for the regeneration is already present in site [A]. Although the cover of *Amorpha* is still very high, it can be reduced to a reasonable level by systematic cutting (Papanastasis *et a.l* 1998). At the present management regime the invasive species seems not directly hinder regeneration except for the negative correlation with *Alopecurus pratensis*. Despite this fact, *Alopecurus* seems to quickly regenerate, and the most important codominant species are also abundant. It is mainly the subordinate species that are much rarer and have a more patchy distribution than in the control site. Their regeneration and dispersion in the whole site probably takes more time. At the present state the recovering habitat seems to be more susceptible to invasions after floods either by the buoyant seeds of *Amorpha* (or another alien species) or natural colonisers. This susceptibility might promote the regeneration through the colonisation of indigenous species only if alien species are managed by proper and systematic control methods. Therefore it is essential to continue mowing twice a year or pursue continuous grazing to prevent *Amorpha's* seed set and regeneration from sprouts.

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Appendix 1. Species list for the two sites [A] and [B]. Species presence is marked by "+"sign.

Species	(A)	(B)
Acer negundo	+	+
Achillea millefolium		+
Agrostis stolonifera	+	+
Alisma plantago-aquatica	+	
Alopecurus pratensis	+	+
Althaea officinalis	+	+
Ambrosia artemisiifolia	+	+
Amorpha fruticosa	+	+
Anthemis arvensis		+
Apera spica-venti	+	+
Arabidopsis thaliana	+	+
Arenaria serpyllifolia		+
Aristolochia clematitis	+	+
Asclepias syriaca	+	+
Asparagus officinalis	+	+
Atriplex prostrata	+	+
Bidens tripartita	+	+
Bolboshoenus maritimus	+	+
Bromus sterilis		+
Bromus tectorum	+	+
Calystegia sepium	+	+
Capsella bursa-pastoris		+
Carduus nutans	+	
Carex acuta	+	+
Carex hirta	+	+

Species	(A)	(B)
Carex melanostachya	+	+
Carex praecox	+	+
Carex riparia	+	+
Centaurea jacea	+	+
Cerastium dubium		+
Cerastium fontanum	+	+
Chenopodium album	+	+
Chenopodium strictum	+	
Cichorium intybus		+
Cirsium arvense	+	+
Cirsium brachycephalum	+	+
Cirsium vulgare	+	+
Convolvulus arvensis		+
Conyza canadensis	+	+
Crepis biennis	+	+
Crepis tectorum	+	
Cynodon dactylon		+
Daucus carota	+	+
Descuriania sophia	+	+
Digitaria sanguinalis	+	+
Echinochloa crus-galli	+	
Echynocystis lobata	+	+
Eleocharis palustris	+	+
Elymus repens	+	+
Epilobium tetragonum		+

Appendix 1. (continued)

Species	(A)	(B)
Equisetum palustre	+	+
Erophila verna ssp. majuscula	+	+
Euphorbia lucida	+	
Fraxinus pennsylvanica	+	+
Fumaria vaillanti	+	
Galium aparine	+	+
Galium palustre	+	+
Glechoma hederacea	+	+
Glyceria maxima	+	
Glycyrrhiza echinata	+	+
Gratiola officinalis	+	+
Holosteum umbellatum		+
Inula britannica	+	+
Iris pseudacorus	+	+
Juncus compressus	+	+
Lactuca saligna		+
Lactuca serriola	+	+
Lamium purpureum	+	+
Lathyrus tuberosus	+	+
Leontodon autumnalis		+
Leucanthemella serotina	+	+
Lotus glaber	+	+
Lychnis flos-cuculi	+	+
Lycopus sp.	+	+
Lysimachia nummularia		+
Lysimachia vulgaris	+	+
Lythrum salicaria	+	+
Lythrum virgatum	+	+
Medicago lupulina	+	+
Mentha aquatica	+	+
Mentha arvensis x (male sterile)	+	+
Myagrum perfoliatum	+	
Myosotis ramosissima	+	+
Myosotis stricta	+	+
Ophioglossum vulgatum	+	+
Picris hieracioides	+	
Plantago lanceolata	+	+
Plantago major	+	+
Poa pratensis	+	+
Poa tivialis	+	+
Potentilla anserina	+	+

Species	(A)	(B)
Potentilla reptans	+	+
Prunella vulgaris	+	+
Pulicaria dysenterica	+	+
Quercus robur	+	+
Ranunculus repens	+	+
Rhinanthus angustifolius		+
Rorippa amphibia	+	
Rorippa astylis		+
Rorippa palustris	+	+
Rumex crispus	+	+
Scirpus lacustris ssp. lacustris	+	
Scutellaria galericulata		+
Scutellaria hastifolia	+	+
Senecio erucifolius		+
Senecio vernalis		+
Setaria pumila	+	+
Sisymbrium orientale		+
Sium latifolium	+	+
Solanum dulcamara	+	+
Sonchus asper	+	+
Stachys palustris	+	+
Stellaria media	+	+
Stenactis annua	+	+
Symphytum officinale	+	+
Taraxacum officinale	+	+
Thalictrum flavum	+	+
Thalictrum lucidum	+	+
Thlaspi arvense		+
Tragopogon dubius	+	+
Trifolium dubium	+	+
Trifolium pratense	+	+
Trifolium repens		+
Valerianella locusta	+	+
Veronica chamaedrys		+
Vicia angustifolia	+	+
Vicia cracca	+	+
Vicia hirsuta	+	+
Vicia tetrasperma	+	
Viola pumila	+	+
Xanthium italicum	+	+