Acta Zoologica Academiae Scientiarum Hungaricae 58 (1), pp. 87–103, 2012

PATTERN OF GENETIC AND MORPHOMETRIC DIFFERENTIATION IN *MACULINEA NAUSITHOUS* (LEPIDOPTERA: LYCAENIDAE) IN THE CARPATHIAN BASIN

HOLLÓS, A.[1], PECSENYE, K.[1], BERECZKI, J.[1], BÁTORI, E.[1], RÁKOSY, L.[2] and VARGA, Z.[1]

[1]*Department of Evolutionary Zoology and Human Biology, University of Debrecen H-4010, Egyetem tér 1, Debrecen, Hungary; e-mail: pecskati@gmail.com*
[2]*Department of Taxonomy and Ecology, Babeş-Bolyai University RO-3400, Str. Clinicilor 5–7, Cluj-Napoca, Romania*

The level of variation and the pattern of differentiation were studied in two Western Hungarian (Transdanubia: Őrség region) and two Romanian (Transylvanian Basin) populations of *Maculinea nausithous* (Dusky Large Blue). The aim was to provide evidence on the genetic differentiation of the Transylvanian populations, which were relegated as *M. nausithous kijevensis* by RÁKOSY *et al.* (2010). In order to analyse genetic variance enzyme polymorphism was studied at 17 loci. The structure of phenotypic variation was investigated by performing morphometric analyses on 11 traits of the wings. Statistical procedures were chosen so, that the results obtained for morphological and genetic data could be compared.
The results of all genetic surveys supported the differentiation of the Transylvanian populations from the Western Transdanubian (Őrség) ones. Hence, genetic results supported the existence of differentiation at the subspecies level in *M. nausithous*. The results of the morphometric analyses, however, were not obvious. In some analyses (phenogram) no clear phenotypic differentiation was observed between the two regions. Nevertheless, the results of hierarchical analysis of variance and Multiple Discriminant Analysis indicated a significant separation of specimens from the two regions. In addition, differences were detected in the level of variation between the two regions. Both genetic and phenotypic variation was higher in the Transylvanian than in the Őrség samples.

Key words: *Maculinea nausithous*, enzyme polymorphism, morphometric variation, geographical differentiation

INTRODUCTION

The Dusky Large Blue *Maculinea nausithous* ([BERGSTRÄSSER], 1779)* has a special life cycle. Imagoes lay eggs on the larger, apical flower heads of *Sanguisorba officinalis* (THOMAS 1984). Larvae develop through three instars in the flowers feeding on green fruits (ELMES *et al.* 1991*a*, *b*, THOMAS & WARDLAW 1992). In the fourth instar they drop to the ground and wait for being discovered by the foraging workers of its host ant mostly *Myrmica rubra* but occasionally *My. scabrinodis* (THOMAS 1984, THOMAS *et al.* 1989, TARTALLY *et al*. 2008) who adopt and take them to the ant nest. Caterpillars live there as social parasites for

about 10 months during the fourth instar (ELMES *et al.* 1991*a*). *M. nausithous* lives in strictly localised populations often with rather limited number of adults (THOMAS 1984, THOMAS *et al.* 1989). Since this species is dependent on the presence of two sequential resources, habitat fragmentation and isolation resulted in severe decline in many of its populations especially in Western Europe. Thus, it is considered to be Near Threatened in Europe and listed in the Annex II of Habitat Directive, IUCN and Hungarian Red Data Books.

*Maculinea nausithous** has a Euro-Siberian distribution with a wide but sporadic range from Western Europe through Kazakhstan and Southern Siberia to Mongolia (LUKHTANOV & LUKHTANOV 1994, TUZOV 1997, WYNHOFF 1998, MUNGUIRA & MARTÍN 1999, RÁKOSY *et al.* 2010). Nevertheless, its distribution has a definite hiatus in the Carpathian Basin. It is locally frequent in the prealpine regions of Austria and Slovenia as well as in the lowland and hilly regions of Western Hungary, but was known to be completely absent in the Great Hungarian Plain, in the Hungarian Northern Middle Range and in Transylvania (RÁKOSY *et al.* 2010) with the next records in East of the Carpathians in Bukovina. However, *M. nausithous* has recently been discovered in Transylvania near Cluj-Napoca at Răscruci and Fânatele Clujului (RÁKOSY *et al.* 2010). The habitat and host ant use of these two isolated populations are different from the Central European ones (TARTALLY *et al*. 2008, RÁKOSY *et al.* 2010). Moreover, their appearance coincides with the original description of *M. nausithous kijevensis* (SHELJUZHKO, 1928). Therefore, RÁKOSY *et al.* (2010) suggested that the two Transylvanian populations belong to *M. nausithous kijevensis* which is widely distributed in humid habitats of the meadow steppic zone in southern Siberia, Kazakhstan and northern Mongolia. Thus, Transylvanian populations can be considered as marginal isolates relative to the continental Transpalaearctic range.

In their paper ALS *et al*. (2004) have already suggested that the great nucleotide divergence observed in *M. nausithous* may represent cryptic species. Thus, the aim of the present work was to study the level of genetic and morphometric differentiation among the *Maculinea nausithous* populations in the Őrség region (West Hungary) and in Transylvania (Romania) in order to unravel possible taxonomic differences between them. Until recently most studies of population structure are based on molecular markers and those concerning morphological traits are much

* According to the priority rule of nomenclature the correct genus name is *Phengaris* (FRIC *et al.* 2010). Nevertheless, BALLETTO *et al.* (2010) has turned to the International Committee of Zoological Nomenclature in order to keep the *Maculinea* genus name. The decision of the Committee has not been published yet. Thus we shall use the *Maculinea* genus name throughout this paper as it has been used in most of the former ecological, conservation biological and genetic studies. It was even applied as an acronyme of an EU project: MacMan. The present paper principally contains results obtained by the support of this project.

scarcer (GARNIER *et al.* 2005). We, therefore, consider it important to analyse these two types of variation in parallel.

MATERIALS AND METHODS

Samples

Maculinea nausithous samples originated from 2 regions: Őrség region (Transdanubia in Western Hungary) and Transylvanian Basin, Romania (close to Cluj Napoca). In both regions we had two populations: Őrség – Magyarszombatfa and Kétvölgy; Transylvania – Răscruci and Fânatele Clujului (Fig. 1). All four populations were sampled in two consecutive years (generations). Thus, altogether we could analyse 8 samples from the 4 populations. The total number of individuals was 256 in the enzyme study, while 127 in the morphometric study (Appendix 1).

In order not to damage the sampled populations mostly males were collected at the end of the flight period after the females laid their eggs. After collection, the individuals were immediately frozen and kept at –80 °C until electrophoresis.

Enzyme studies

Allozyme polymorphism was studied at 17 different loci by vertical polyacrylamide gel electrophoresis: aconitase (*Acon*), acid phosphatase (*AcphB*) aldehyde oxidase (*Aox*), esterase (*Est*), glutamate dehydrogenase (*Gdh*) glutamate oxalacetate transaminase (*GotA* and *GotB*), glucose-6-phosphate dehydrogenase (*G6pdh*), α-glycerophosphate dehydrogenase (α*Gpdh*), hexokinase (*Hk*), isocitrate

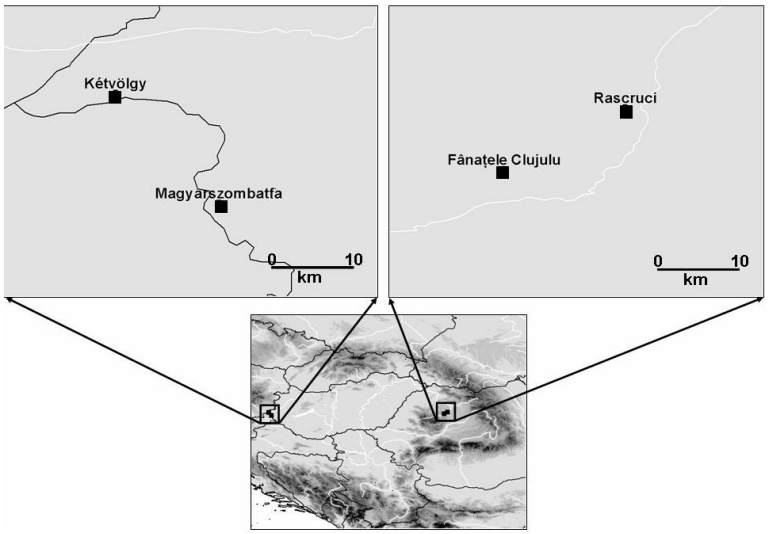

Fig. 1. Sample sites. Őrség region (West Hungary): Kétvölgy (Kv) and Magyarszombatfa (Mfa). Transylvania (Romania): Răscruci (Ras) and Fânatele Clujului (Fan)

dehydrogenase (*IdhA* and *IdhB*), malate dehydrogenase (*Mdh*), malic enzyme (*Me*), phosphoglucose isomerase (*Pgi*), phosphoglucomutase (*Pgm*) and superoxid dismutase (*Sod*). Thoraxes and abdomens were homogenized separately in 350–400 µl extraction buffers. Thorax samples were used to study *GotA, GotB*, α*Gpdh, Hk*, *IdhA, IdhB*, *Mdh*, *Me*, *Pgi*, *Pgm*, and *Sod*, while abdomen extracts were used to analyse *Acon*, *AcphB*, *Aox*, *Est* and *6Pgdh*. The extraction buffer, the electrophoresis buffer systems and running conditions together with the staining solutions were slightly modified after BERECZKI *et al.* (2005).

Morphometric studies

Before electrophoresis, the wings of the individuals were cut and analysed separately in the morphometric study. Wings were fixed on transparency films and photographed by Sony DSC-H2 digital camera. Measurements were completed on the high resolution digital photos by computer using the Image J 1.36 programme (KIZIC & BOROVAC 2001). Eleven traits were measured on the forewings and the hindwings (Fig. 2). Five traits characterised the size and the shape of the wings, while six concerned the pattern of the hind wing. Some of these traits especially distances were also

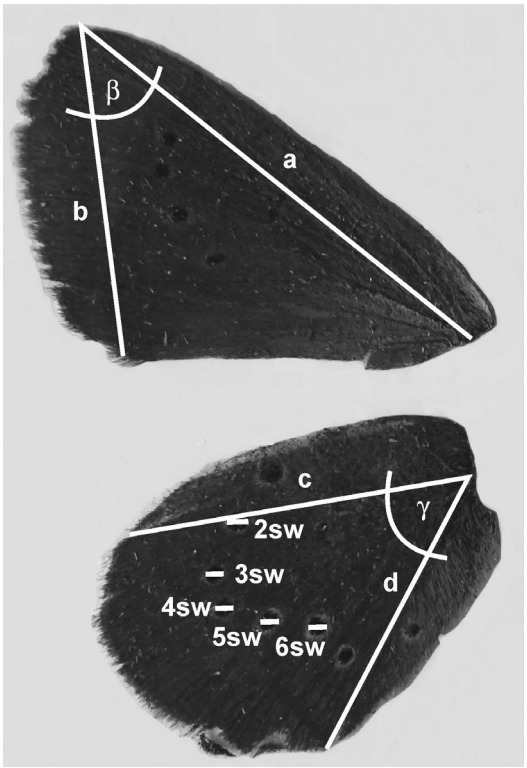

Fig. 2. Measured traits on the wings of *Maculinea nausithous*. Forewing: anal length (a), length of the outer margin (b), apical angle (β). Hindwing: anal length (c), costal length (d), basal angle (γ), widths of spots 2–6 (2sw–6sw)

used in other morphometric studies (WYNHOFF 2001, PRIETO *et al.* 2009). Distances were determined between fix points indicated by the veins. Costal length (d – hindwing): between the basal end of the discal cell and the outer end of the radial vein ($Sc+R_1$); anal length (a – forewing and c – hindwing): between the basal end of the discal cell and the outer end of the anal veins (1A+2A on the forewing and 3A on the hindwing); length of the outer margin (b – forewing): between the outer end of the radial vein (R_3) and the outer end of the anal veins (1A+2A). In addition two angles were also measured. Basal angle (gr – hindwing) enclosed by the costal and anal margins and apical angle (br – forewing) enclosed by the costal and outer margins. The pattern of the hindwing was characterised by the diameter of the spots on the underside. It was possible to measure 5 spots (s2–s6) consistently on all individuals (Fig. 2). Morphometric study was only carried out on males, thus the sample sizes were slightly lower in these analyses than in the enzyme studies (Appendix 1).

Statistical analyses

Similar statistical procedures were applied on the genetic and morphometric data in parallel enabling us to compare the results. Variation was studied in two ways: both the amount and the structure of it was analysed. Genetic data – Genotype and allele frequencies were calculated on the basis of banding patterns. The classical parameters of genetic variation (average number of alleles, average observed heterozygosity and proportion of polymorphic loci) were computed for each sample (Appendix 2). These parameters were compared between the regions by permutation test using FSTAT ver. 1.2 (GOUDET 1995). Allele frequencies were used to estimate CAVALLI-SFORSA and EDWARDS chord distances (CAVALLI-SFORSA & EDWARDS 1967) and an UPGMA dendrogram (SNEATH & SOKAL 1973) was constructed on the basis of these data. Bootstrap values were calculated from 2000 replicates. The computation of chord distances was performed by PAST ver.1.56 (HAMMER *et al.* 2006), and this program was also used to process the dendrogram and the bootstrapping. The distribution of total genetic variation at various levels of the hierarchy was studied by AMOVA (EXCOFFIER *et al.* 1992, WEIR 1996). In this analysis, the total genetic variation was partitioned into five components: between regions, among populations within a region, among samples (generations) within a population and among individuals within a sample. AMOVA was carried out by Arlequin software version 3.11 (SCHNEIDER *et al.* 2000). Finally, the genetic structure of populations was analysed by Bayesian-clustering method (PRITCHARD *et al.* 2000). Here, we estimated the most probable number of genetically differentiated groups (K) in our samples and assigned the individuals to these groups. STRUCTURE 2.3.2 was run to carry out these analyses with initial burn in 20000 and running length 100000.

Morphological data – The phenotypic variation of the samples was characterized by the mean value of Leven's variables (MANLY 1986), which are the deviations between the individual trait values and the sample average of the given trait. In order to be able to compare these values for the different traits we expressed them as percentages of the trait averages (L%). We also calculated the coefficient of variation for the two regions separately (SOKAL & ROHLF 1995). Euclidean distances among the average canonical variables were computed and used as morphometric distances. UPGMA phenogram was constructed on the basis of this distance matrix. Bootstrap values were calculated from 2000 replicates. The analyses were computed by PAST ver.1.56. The correlation between genetic and morphometric distances was analysed by MANTEL test (MANTEL 1967) with 999 permutations. GenAlEx6 (PEAKALL & SMOUSE 2006) was used to carry out the test. The distribution of phenotypic variation at different levels of the hierarchy was analysed by a hierarchical ANOVA using the programme GLIM 4 (FRANCIS *et al.* 1994). In this analysis all traits were analysed separately and then the percentages were averaged over the traits. The levels of hierarchy were similar to

Table 1. Parameters of genetic and morphometric variation in the samples of the Őrség region and Transylvania. n_A: average number of alleles per locus; H: average observed frequency of heterozygotes; P%: portion of polymorphic loci; F_{IS}: heterozygote deficiency; CV: coefficient of variance; L%: average Levene's values expressed as portions of trait averages.

		Genetic variation				Morphological variation	
Region	Sample	nA	H	P	FIS	CV	L%
	02Kv	1.53	0.085	0.294	0.177	12.94	0.098
	03Kv	1.65	0.107	0.294	0.142	10.11	0.088
	Kétvölgy	1.59	0.096	0.294	0.159	11.53	0.093
	02Mfa	1.59	0.091	0.353	0.209	10.95	0.084
	03Mfa	1.82	0.111	0.412	0.063	12.54	0.095
	Magyarszombatfa	1.71	0.101	0.382	0.136	11.75	0.089
Őrség		1.65	0.099	0.338	0.149	12.03	0.086
	07Răs	1.88	0.148	0.588	0.181*	11.21	0.076
	08Răs	2.35	0.177	0.706	0.121	13.39	0.090
	Răscruci	2.12	0.163	0.647	0.151	12.30	0.083
	06Fan	2.18	0.168	0.588	0.172*	12.77	0.101
	07Fan	2.29	0.166	0.647	0.163*	11.55	0.082
	Fânaţele	2.24	0.167	0.618	0.167*	12.16	0.092
Transylvania		2.18	0.165	0.632	0.167*	13.01	0.095

those in AMOVA. Multiple Discriminant analysis (MDA) was computed in order to determine the most probable sample to which the individuals belonged. SPSS 16.0 programme package was used for the computation of this analysis.

RESULTS

Level of variation

We have compared the classical parameters of polymorphism between the two regions performing 1500 permutations of the samples. Significant differences were detected in two parameters: average number of alleles (P = 0.016) and observed frequency of heterozygotes (P = 0.026). This indicated a higher level of variation in the Transylvanian populations compared to those in the Őrség region (Table 1: Genetic variation).

The level of phenotypic variation also tended to be higher in the Transylvanian populations compared to the Őrség ones (Table 1: Morphological variation). Though, the differences in Levene's variables were only significant in case of two

traits (rb: $F_{1,126} = 4.14$, $P < 0.05$; rg: $F_{1,126} = 6.8$, $0.025 < P < 0.05$), the L% value for the sixth spot diameter was more than 80% larger in the Transylvanian samples than in the Őrség ones ($F_{1,126} = 3.56$, $P > 0.05$).

Pattern of differentiation – UPGMA dendrogram and phenogram

In the study of the structure of genetic variation, an UPGMA dendrogram was constructed first on the basis of CAVALLI-SFORSA and EDWARDS chord distances. The high bootstrap value indicated clear separation between the two regions (Fig. 3A). Moreover, the bootstrap value supported an evident differentiation even within the Őrség region. Nevertheless, the differentiation between the two populations was not clear in Transylvania.

In order to see the level of genetic differentiation between the two regions relative to that among closely related species we constructed a new dendrogram including a *Maculinea teleius* sample (3tKv) from the Őrség region as an out group (Fig. 4). Comparing the level of genetic differentiation between the two species to that between the *M. nausithous* samples of the two regions we found that differentiation at the species level was far larger than at the regional level.

When surveying morphometric variation an UPGMA phenogram was first built using the Euclidean distances between the canonical variables (Fig. 3B). The phenogram, however, did not suggest a clear phenotypic differentiation between the two regions. One Transylvanian sample (7Fan) was separated clearly from all others supported by a high bootstrap value (Fig. 3B). Though the other samples tended to cluster according to their regional origin the low bootstrap values did not support evident regional differentiation among them.

In the analysis of the association between genetic and morphometric distances a Mantel test was carried out. The results showed significant correlation between the two distance matrices ($R = 0.566$, $P = 0.028$, $N = 28$). Moreover, the points composed of two clouds corresponding to the lower within and higher between region distances (Fig. 5). This finding again implied an apparent differentiation between the two regions.

Pattern of differentiation – AMOVA and hierarchical ANOVA

The next step in the analysis of genetic variation was a series of AMOVA. In the first analysis, all data of both regions were involved. The results showed that far the highest portion of variation (86.7%) could be attributed to the within sample component, that is the variation among the individuals (Fig. 6A). A fairly high percentage of genetic variation (10.3%) was explained by the differences between the

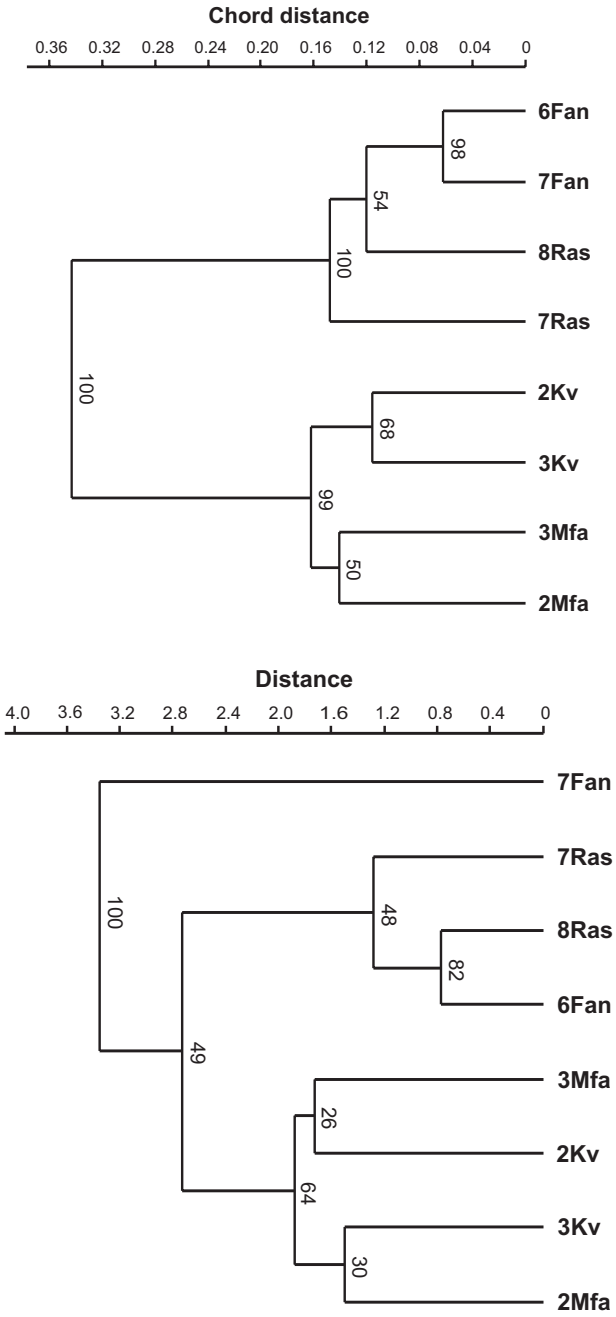

Fig. 3. UPGMA dendrogram constructed using CAVALLI-SFORSA & EDWARDS chord distances (A) and UPGMA phenogram built on the basis of the Euclidean distances among the average canonical variables of the samples (B). Bootstrap values were obtained using 2000 replicates

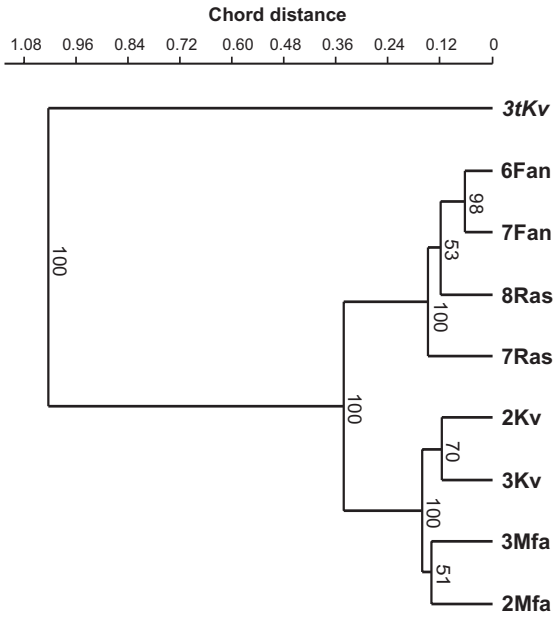

Fig. 4. UPGMA dendrogram constructed using CAVALLI-SFORSA & EDWARDS chord distances with a *Maculinea teleius* sample (3tKv) as out group. Bootstrap values were obtained using 2000 replicates

two regions (Fig. 6A). In the next part of AMOVA, the data of the two regions were analysed separately. In this way we could compare the distribution of variation between the two regions. Though the outcome of these analyses indicated a higher level of differentiation between the two populations in the Őrség region

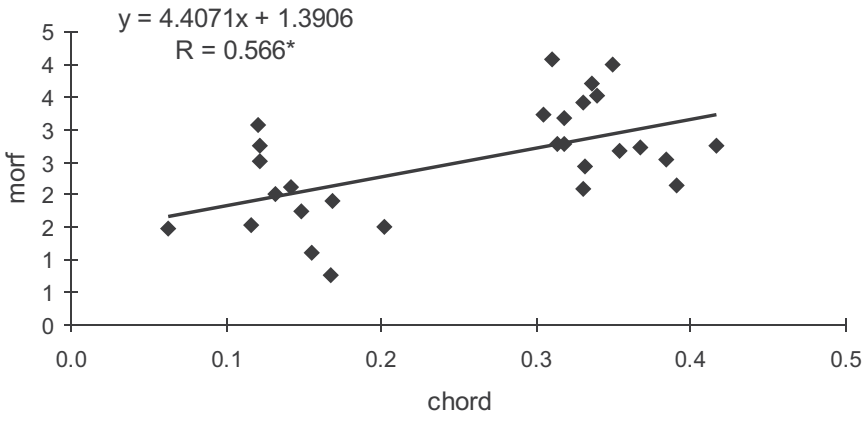

Fig. 5. Results of Mantel test. Chord: Cavalli-Sforsa and Edwards chord distances among the samples; morf.: Euclidean distances among the average canonical variables of the samples

(3.5%) than in Transylvania (1.4%) this difference was not significant in the Fisher exact test (P = 0.31) (Fig. 6B).

Simultaneously a hierarchical ANOVA was carried out on the morphometric data. This analysis was also performed in two steps. First, the data of all individuals from both regions were included. In agreement with the distribution of genetic variation, these results also suggested that the differences among the individuals contributed most (75.4%) to phenotypic variation (Fig. 4C). Like in AMOVA, the differences between the two regions explained a sizeable amount of phenotypic

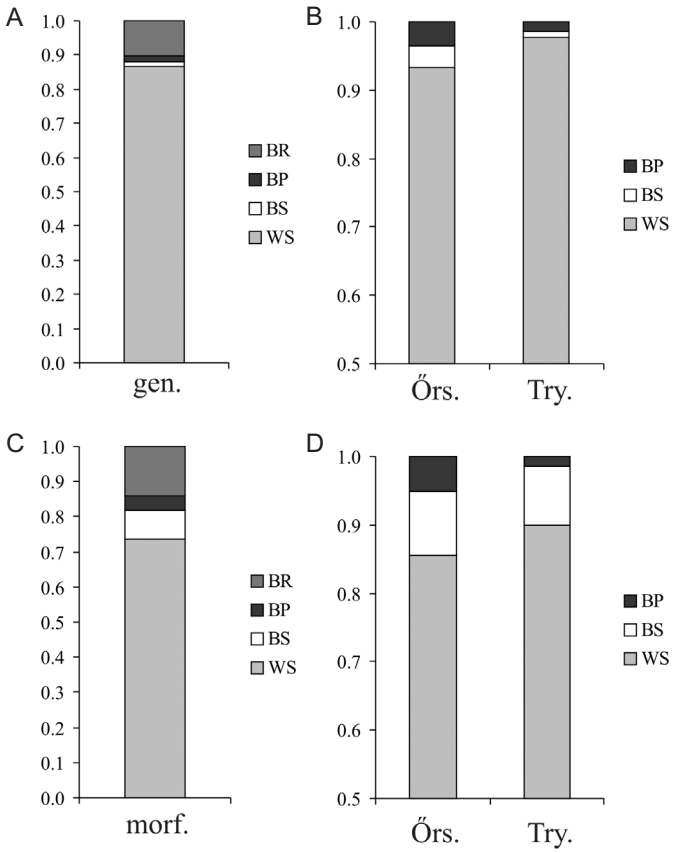

Fig. 6. The results of AMOVA computed on the genetic data and hierarchical ANOVA of the morphometric data. A = AMOVA of both regions together. B = AMOVA of the two regions separately. C = Hierarchical ANOVA of both regions together. D = Hierarchical ANOVA of the two regions separately. The patterns of the columns are consistent in all charts. BR: between region component of variance (dark grey); BP: variation among the populations within the regions (black); BS: variation among the samples/generations within the populations (white); WS: within sample component of variance (light grey). Őrs: Őrség; Try: Transylvania

variation (14.0%) (Fig. 6C). The next step was the computation of hierarchical ANOVA for the two regions separately. The outcome of these analyses was similar to that of AMOVA as morphometric differentiation between the populations was also higher in the Őrség region (5.2%) than in Transylvania (1.4%) (Fig. 6D). Nevertheless these differences were not significant either in the Fisher exact test (P = 0.11).

Pattern of differentiation – Bayesian clustering and multiple discriminant analysis

In the last part of the genetic analyses we estimated the most probable number of genetic clusters in our data set. Hence, we run Structure assuming K between 1 and 4. In runs with K = 3 the probability values were a bit higher (ln(PD) = –2757–2758.8) than in those with K = 2 (ln(PD) = –2704.7–2708.5). Nevertheless

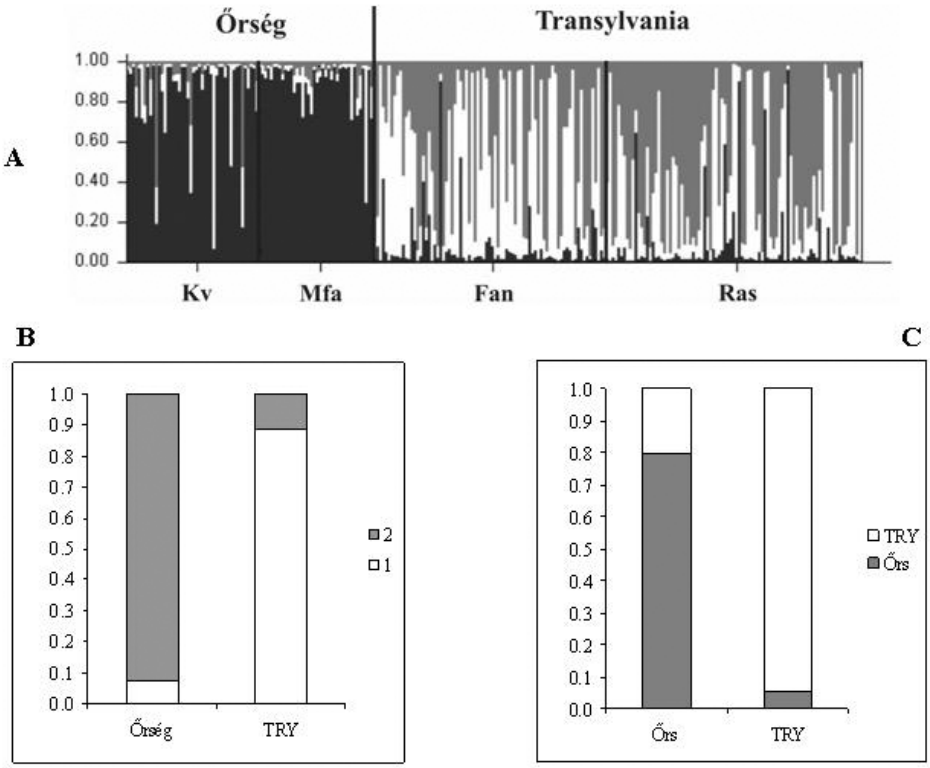

Fig. 7. Results of the classification of individuals. A: Bar plot of the individuals as a result of the Bayesian clustering analysis. B: Distribution of the two genetic clusters in the two regions. 1: genetic cluster 1; 2: genetic cluster 2. C: Allocation of the individuals at the regional level on the basis of their morphometric data. Őrs = Őrség region; TRY = Transylvania

the variance of the probability values were much lower (var[lnp(D)] = 95.4–99.4) in runs with K = 2 compared to those runs where K was 3 (var[lnp(D)] = 208.9–216.3). Comparing the probabilities of the two K values and their variances we have chosen K = 2 as the most likely number of genetic clusters in our data set. In these runs, cluster 1 mostly contained Transylvanian individuals (88.5%), while cluster 2 was mainly composed of Őrség ones (92.9%) (Fig. 7B). Nevertheless, the outcome of runs with K = 3 was also interesting. The Transylvanian populations had two distinct genetic clusters, while those of the Őrség region mostly contained one (Fig. 7A). This result indicated a higher level of variation in Transylvania.

Concurrently a multiple Discriminant analysis (MDA) was performed on the morphometric data. The two regions were considered as separate groups in the computation. The Discriminant function proved to be significant (P = 0.0005) though the high Wilk's λ value (0.83) indicated a relatively poor classification power. The outcome of the classification was slightly asymmetrical. Most of the Transylvanian individuals were correctly assigned (94.3%), while 79.5% of the Őrség individuals were only properly allocated (Fig. 7C). The diameters of the spots (s1, s6 and s5) proved to be the most important traits in the classification. All spots were smaller in the Transylvanian samples.

DISCUSSION

The main goal of the present work was to unravel the pattern of differentiation in the *M. nausithous* populations of the Őrség region (West Hungary) and Transylvania (Romania). When carrying out this survey we had two objectives. First, *M. nausithous* has a disjunct distribution in Europe with a clear hiatus in the Hungarian lowland and Hungarian Northern Middle Range. The Western margin of this hiatus runs in central Transdanubia, north of the Lake Balaton. At the same time the newly discovered Transylvanian populations are situated at the Eastern border of the hiatus (RÁKOSY *et al.* 2010). The genetic similarities or differences among these populations are, therefore, of great interest. Second, ALS *et al.* (2004) suggested the presence of cryptic species in *M. nausithous*. Moreover, RÁKOSY *et al.* (2010) relegated the Transylvanian populations to the eastern Euro-Siberian *M. nausithous kijevensis* mostly on the basis of their ecological and external morphological characteristics. Thus it was important to estimate the level of genetic and also phenotypic differentiation of these Transylvanian populations from those of the typical *M. nausithous* in Hungary.

The results of all genetic surveys supported the differentiation between the Transylvanian and Western Transdanubian (Őrség) populations. Their samples

clustered in different branches of the dendrogram and the outcome of the classification also suggested clear separation between them. Nevertheless, it was also important to find out whether this differentiation indicated distinct species or subspecies. The dendrogram including a *Maculinea teleius* sample showed that the level of differentiation between the *M. nausithous* samples originating from the two regions was relatively low compared to that between *M. nausithous* and *M. teleius.* Thus, Transylvanian populations seem to be differentiated from the Western Hungarian (Őrség) populations of *M. nausithous* at the level of subspecies. Nevertheless we need further genetic evidence using more samples to decide whether Transylvanian (and more eastern) populations belong to *M. nausithous kijevensis*.

The results of the morphometric analyses were pretty similar to those of the genetic studies. In hierarchical ANOVA and MDA the level of phenotypic differentiation between the two regions was similar to that of genetic differentiation. Although there were certain differences in the topology and bootstrap support of the dendrogram and phenogram the Őrség samples and three of the Transylvanian samples were clustered in separate branches in both cases. It thus appears that genetic and phenotypic variation has a similar pattern in *M. nausithous*.

The congruence between enzyme polymorphism and morphological variation was also supported by the significant correlation between the genetic and morphometric distance matrices. Both genetic and morphometric variation was widely used to study the level and structure of variation in natural populations (e.g. morphometric: WYNHOFF 2001, FORDYCE *et al.* 2002, PRIETO *et al.* 2009; genetic: AAGARD *et al.* 2002, SCHMITT *et al.* 2003, BERECZKI *et al.* 2005, PECSENYE *et al.* 2007). These analyses were, however, rarely carried out in parallel (e.g.GARNIER *et al.* 2005, FIORENTINO *et al.* 2008, FRANCOY *et al.* 2009, SUWANVIJITR *et al.* 2010). The correlation between genetic and morphometric variation varied in the different organisms. In snails, analysis of mtDNA sequence and morphometric traits revealed different phylogenetic relationship between the species studied (FIORENTINO *et al.* 2008). On the contrary, a similar population structure was found in *Carabus soleri* when using microsatellites and morphometric traits (GARNIER *et al.* 2005).

Another remarkable result of this study concerns the difference in the level of genetic and also phenotypic variation between the two regions. Both types of variation were higher in the Transylvanian than in the Őrség samples. Moreover, the differences in the parameters of genetic variation (nA and H) and in some morphological traits (apical angle of the forewing and basal angle of the hindwing) proved to be significant. One possible explanation of this phenomenon can be the marginal position of the Őrség populations relative to the hiatus in the distribution of *M. nausithous* in the Carpathian Basin. It is well known that marginal populations

tend to be less variable than central ones (HEWITT 2000, 2004, IBRAHIM *et al.* 1996, SCHMITT & HEWITT 2004, SCHMITT 2007, THOMAS *et al.* 2001). This assumption is also supported by the differences in the level of differentiation between the two regions. Both phenotypic and also genetic differentiation seemed to be higher in the Őrség region than in Transylvania. Thus, the Őrség populations exhibited lower level of variation coupled with a higher level of differentiation compared to the Transylvanian ones. Nonetheless, it seems to be contradicting that the occurrence of *M. nausithous* in Transylvania was not known until recently (RÁKOSY *et al.* 2010). This implies that the Transylvanian populations might also be marginal relative to the huge continental distribution of *M. nausithous* through Eastern Europe to Southern Siberia, Kazakhstan and Mongolia. It thus appears that populations of both regions can be regarded as marginal ones though they probably belong to biogeographically different *M. nausithous* population groups. Transylvanian populations are part of a large group of continental populations with a more or less continuous distribution, while the Őrség region belongs to the declining Central and Western European group of populations with patchy, disjunct distribution. Considering this latter approach our data seem to support that Transylvanian and Őrség populations might belong to distinct subspecies with specific history in the past and therefore different genetic variation and differentiation pattern. Nevertheless, further surveys are required at a trans-continental scale to find the true explanation of the differences between the two regions, including more samples from the typical locality of *M. nausithous kijeviensis* and also from the eastern part of the distribution of the species.

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Acknowledgements – The study was supported by the MacMan EVK2-CT-2001-00126 project. The authors are grateful to J. V. SIPOS and Z. ILONCZAY who effectively contributed to the sampling at several sites. The technical assistance of V. MESTER in the electrophoretic work is very much respected. The contribution of the anonym reviewers is highly appreciated. The support of the Nature Conservation Authorities of Hungary is greatly acknowledged.

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Received November 3, 2010, accepted August 24, 2011, published May 10, 2012

Appendix 1.

Maculinea nausithous samples. N gen: sample sizes in the survey of enzyme polymorphism; N morf: sample sizes in the analyses of morphometric traits.

Regions	Population	Year	N gen	N morf
Őrség	Kétvölgy	2002	20	10
		2003	20	11
	Magyarszombatfa	2002	25	10
		2003	21	8
Transylvania	Răscruci	2007	33	15
		2008	56	29
	Fânațele	2006	41	31
		2007	40	13