Journal of Insect Conservation **7:** 207–213, 2003.

Pattern of genetic differentiation in two *Isophya* species (Orthoptera: Tettigonoidea) in north-east Hungary

Katalin Pecsenye[1,*], Edit Vadkerti[2] and Zoltán Varga[1]
*[1]Department of Evolutionary Zoology and Human Biology, University of Debrecen; [2]Department of Zootaxonomy and Synzoology, Institute of Biology, University of Pécs; *Author for correspondence (e-mail: pecskati@tigris.klte.hu)*

Received 22 October 2002; accepted in revised form 24 October 2003

Key words: Enzyme polymorphism, Genetic differentiation, Hungary, *Isophya*

Abstract

Allozyme polymorphism was studied in two populations of *Isophya kraussi* and *Isophya stysi*. Both species are flightless and have low dispersal ability. As a consequence, we expected high level of genetic differentiation among their local populations. Samples were collected in three regions of Hungary. Enzyme polymorphism was investigated at 10 loci (*Aldox*, *Est*, *Got*, α*Gpdh*, *Hk*, *Idh*, *Mdh*, *Me*, *Pgi* and *Pgm*) in both species. High levels of polymorphism were detected in all samples. α*Gpdh* proved to be diagnostic as there were no common alleles in the two species. At four further loci (*Got*, *Hk*, *Mdh* and *Me*), the two species had one common allele together with one or more differentiating alleles. We detected high F_{IT} values implying a high level of genetic variation. The positive F_{IS} values suggested a tendency of heterozygote deficiency in both species. The highly significant overall F_{ST} values indicated clear genetic differentiation among the local populations. Thus our results confirmed the taxonomic status of these two species. The dendrogram constructed on the basis of Nei's genetic distances and the results of the PCA analyses fully confirmed those obtained by *F*-statistics.

Introduction

Survival of natural populations in fragmented landscapes is one of the central problems in conservation biology (Opdam 1988; Settele et al. 1996; Thomas 2000). Habitat fragmentation results in population subdivision and isolation. Consequently, it impacts on the genetic structure of populations resulting in decreasing effective population size and loss of genetic variation (Gilpin 1991; Hanski and Gilpin 1991). Nevertheless, different species may experience the same fragmented habitat in a different way (Rolstad 1991). Thus, surveys of genetic differentiation among local populations within a species or between closely related species became more and more established in conservation studies (Vogler and Desalle 1994; Newton et al. 1999; Morgan-Richards et al. 2000). Monitoring allozyme polymorphism proved to be appropriate to estimate the level of polymorphism and the structure of genetic variation in the populations of many species (Lewontin 1991; Meglécz et al. 1999; Schmitt and Seitz 2001; Aagaard et al. 2002).

The main goal of the present study was to analyze the structure of genetic variation in two *Isophya* species (Orthoptera: Tettigonoidea) in Hungary: in *I. kraussi* (Brunner von Wattenwylý 1878) and in *I. stysi* (Čejhaný 1957). Six *Isophya* species are recognized in Hungary. They are all flightless and consequently they have low dispersal rate and week colonizing ability (Szövényi et al. 2001). As a consequence, all six species are vulnerable, four of them are protected (Ministry of environment: List of protected species in Hungary,

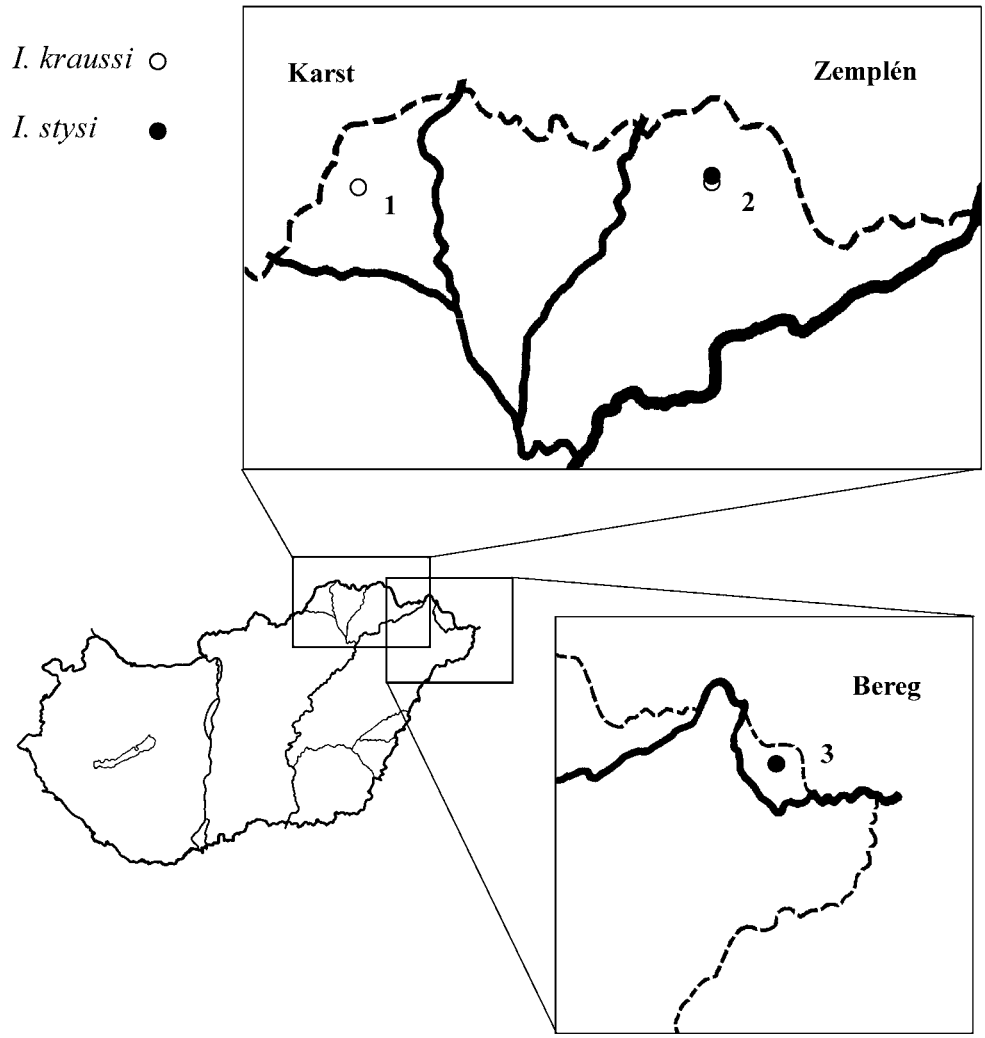

Figure 1. Sample sites. (1) Jósvafö (Aggtelek Karst region); (2) Gyertyánkút meadows (Zemplén Mts.); (3) Kaszonyi hill (Bereg lowland).

2000) and two have been added to the Annexes II–IV of the Habitat Directive. *I. kraussi* has a Central and South-East European distribution (Rácz 1998; Rácz et al. 1996). In Hungary, it is widespread in the edges, skirts and clearings of the mountainous areas (Nagy and Rácz 1996). *I. stysi* is endemic in the Carpathian basin (Nagy and Szövényi 1999). It occurs in different types of mesic and semi-dry meadows of the East Carpathians, Transylvanian Island Mountains, Hungarian Central Range and the Eastern margin of the Pannonian lowland. The species is protected in Hungary (Ministry of environment: List of protected species in Hungary, 2000). Like *I. kraussi*, *I. stysi* also occurs in the dense grassy-scrubby vegetation of the forest edges and clearings. As a consequence, the two species may co-occur in certain habitats, e.g., in the Zemplén Mountains.

Methods

Samples

Two populations were sampled for each species from three distinct geographical regions in Hungary (Figure 1). *I. kraussi* was collected in two regions: in Jósvafö (Aggtelek Karst) and in Gyertyánkút near

Telkibánya (Zemplén Mts.). *I. stysi* samples originated from the Kaszonyi Hill (Bereg lowland) and also from Gyertyánkút (Zemplén Mts.). In this way, we could determine the level of differentiation both within and between species.

Study of enzyme polymorphism

Imagines were collected in 1998–1999 and stored at −80 °C until the electrophoresis. Muscles dissected from the thorax and abdomen of the specimens were homogenised separately in ~4 μl/mg of the extraction buffer. Enzyme polymorphism was investigated by polyacrylamide gel electrophoresis. Fourteen enzymes were studied in all four samples. Thorax samples were used to detect glutamate–oxalacetate transaminase (GOT), α-glycerophosphate dehydrogenase (αGPDH), hexokinase (HK), isocitrate dehydrogenase (IDH), lactate dehydrogenase, (LDH), malate dehydrogenase (MDH), malic enzyme (ME), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM) and superoxid dismutase (SOD). The other four enzymes (aconitase (ACON), aldehyde oxidase (ALDOX), esterase (EST) and 6-phophogluconate dehydrogenase (6PGDH)) were detected from the abdomen of the specimens. Out of the total 14 loci, 10 (*Aldox*, *Est*, *Got*, *αGpdh*, *Hk*, *Mdh*, *Me*, *Pgi* and *Pgm*) were scored consistently in each sample.

Statistical analyses

Genotype and allele frequencies were calculated on the basis of banding patterns. Measures of genetic variation (average number of alleles, proportion of polymorphic loci, average observed and expected heterozygosity) were calculated for each population. The Markov chain method was used to estimate the exact Hardy–Weinberg probability without bias (Guo and Thompson 1992). An exact test for population differentiation (Raymond and Rousset 1995a) was conducted to test for independence of the allelic composition of the populations. Genepop, version 1.0 (Raymond and Rousset 1995b) was used to perform the Hardy–Weinberg test, and the exact test of population differentiation. Genetic differentiation among the populations was also analysed by Wright's *F*-statistics (Wright 1978; Weir 1990). In this analysis, the total genetic variation of the samples (F_{IT}) is partitioned into within (F_{IS}) and between population components (F_{ST}). These analyses were conducted by FSTAT version 1.2 (Goudet 1995). Allele frequencies were used to estimate Nei's genetic distances (Nei 1975) and an UPGMA dendrogram (Sneath and Sokal 1973) was constructed on the basis of these data. The computation was performed by Biosys-1, Release 1.7 (Swofford and Selander 1981). In the last part of the study, we carried out a principal component analysis (PCA) using the genotypic composition of the individuals to show the size of overlap in the genetic variation of the populations in a reduced space of variables. PCA analyses were performed running R Package Version 4.0 (Casgrain and Legendre 2001).

Table 1. Some important parameters of genetic variability estimated for the four samples of the two *Isophya* species at the 10 investigated loci

	I. kraussi			*I. stysi*		
	Zemplén	Karst	Average	Zemplén	Bereg	Average
N	7.2	9.9	8.55	13	11	12
n	1.9	2.0	1.95	2.0	2.2	2.1
P	0.70	0.70	0.70	0.60	0.80	0.70
H_o	0.170	0.203	0.187	0.208	0.264	0.236
H_e	0.288	0.292	0.290	0.290	0.389	0.340
F_{IS}	0.437	0.311	0.366	0.293	0.333	0.314

N: average sample size, *n*: average number of alleles, *P*: proportion of polymorphic loci; H_o: observed frequency of heterozygotes; H_e: expected frequency of heterozygotes; F_{IS}: the average size of the departure of heterozygote frequency from the Hardy–Weinberg expectation.

Results

Level of polymorphism

Although the sample sizes were rather low we detected a high level of polymorphism in both species. Except for the *Idh* locus in *I. stysi*, all 10 loci exhibited one or more alternative alleles in at least one sample. The average portion of polymorphic loci was 70% in both species (Table 1: P) and the average heterozygote frequencies were also high (Table 1: H_o). The observed frequency of heterozygotes was consistently lower than the expected one in every population (Table 1: H_o vs. H_e). All further analyses were based on these 10 loci.

To confirm the taxonomic status of the two species we were looking for obvious genetic differences

Table 2. Results of *F*-statistics and the exact test of population differentiation computed on the data of all four populations

Loci	*F*-statistics			Exact test
	F_{IT}	F_{ST}	F_{IS}	
Aox	0.424**	0.209**	0.272*	***
Est	0.438**	0.108*	0.371*	***
Got	0.668**	0.347**	0.491**	***
Gpdh	0.735**	0.646**	0.252	***
Hk	0.842**	0.686**	0.497*	***
Idh	0.001	0.003	−0.003	ns
Mdh	0.209	0.100**	0.122	***
Me	0.810**	0.704**	0.359	***
Pgi	−0.023	0.011	−0.034	ns
Pgm	0.595**	0.092*	0.553**	*
Total	0.585**	0.378**	0.334**	***

F_{IT} measures of total genetic variation; F_{IS} measures of the genetic variability within the populations; F_{ST} measures of the genetic variation among the populations.
*Significant at 0.05 level; **significant at 0.01 level; ***significant at 0.001 level.

between them. α*Gpdh* proved to be absolutely diagnostic, as it had not any common allele in the two species. At four further loci (*Got*, *Hk*, *Mdh* and *Me*), the two species had one common allele together with one or more differentiating alleles in the surveyed populations. Moreover, *I. stysi* populations had additional rare alleles at the *Pgi* and *Pgm* loci, which did not occur in *I. kraussi* populations.

Structure of genetic variation

The results of the *F*-statistics also showed a high level of genetic variation in the two species (Table 2: F_{IT}). This variation was more or less equally partitioned into within (F_{IS}) and between population (F_{ST}) components. The high and positive overall F_{IS} values indicated heterozygote deficiency in both species (Table 2).

The highly significant overall F_{ST} values indicated strong genetic differentiation among the populations (Table 2). These results are in good agreement with those of the exact test for population differentiation (Table 2). When the F_{ST} values were calculated for the two species separately, the level of differentiation was lower among the local populations within both species than that among all four populations (F_{ST} values in Table 3 vs. in Table 2). Nevertheless, it is remarkable that the

Table 3. F_{ST} values and the results of the exact test of population differentiation estimated for the local populations within each species

Loci	*I. kraussi*		*I. stysi*	
	F_{ST}	Exact prob.	F_{ST}	Exact prob.
Aox	−0.052	ns	−0.056	ns
Est	−0.097	ns	−0.051	ns
Got	0.027	ns	0.327**	***
Gpdh	0.043	ns	0.586**	***
Hk	−0.020	ns	0.455**	***
Idh	−0.012	ns	–	–
Mdh	0.091	ns	−0.047	ns
Me	0.267*	*	0.593**	***
Pgi	–	–	−0.006	ns
Pgm	0.412*	*	−0.056	ns
Total	0.099*	*	0.232**	***

*Significant at 0.05 level; **significant at 0.01 level; ***significant at 0.001 level.

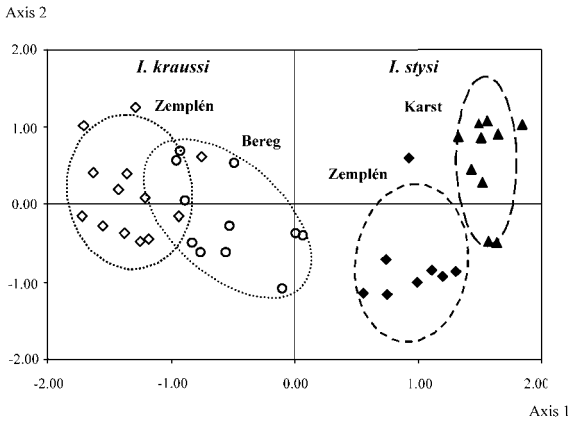

Figure 2. Results of the PCA analysis. The points represent the genotypic composition of the individuals along the first two axes in a reduced space of variables.

average F_{ST} value estimated for *I. kraussi* was less than half of that calculated for *I. stysi* (Table 3). Moreover, the number of differentiating loci was also larger in *I. stysi* than in *I. kraussi*. Nei's genetic distance calculated on the basis of allele frequencies was also higher in *I. stysi* than in *I. kraussi*.

The results of the PCA analyses confirmed the relatively high level of genetic differentiation among the surveyed populations. For both species, the individuals of the populations comprised two more or less distinct clouds of points along the first two axes (Figure 2). It is remarkable that the two

ellipses partly covered each other for *I. kraussi*, while they had no overlap for *I. stysi*. Since the ellipses represent the size of variation among the individuals within the populations this figure also indicates that the level of variation was fairly similar in the four investigated populations. The individuals of the two species were completely separated along the first axis (Figure 2). This axis explained more than 37% of the total genetic variation and the α*Gpdh*, *Hk* and *Me* loci contributed most to it.

Discussion

We observed a high level of polymorphism in the two *Isophya* species at the investigated enzyme loci. This level is slightly higher than that described in other Orthoptera species. Oudman and his co-workers (1990) found that the mean ratio of polymorphic loci was slightly higher than 20% with a heterozygote frequency ranging from 0.03 to 0.14 in 20 local populations of *Ephippiger ephippiger* in Southern France. In six South American populations of a grasshopper (*Trimerotropis palldipennis*), the average portion of polymorphic loci was about 0.35 and the average heterozygosity was over 0.15 (Matrajt et al. 1996). Similar level of polymorphism ($P \approx 0.34$, $H_{av} \approx 0.07$–0.16) was detected in 10 Italian populations of a cave cricket (*Dolichopoda schiavazzii*) by Allegrucci et al. (1997). It thus appears that the high level of genetic variation detected by enzyme electrophoresis is a general feature of various orthopteroid insect species.

The two investigated populations of both species exhibited an overall heterozygote deficiency, which often resulted in significant Hardy–Weinberg disequilibrium. Heterozygote deficiency was also found in other Orthoptera species (Allegrucci et al. 1997; Orr et al. 1994). In *Dolichopoda* populations, this phenomenon was due to the presence of 2-yearly cohorts in the populations (Allegrucci et al. 1997). Since *Isophya* species have 1–4 year diapausa in the egg stage (Nagy and Szövényi 1999; Szövényi et al. 2001) their populations might be comprised of different cohorts as well. Nevertheless, heterozygote deficiency can be the consequence of several other evolutionary forces in addition to population subdivision (e.g., inbreeding, selection, etc.). At present, our data are not sufficient to exclude the influence of any possible force and therefore further investigations are required.

Since *Isophya* species are flightless and have poor dispersal ability (Nagy and Szövényi 1999; Szövényi et al. 2001) we expected a high level of differentiation among their local populations. Both the F_{ST} values and the results of the exact test of population differentiation fulfilled our expectations. Other studies have described various F_{ST} values for different Orthoptera species. Matrajt and his co-workers (1996) have detected an F_{ST} value of around 0.18 in six populations of *Trimertropis pallidipennis* in South America. Orr et al. (1994) have found an exceptionally high level of differentiation ($F_{ST} = 0.449$) in 14 populations of *Melanoplus sanguipes* in California. It is, however, interesting that they reported a much lower level of differentiation ($F_{ST} = 0.084$) among four Californian populations of *M. devastator*, which is a close relative to *M. sanguipes* (Orr et al. 1994). Oudman et al. (1990) have described a relatively high level of differentiation in the *Ephippiger ephippiger* species complex as well. They analysed 20 populations in Southern France, and found that Nei's genetic distances were ranging from about 0.01 to 0.1. In a study by Allegrucci et al. (1997) the F_{ST} value computed for nine populations of a cave cricket (*Dolichopoda sciavazzii*) was 0.34 and Nei's genetic distances were ranging from 0.01 to 0.125. All these data suggest that local populations of various Orthoptera species with different life history and habitat preference tend to be strongly differentiated. Nevertheless, most of these species have poor dispersal ability, which probably accounts for the high level of genetic differentiation among their populations.

Similarly to Orr et al. (1994), we also found some difference in the level of genetic differentiation between two closely related Orthoptera species. Local populations of *I. kraussi* seemed to have a more similar genetic composition than those of *I. stysi*. One possible explanation of this difference between the two *Isophya* species arises when contrasting their sampled populations to their general distribution. As the distribution of *I. stysi* is mostly confined to the mountains of Transylvania the sampled Hungarian populations can be considered peripheral representing the western boundary of the range of the species. On the contrary, *I. kraussi* is widely distributed in the entire Carpathian basin and therefore the sampled populations might be

regarded as central ones. Marginal populations are known to be more exposed to the effect of genetic drift than central ones (e.g., Saura et al. 1973 and references therein). As a consequence, the level of differentiation among such peripheral populations is expected to be higher.

I. kraussi and *I. stysi* are rather similar to each other morphologically. Therefore, the identification of single specimens especially females is often difficult. Their songs are, however, clearly distinct (Orci 2002). Our data suggest a very strong genetic differentiation between these two species at the investigated enzyme loci.

In summary, we can conclude that the two *Isophya* species exhibit a similarly high level of genetic variation. Their local populations are clearly differentiated. This suggests that migration can hardly counterbalance the differentiating effect of genetic drift in their populations. Ineffective migration among local populations was also expected considering the low dispersal rate characteristic for the *Isophya* species. Since both species have a low colonising ability there is a very low chance of recolonisation after population extinction. As a consequence, it is of great conservation interest to have their populations and consequently their habitats (dense grassy vegetation of forest skirts and clearings) undisturbed.

Acknowledgements

The authors are especially grateful to the Hungarian Nature Conservation Authorities for the permissions of sample collections. The authors are also obliged to István Rácz who collected some of the samples. The technical assistance of Valéria Mester is highly appreciated. The project was supported by FKFP 0473/200 grant of the Ministry of Education.

References

Aagaard K., Hindar K., Pullin A.S., James C.H., Hammarstedt O., Balstad T. and Hanssen O. 2002. Phylogenetic relationships in brown argus butterflies (Lepidoptera: Lycaenidae: *Aricia*) from north-western Europe. Biol. J. Linn. Soc. 75: 27–37.

Allegrucci G., Minasi M.G. and Sbordoni V. 1997. Patterns of gene flow and genetic structure in cave-dwelling crickets of the Tuscan endemic, *Dolichopoda schiavazzii* (Orthoptera, Rhaphidophoridae). Heredity 78: 665–673.

Casgrain P. and Legendre P. 2001. The R package for multivariate and spatial analysis, Version 4.0 d5 User's manual. Web site ⟨http://www.fas.unmontreal.ca/BIOL/legendre/⟩.

Gilpin M.E. 1991. The genetic effective size of a metapopulation. Biol. J. Linn. Soc. 42: 165–175.

Goudet J. 1995. FSTAT version 1.2: a computer program to calculate *F*-statistics. J. Heredity 86(6): 485.

Guo S.W. and Thompson E.A. 1992. Performing the exact test of Hardy–Weinberg proportions for multiple alleles. Biometrics 48: 367–372.

Hanski I. and Gilpin M. 1991. Metapopulation dynamics: brief history and conceptual domain. Biol. J. Linn. Soc. 42: 3–16.

Lewontin R.C. 1991. Electrophoresis in the development of evolutionary genetics: milestone or millstone. Genetics 128: 657–662.

Matrajt M., Confaloineri V. and Vilardi J. 1996. Parallel adaptive patterns of allozyme and inversion polymorphisms on an ecological gradient. Heredity 76: 346–354.

Meglécz E., Nève G., Pecsenye K. and Varga Z. 1999. Genetic variations in space and time in *Parnassius mnemosyne* (Lepidoptera) populations in northeast Hungary. Biol. Conserv. 89: 251–259.

Morgan-Richards M., Trewick S.A. and Wallis G.P. 2000. Characterisation of a hybrid zone between two chromosomal races of the weta *Hemideina thoracica* following a geologically recent volcanic eruption. Heredity 85: 586–592.

Nagy B. and Rácz I. 1996. Orthopteroid Insects in the Bükk Mountain. In: Mahunka S. (ed.), The Fauna of the Bükk National Park, Hungarian Natural History Museum, Budapest, pp. 95–123.

Nagy B. and Szövényi G. 1999. A Körös–Maros Nemzeti Park állatföldrajzilag jellegzetes Orthoptera fajai és konzervációökológiai viszonyaik. Természetvédelmi Közlemények 8: 137–160.

Nei M. 1975. Molecular Population Genetics and Evolution. North-Holland Publishing Company, Amsterdam.

Newton A.C., Allnutt T.R., Gilles A.C.M., Lowe A.J. and Ennos R.A. 1999. Molecular phylogeography, intraspeciphic variation and the conservation of tree species. TREE 14: 140–145.

Opdam P. 1988. Populations in fragmented landscape. In: Schreiber K.F. (ed.), Connectivity in Landscape Ecology, Münstersche Geographische Arbeiten 29, Münster, pp. 75–77.

Orci K.M. 2002. On the Bioacustics and Morphology of some Species-Groups of Orthoptera. PhD Thesis, Debrecen.

Orr M.R., Porter A.H., Mousseau T.A. and Dingle H. 1994. Molecular and morphological evidence for hybridization between two ecologically distinct grasshoppers (*Melanoplus sanguinipes* and *M. devastator*) in California. Heredity 72: 42–54.

Oudman L., Duijm M. and Landman W. 1990. Morphological and allozyme variation in the *Ephippiger ephippiger* complex (Orthoptera, Tettigonioidea). Netherlands J. Zool. 40(3): 454–483.

Rácz I., Varga Z., Mezö H. and Parragh D. 1996. Studies on the Orthoptera fauna of the Aggtelek Karst. In: Tóth E. and Horváth R. (eds), Research in Aggtelek National Park and Biosphere Reserve II. ANP Füzetek 1. Aggtelek National Park, Aggtelek, pp. 99–107.

Rácz A. 1998. Zoogeographical analysis of the Orthoptera fauna from the Bükk Mountains (N Hungary). Folia Entomologica Hungarica LIX: 5–16.

Raymond M. and Rousset F. 1995a. An exact test for population differentation. Evolution 49: 1280–1283.

Raymond M. and Rousset F. 1995b. GENEPOP ver.1.2, a population genetics software for exact tests and ecumenicism. J. Heredity 86: 246–249.

Rolstad J.S. 1991. Consequences of forest fragmentation for the dynamics of bird populations: conceptual issues and the evidence. Biol. J. Linn. Soc. 42: 123–134.

Saura A., Lakovaara S., Lokki J. and Lankinen P. 1973. Genetic variation in central and marginal populations of *Drosophila subobscura*. Hereditas 75: 33–46.

Settele J., Margules C., Poschlod P. and Henle K. (eds), 1996. Species Survival in Fragmented Landscapes. Kluwer, Dordrecht.

Schmitt Th. and Seitz A. 2001. Intraspecific allozymatic differentiation reveals the glacial refugia and the postglacial expansions of European *Erebia medusa* (Lepidoptera: Nymphalidae). Biol. J. Linn. Soc. 74: 429–458.

Sneath P.H. and Sokal R.R. 1973. Numerical Taxonomy, W.H. Freeman, San Francisco.

Szövényi G., Nagy B. and Orci K.M. 2001. Isophya szöcskepopulációk Magyarországon. In: Isépy I., Korsós Z. and Papp L. (eds), II. Kárpát-medencei Biológiai Szimpózium. Magyar Biológiai Társaság and Magyar Természettudományi Múzeum, Budapest, pp. 255–258.

Swofford D.L. and Selander R.B. 1981. Biosys-1: A FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. J. Heredity 72: 281–283.

Thomas C.D. 2000. Dispersal and extinction in fragmented landscapes. Proc. R. Soc. Lond. B 267: 139–145.

Vogler A.P. and Desalle R. 1994. Diagnosing units of conservation management. Conserv. Biol. 8: 354–363.

Weir B.S. 1990. Genetic Data Analysis. Sinauer Associates, Inc., Sunderland.

Wright S. 1978. Evolution and the Genetics of Populations, vol. 4. Variability Within and Among Natural Populations. University of Chicago Press, Chicago.