



Evolutionary significant units (ESUs) and functional conservation units (FCUs) in the *Phengaris (Maculinea) nausithous* populations of East-Central Europe

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Abstract

The aim of this study was to analyse the level and structure of genetic and morphometric variation in the East-Central European populations of *Phengaris (Maculinea) nausithous*. On the basis of the life cycle of the species, we expected a low level of variation within the populations coupled with a relatively high level of differentiation among them. We also assumed that the differentiation among the populations has a regional pattern. In order to confirm these assumptions, we collected population samples from two regions within the Carpathian Basin (western Transdanubia and Transylvania) and from a region east of the Carpathian Mountains (Bukovina). The level of enzyme polymorphism and the amount of morphometric variation were investigated in the sampled populations. As the western (Transdanubia) and the eastern populations (Transylvania plus Bukovina) were suggested to belong to different subspecies (Rákossy et al. in *Nota Lepidopterol* 33:31–37, 2010), our working hypothesis was that these two groups of populations belong to different evolutionary significant units (ESUs). The results of all genetic analyses (PCA, Bayesian-clustering analysis and especially the UPGMA dendrogram) confirmed our assumption. The level of differentiation was higher between the western and eastern populations resulting in two monophyletic lineages of *Phengaris (Maculinea) nausithous* in East-Central Europe. This result suggests that these lineages can be considered as two different ESUs.

Keywords Evolutionary significant unit (ESU) · *Maculinea nausithous* · Enzyme polymorphism · Genetic differentiation

Introduction

Professor Zoltán Varga is one of the most enthusiastic conservation biologists in Hungary. He recognised fairly early the significance of studying genetic variation in natural populations. In the late 70's, he established a molecular population genetic laboratory in his department. At that time the study of enzyme polymorphism was the most popular molecular method all over the scientific world. The first author of this paper has been working in this laboratory from the beginning. Later, generations of PhD students were educated

there. The main goal of Z. Varga, on the one hand, was to carry out various research projects in natural populations of endangered or vulnerable butterfly species; but, on the other hand, he intended to provide data for everyday nature conservation on the level and structure of genetic variation in these species. His aims were completely fulfilled and the results obtained in this population genetic laboratory were published in several scientific papers. The present study was also carried out in this laboratory. Moreover, some aspects of the results, especially regarding the *Phengaris (Maculinea)* species were used in practical nature conservation and helped to work out conservation strategies for these species.

In order to develop efficient conservation strategies for endangered butterfly species, it is of great importance to outline proper conservation units (Allendorf and Luikart 2009). The two most commonly discussed conservation units are evolutionarily significant unit (ESU) and management unit (MU) or functional conservation unit (FCU) (Funk et al. 2012). Out of these, the ESU was introduced by Ryder (1986) and characterised as a group of genetically

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and ecologically differentiated populations. Nonetheless, the definition of an ESU has changed over the past three decades. Moritz (1994) regarded this as a reciprocally monophyletic unit. A further operational concept of an ESU was introduced by Crandall et al. (2000) who emphasised the importance of ecological and genetic differentiation between different entities. With this in mind, the other important unit in conservation biology is FCU or MU, a group of demographically and ecologically independent populations (Maes et al. 2004; Allendorf and Luikart 2009; Funk et al. 2012). Thus, FCUs/MUs are often defined within ESUs and represent populations that are important for the long-term persistence of an ESU (Hughes et al. 1997; Vila et al. 2006; Funk et al. 2012). This implies that FCUs must be genetically, and perhaps adaptively, differentiated within an ESU (Palsbøll et al. 2007; Funk et al. 2012); it is therefore of utmost importance in practical nature conservation to delineate the FCUs of endangered species.

The dusky large blue *Phengaris (Maculinea) nausithous* (Bergsträsser 1779) has a highly specialised life cycle. Imagoes lay eggs on the larger, apical flower heads of *Sanguisorba officinalis* (Thomas 1984). Larvae develop through three instars in the flowers and green fruits (Elmes et al. 1991; Thomas and Wardlaw 1992). Then they drop on the ground and wait for being discovered by the foraging workers of the ant *Myrmica rubra* (Thomas 1984; Thomas et al. 1989; Tartally et al. 2008) who adopt and transport them to the ant nest. Caterpillars live there as social parasites for about 10 month during the fourth instar (Elmes et al. 1991). The species has a Euro-Siberian distribution with a wide but sporadic range from Western Europe through Southern Siberia to Mongolia (Lukhtanov and Lukhtanov 1994; Tuzov 1997; Wynhoff 1998; Munguira and Martin 1999; Rákósy et al. 2010). The European distribution of the species has a definite gap in the Carpathian Basin. It is locally frequent in the western and northern hilly regions of Transdanubia, but was known to be completely absent in the Pannonian lowland as well as in Transylvania (Rákósy et al. 2010). However, *P. nausithous* has recently been discovered in Transylvania near Cluj-Napoca at Răscrući and Fănațele Clujului (Rákósy et al. 2010). The ecology and host ant use of these two isolated populations are different from the Central European ones. Moreover, their appearance coincides with the original description of *P. nausithous kijevensis* (Sheljuzhko 1928). Therefore, Rákósy et al. (2010) suggested that the two Transylvanian populations belong to *P. nausithous kijevensis*.

Since *P. nausithous* is dependent on the presence of two sequential resources and lives in strictly localised small populations (Thomas 1984; Thomas et al. 1989), habitat fragmentation and isolation resulted in severe decline in many of its populations. Therefore, the aim of our study was to analyse the level and pattern of genetic and phenotypic

differentiation among the *P. nausithous* populations of the Carpathian Basin and those of Bukovina, east of the Carpathian Mountains. We were especially interested in the differentiation between the populations located west (western Transdanubia) and east (Transylvania and Bukovina) of the Pannonian lowland, i.e. of the gap in the distribution of the species. Based on the pattern of differentiation among these populations, we intended to identify the conservation units of *P. nausithous* in East-Central Europe. Our working hypothesis was that the eastern populations belong to a different subspecies (Rákósy et al. 2010), therefore these populations can be considered as different ESU-s. In agreement with our aims, we collected population samples from a western region (Western Transdanubia) and two eastern regions (Transylvania and Bukovina) and analysed the level enzyme polymorphism together with the amount of morphometric variation in these samples.

Materials and methods

Samples

P. nausithous samples originated from 3 regions: Hungary (Őrség region in Western Transdanubia: NYDT), Transylvania (close to Cluj Napoca: TRY) and Bukovina (east of the Carpathian Mountains: BUK). In the Transdanubian region we had 6, in Transylvania 3, and in Bukovina also 3 population samples (Table 1 and Fig. 1). On average, we obtained about 24 individuals per sample though the sample sizes varied greatly. 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 centigrade until electrophoresis.

Enzyme studies

Allozyme polymorphism was studied in all 12 populations at 17 different loci by vertical polyacrylamide gel electrophoresis: aconitase (*Acon*), acid phosphatase (*AcphB*), aldehyde oxidase (*Aox*), esterase (*Est*), glucose-6-phosphate dehydrogenase (*G6pdh*), glutamate dehydrogenase (*Gdh*), glutamate oxalacetate transaminase (*GotA* and *GotB*), α -glycerophosphate dehydrogenase (*α Gpdh*), hexokinase (*Hk*), isocitrate dehydrogenase (*IdhA* and *IdhB*), malate dehydrogenase (*Mdh*), malic enzyme (*Me*), phosphoglucose isomerase (*Pgi*), phosphoglucomutase (*Pgm*) and superoxide dismutase (*Sod*). Thoraxes and abdomens were homogenised separately in 350–400 μ l extraction buffers. Thorax samples were used to study *G6pdh*, *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 *Gdh*. The

Table 1 Sample sites of the *M. nausithous* populations

Region	Population	Abbrev.	Longitude	Latitude	N_{enz}	N_{morph}
NYDT	Nagygörbő	Ng	17.1852	46.9318	18	–
	Orfalu	Orf	16.2695	46.8789	12	–
	Szomoróc	Szo	16.3193	46.7819	36	–
	Óriszentpéter	Oszp	16.3955	46.8587	24	–
	Magyarszombatfalva	Mfa	16.3335	46.7579	46	18
	Kétvölgy	Kv	16.2267	46.8793	74	28
TRY	Fanatele A	FanA	23.6272	46.8313	22	–
	Fanatele B	FanB	23.6321	46.8414	59	25
	Rascruci	Ras	23.7290	46.9236	89	23
BUK	Radauti	Rad	25.9192	47.8425	24	–
	Solca	Sol	25.8367	47.7000	27	18
	Sucevita	Suc	25.7167	47.7833	32	–

N_{enz} sample sizes in the enzyme studies, N_{morph} number of males used in the morphometric studies. *NYDT* Western Transdanubia, *TRY* Transylvania, *BUK* Bukovina

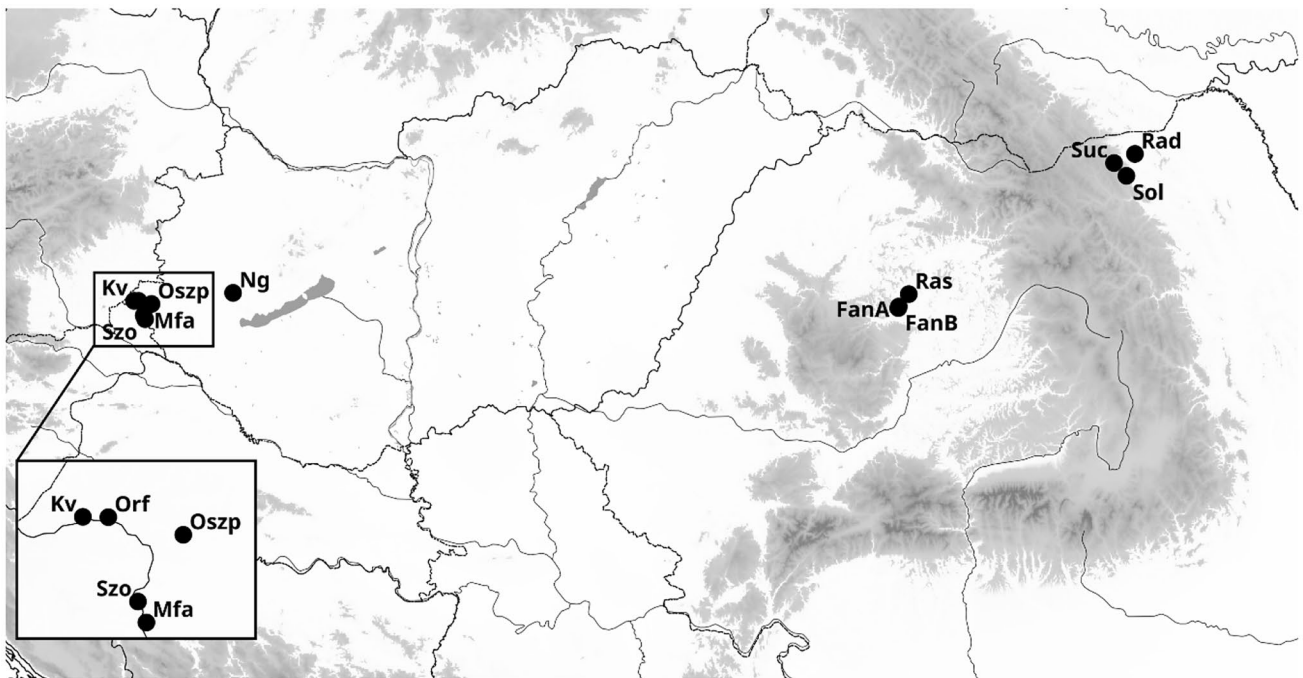


Fig. 1 *M. nausithous* samples in the Carpathian Basin and in Bukovina. Western Transdanubia (NYDT), Nagygörbő (Ng), Orfalu (Orf), Szomoróc (Szo), Óriszentpéter (Oszp), Magyarszombatfa (Mfa),

Kétvölgy (Kv). Transylvania (TRY), Fanatele A (FanA), Fanatele B (FanB), Rascruci (Ras). Bukovina (BUK), Radauti (Rad), Solca (Sol), Sucevita (Suc)

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 later separately. Not all samples were used in the morphometric analyses. Measurements were conducted

for two samples from Transdanubia, for two samples from Transylvania and for one sample from Bukovina (Table 1). Wings were fixed on transparency films and photographed with a Sony DSC-H2 digital camera. Measurements were completed on high-resolution digital photographs by computer using the IMAGE J 1.36 programme (Kizic and Borovac 2001). Four traits were measured both on the forewings and the hindwings. Two traits were distance metrics describing the size of the wings, while the other two were angle metrics

indicating the shape of them. Distances were determined between fix points. Anal length: 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: between the outer end of the radial vein (R3 on the forewing and Sc + R1 on the hindwing) and the outer end of the anal veins (1A + 2A on the forewing and 3A on the hindwing). Two angles were also measured both on the forewings and hindwings. Basal angle enclosed by the costal and anal margins and apical angle enclosed by the costal and outer margins. The morphometric study was only carried out on males, thus the sample sizes were slightly lower in these analyses than in the enzyme studies.

Statistical analyses

Statistical procedures aimed to compare the results obtained for morphological and genetic data could partly be compared. Variation was studied in two ways: both the amount and the structure of it were analysed.

Genetic data

Genotype and allele frequencies were calculated on the basis of banding patterns. Measures of genetic variation were calculated for each population sample using GENALEX 6.5 (Peakall and Smouse 2006) and FSTAT v.1.2 (Goudet 1995): average number of alleles per locus (n_A), allelic richness (A_r), proportion of fixed alleles (%Fix), average observed heterozygosity (H_o) and proportion of polymorphic loci using the 95% criterion (P_{95}). These parameters were compared among the geographic regions by performing ANOVA running GLIM 4 (Francis et al. 1994). The genetic structure of the populations was first analysed by applying principal component analysis (PCA) using the allele frequency data of the samples. The computation of allele frequencies was performed by GenAlEx6 (Peakall and Smouse 2006), while PAST ver.1.56 (Hammer et al. 2001) was used to carry out PCA. As a next step, Bayesian-clustering method was used (Pritchard et al. 2000) to estimate the most probable number of genetically differentiated groups (K) in our samples and assigned the individuals to these groups. STRUCTURE 2.3.2 (Pritchard et al. 2010) was run to carry out these analyses without population priors, using admixture model, an initial burn-in value of 100,000 and running length of 500,000 iterations. K was assumed between 1 and 12 repeating each run ten times. In the next step, we calculated the change in log-scaled probability between successive K values (ΔK) (Evanno et al. 2005), using STRUCTURE HARVESTER 0.6.91 (Earl and VonHoldt 2011). The distribution of total genetic variation at various levels of the hierarchy was also studied by AMOVA (Excoffier et al. 1992; Weir 1996). In this analysis, the total genetic variation was partitioned into

three components: between regions (BW), among populations within a region (WR/BP), and among individuals within a population (WP). AMOVA was carried out by ARLEQUIN version 3.11 (Schneider et al. 2000). Finally an UPGMA dendrogram was constructed (Sneath and Sokal 1973) on the basis of the Cavalli-Sforza and Edwards chord distances derived from the allele frequencies of the samples (Cavalli-Sforza and Edwards 1967). The statistical support of the branching pattern in the dendrogram was estimated with 3000 nonparametric bootstrap replicates (Felsenstein 1985). The percentages of replicates where the nodes are still supported are shown by the dendrogram. PAST ver.1.56 (Hammer et al. 2001) was used to process the dendrogram and carry out the bootstrap.

Morphological data

Phenotypic variation of the samples was characterised by the coefficients of variation calculated for each population (Sokal and Rohlf 1981). The average values were used to compare the level of variation among the regions. Pearson product moment correlation was computed between the genetic (%Fix and n_A) and morphometric (CV) measures of variation (Sokal and Rohlf 1981) using the programme GLIM4 (Francis et al. 1994). Multiple discriminant analysis (MDA) was computed in order to determine the most probable number of groups and to compare the distribution of canonical variates among these groups. SPSS 16.0 programme package was used for the computation of this analysis. The distribution of phenotypic variation at different levels of the hierarchy was analysed by a hierarchical ANOVA using 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 those in AMOVA.

Results

Level of variation in the populations

Genetic variation characterised by different parameters of enzyme polymorphism indicated a relatively low level of variation in the *P. nausithous* populations (Table 2: Total). Comparing these parameters among the regions the highest values were found for the Transylvanian populations. Nevertheless, none of the regional differences proved to be significant. (Table 2: ANOVA and P).

Morphometric variation was characterised by the coefficients of variation (CV) which were averaged separately over the traits characterising the size (distances) and the shape (angles) of the wings. The average CV values computed for the size measurements were 65% higher than those

Table 2 Average measures of enzyme polymorphism in the *M. nausithous* populations

Region	<i>Np/r</i>	<i>N/s</i>	<i>n_A</i>	<i>Ar</i>	%Fix	<i>Ho</i>	<i>P₉₅</i>
NYDT	6	34.4	1.94	1.42	0.395	0.095	0.412
TRY	3	54.9	1.88	1.64	0.166	0.173	0.588
BUK	3	27.4	1.65	1.41	0.322	0.099	0.471
Total	12	37.8	1.79	1.48	0.319	0.116	0.461
ANOVA	<i>F_{2,10}</i>	–	0.39	0.40	1.94	0.26	0.15
	<i>P</i>	–	0.687	0.683	0.194	0.776	0.864

NYDT Western Transdanubia, *TRY* Transylvania, *BUK* Bukovina. *Np/r* number of populations within the region, *N/s* average sample size, *n_A* observed number of alleles, *Ar* allelic richness (*N*=6), *Ho* observed heterozygosity, %Fix proportion of fixed alleles, *P₉₅* proportion of polymorphic loci using the 95% criterion. ANOVA comparison of the average measures estimated for the regions, *P* probability

processed for the angles (Fig. 2A). Moreover, the CV values of the size measurements averaged for the three regions differed considerably, indicating a lower variation of wing size in the Transylvanian region, compared to the other two ones. Nonetheless, the average CV values of the angles did not differ among the regions (Fig. 2A). These latter CV values, however, were different between the two wings, namely the averages of the hindwings were higher than those of the forewings. It thus appears that the shape of the hindwings is more variable than that of the forewings.

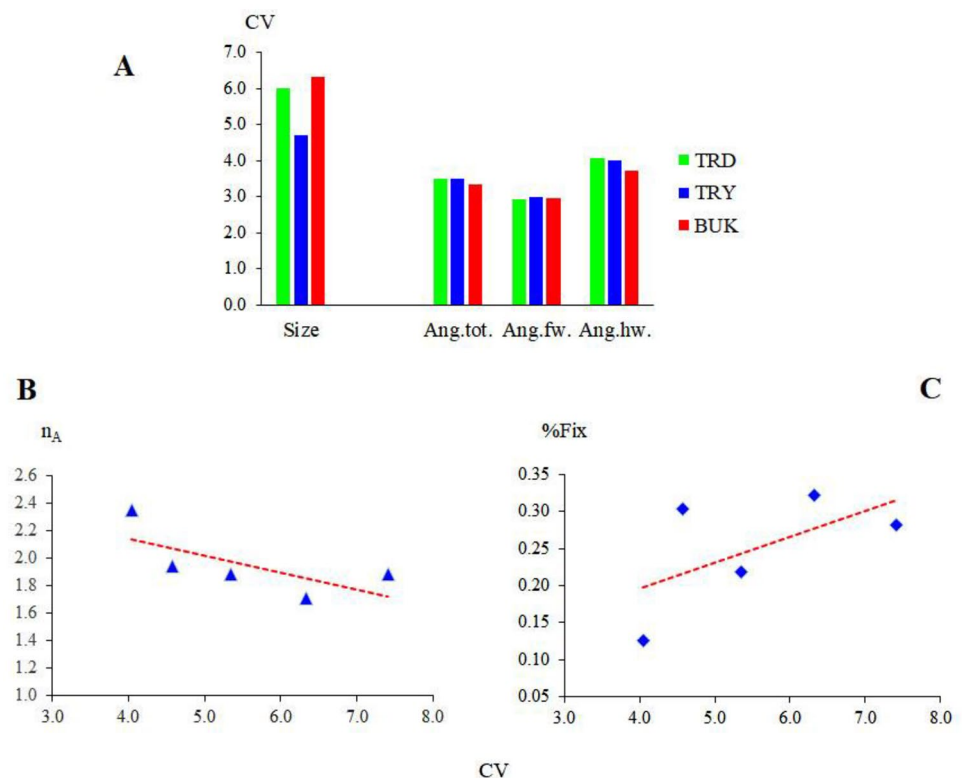
We have analysed the correlation between the parameters of enzyme polymorphism and the CV values averaged over the size and the shape measurements separately. None of these analyses resulted in a significant correlation, but all associations between the measurements of enzyme

polymorphism and the average CV values calculated for the size of the wings indicated a trend. Namely, as the level of genetic variation increased the amount of morphometric variation decreased. This trend was the strongest between the CV values computed for the size of the wings and the proportion of fixed alleles (Fig. 2C: %fix) as well as the average number of alleles per locus (Fig. 2B: *n_A*).

Structure of variation

In the analysis of the genetic structure of variation, a principal component of analysis was first computed. The results showed a clear differentiation among the three regions: the populations of western Transdanubia (NYDT) were clearly separated from those of Bukovina (BUK) and Transylvania

Fig. 2 Average values of variation coefficients (CV) estimated for the *M. nausithous* populations. **A** CV values were averaged over the regions for the measurements indicating the size of the wings (Size), for all angles (Ang.tot.), for the angles of the forewing (Ang.fw.) and for those of the hindwing (Ang.hw.). *NYDT* Western Transdanubia, *TRY* Transylvania, *BUK*: Bukovina. **B** Correlation between the observed number of alleles (*n_A*) and the CV values averaged over the size measurements of the wings in the five populations (*R*=0.692; *P*=0.196). **C** Correlation between the proportion of fixed alleles (%Fix) and the CV values averaged over the size measurements of the wings in the five populations (*R*=0.594; *P*=0.291)



(TRY) along the first axis (Fig. 3). This axis explained 66.8% of the total variation in allele frequencies and the most significant loci contributing to it were *Aox* and *Pgm*. The populations of Transylvania and Bukovina were differentiated along the second axis which explained 19.5% of the variation and was determined by *Idh* and *Pgi*.

Next, we carried out a Bayesian-clustering analysis in order to (i) estimate the most probable number of genetic clusters (K) and (ii) to assess the importance of the geographic origin of the populations. Accordingly, we ran STRUCTURE 2.3.2 assuming K between 1 and 12 repeating each run ten times. The most probable K values were selected using STRUCTURE HARVESTER 0.6.91. On the basis of the ΔK values, $K=3$ was estimated as the most likely number of genetic clusters in the *P. nausithous* populations. As a next step, the distribution of these genetic clusters was calculated for all individuals in all populations. We observed a considerable heterogeneity in the frequencies of the clusters. The distribution of the three clusters specified three genetic groups ('genetic regions') corresponding to the three geographic regions in the investigated populations (Fig. 4). The triangle plot of the cluster probabilities indicated a stronger differentiation between the Transdanubian populations and those of Transylvania together with Bukovina supporting the results of PCA. Moreover, certain alleles of the *Aox* and *Pgm* loci proved to be cluster specific: *Pgi2* for cluster

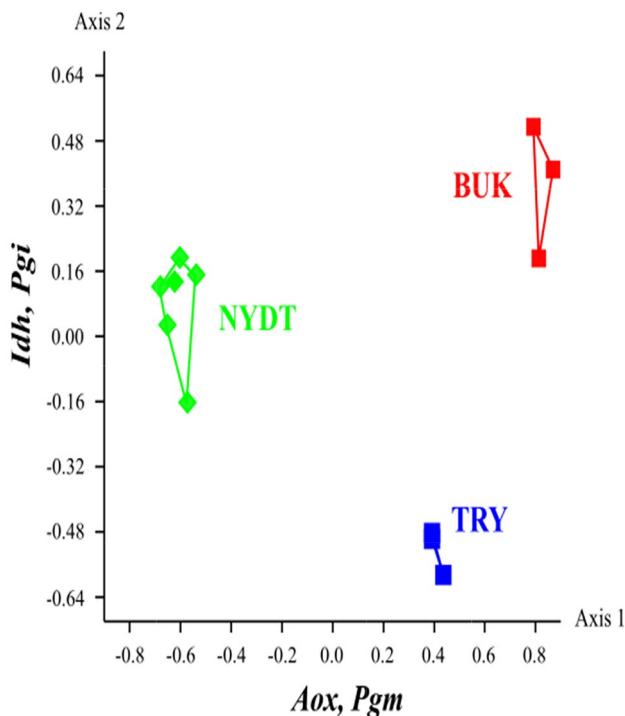


Fig. 3 The results of PCA in the *M. nausithous* populations. NYDT Western Transdanubia, TRY Transylvania, BUK Bukovina. The first two axes explained 86.3% of the total genetic variance. The loci contributing most to the axes are presented

1 (characteristic for the Transdanubian populations), while *Aox4* and *Aox5* for cluster 3 (typical for the populations of Bukovina).

Phenotypic differentiation was first analysed by a multiple discriminant analysis using the morphometric data of the individuals. The results revealed that the regions were significantly differentiated along the first root ($\chi^2=49.7$; $DF=20$; $P<0.001$). The apical angle of the forewing contributed most to this root. The distribution of the canonical variates along this root clearly showed the differentiation of the Transdanubian individuals from those of the other two regions (Fig. 5).

The next step in the analysis of genetic variation was to compute an AMOVA. The results showed that the highest portion of variation (75.1%) could be attributed to the within-sample component, defined as the variation among the individuals (Fig. 6: Gen.). A fairly high percentage of the genetic variation (23.1%) was explained by the differences among the regions indicating a clear differentiation among them (Fig. 6: Gen.).

Simultaneously, a hierarchical ANOVA was carried out on the morphometric data. In agreement with the distribution of genetic variation, these results also suggested that the differences among the individuals contributed most (90.7%) to the phenotypic variation, whereas the differences among the three regions only explained 5.6% of the total phenotypic variation (Fig. 6: Morph.). Thus, in accordance with the results of the multiple discriminant analysis, hierarchical ANOVA also indicated a clear but rather low level of morphometric differentiation among the regions.

Finally, an UPGMA dendrogram was constructed on the basis of the Cavalli-Sforza and Edwards chord distance matrix. In this analysis, three samples of *Phengaris (Maculinea) teleius* were used as outgroups. These outgroup samples were clustered in a separate branch of the dendrogram supported by a high bootstrap value (Fig. 7). The *P. nausithous* samples were clustered in two main branches of the dendrogram also supported by a high bootstrap value (Fig. 7). These two *P. nausithous* branches corresponded to the western and eastern geographic regions: western Transdanubia (NYDT) and Transylvania (TRY) together with Bukovina (BUK). The populations of these two eastern regions were also separated in two branches but this separation was supported by a lower bootstrap value (Fig. 7). This result, similarly to that of PCA and the Bayesian-clustering analysis, indicated a stronger differentiation between the populations situated on the West and East side of the gap in the East-Central European distribution of this species.

Discussion

Phengaris (Maculinea) nausithous is an obligate myrmecophilous butterfly i.e. its larvae can only develop in the nests of *Myrmica* species (Thomas 1984; Thomas et al. 1989). In

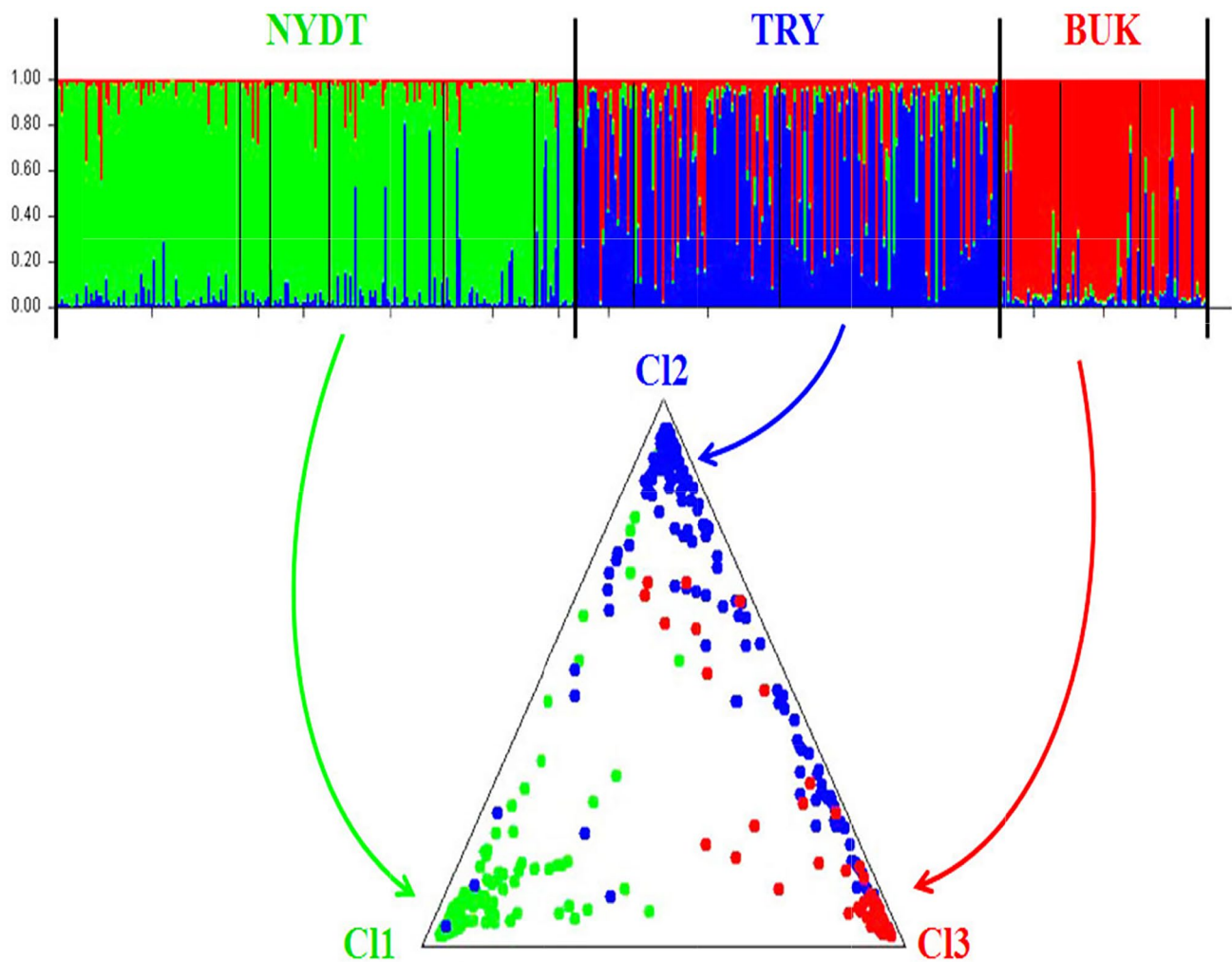


Fig. 4 The results of the Bayesian-clustering analyses in the *M. nausithous* populations. The most probable number of clusters was $K=3$. *NYDT* Western Transdanubia, *TRY* Transylvania, *BUK* Buko-

vina. CI1, CI2 and CI3: the assumed clusters. The columns (Upper part of the figure) and the dots (lower part of the figure) represent the cluster probabilities estimated for the individuals

Europe, *P. nausithous* mostly uses *M. rubra* as a host ant (e.g. in Transdanubia and Bukovina) but in Transylvania it parasitizes *M. scabrinodis* (Tartally et al. 2008; 2019). Since females lay eggs on the food plants regardless of the distance to the nests of the host ants (Thomas and Elmes 2001; Árnys et al. 2006), larvae drop to the ground at random. Considering that a large portion (approx. 90%) of larvae die not discovered by the ant workers we can assume that *P. nausithous* populations are strongly affected by genetic drift. As a consequence, a relatively low amount of variation together with a fairly high level of differentiation is expected in its populations (Pecsenye et al. 2007). Our results fulfilled these expectations as we found a relatively low level of enzyme polymorphism in the investigated populations with an average number of alleles of $n_A=1.79$ (average of other lycaenid species $n_A=2.23$ (Pecsenye et al. 2015)), and an average heterozygote frequency of $H_o=0.116$ (average of

other lycaenid species $H_o=0.209$ (Pecsenye et al. 2015)). The low level of enzyme polymorphism was coupled with a high level of differentiation. The overall fixation index was $F_{ST}=0.195^{**}$ which is close to the value estimated by Figurny-Puchalska et al. (2000) for the Polish populations ($F_{ST}=0.153^{**}$).

The results of all analyses indicated a regional pattern of differentiation in the investigated *P. nausithous* populations (Hollós et al. 2012). At the same time, the outcome of the PCA and the Bayesian-clustering analysis of the genetic data together with the results of the multiple discriminant analysis of the morphometric data suggested a stronger differentiation between the western (Western Transdanubia) and eastern (Transylvania plus Bukovina) regions. Based on the differences in morphology and ecology observed in the Transylvanian *P. nausithous* populations, Rákósy et al. (2010) suggested

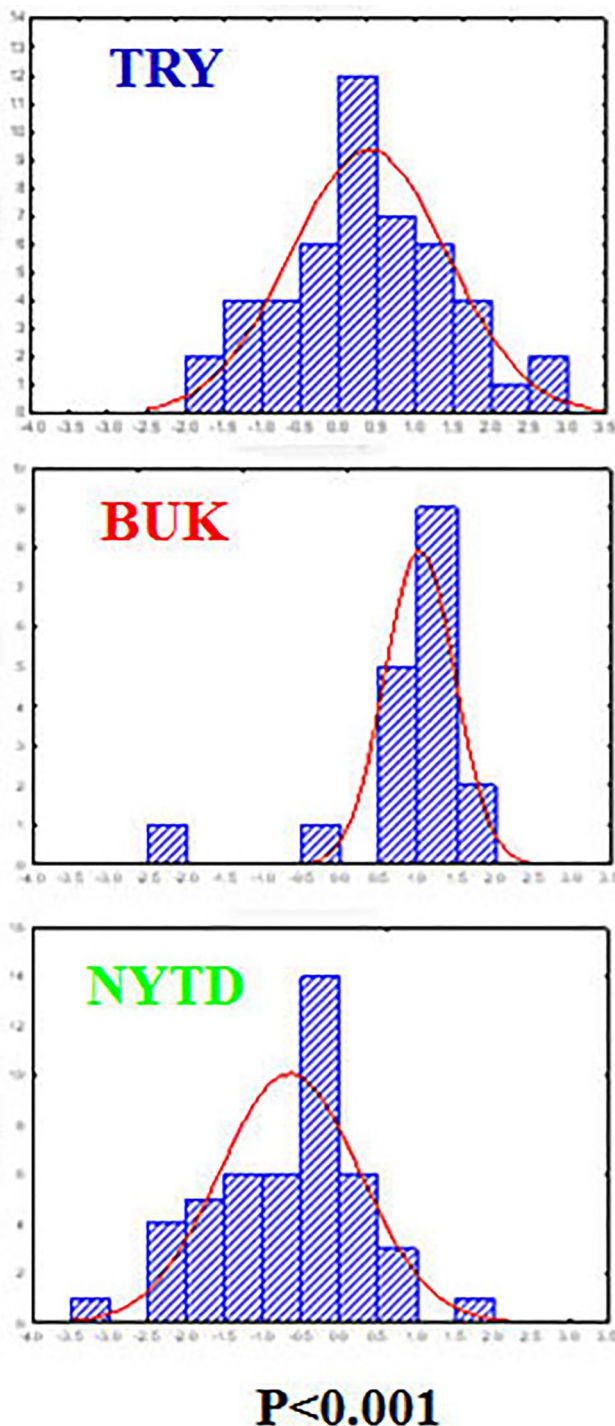


Fig. 5 The results of the linear discriminant analysis in the populations of *M. nausithous*. The distribution of the canonical variables along the first root indicated significant differences among the three regions. *NYTD* Western Transdanubia, *TRY* Transylvania, *BUK* Bukovina

that these populations belong to a different subspecies, namely to *P. nausithous kijevoensis* described by Sheljuzhko (1928). The dendrogram constructed on the basis of

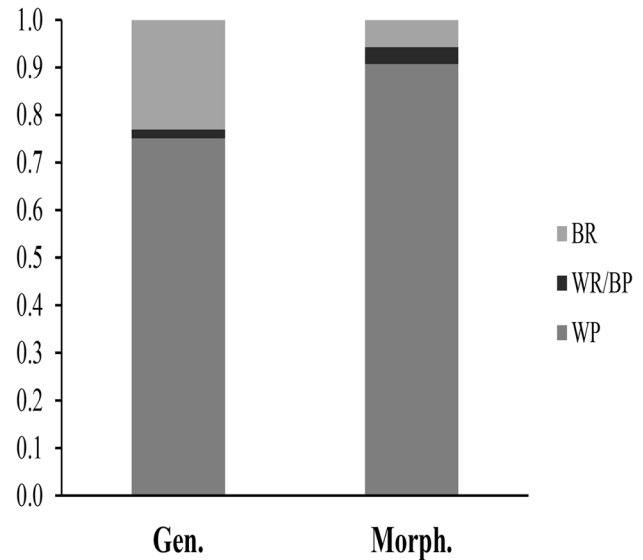
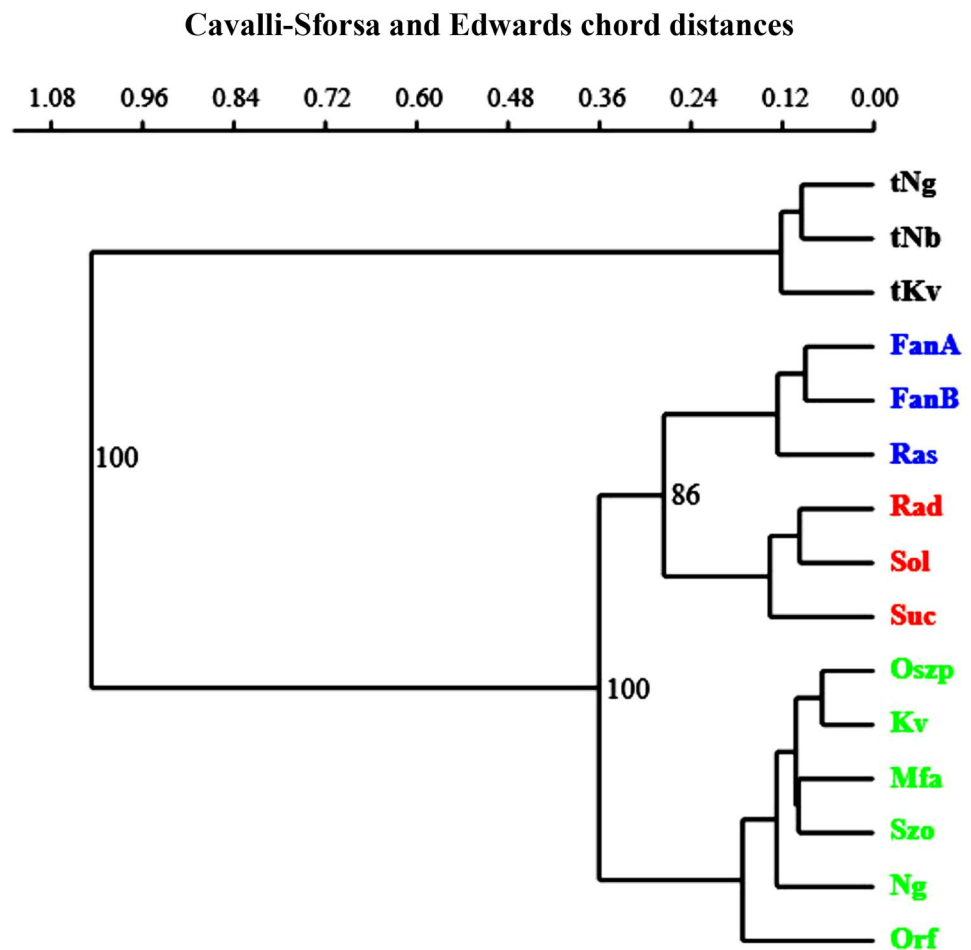


Fig. 6 The results of AMOVA (Gen.) and ANOVA (Morph.) in the *M. nausithous* populations. Levels of hierarchy: between regions (BR), among populations within a region (WR/BP) and among individuals within a population (WP)

the Cavalli-Sforza and Edwards chord distances showed most explicitly the differentiation between the western and eastern *P. nausithous* regions of East-Central Europe, as a few samples of *Phengaris (Maculinea) teleius*, another predator *Phengaris* species, were also included in this analysis. Three main branches of this dendrogram were supported by a high bootstrap value: *P. teleius* samples, the *P. nausithous* populations of western Transdanubia and those of the two eastern regions (Transylvania and Bukovina). It thus appears that the western and eastern populations of *P. nausithous* can be described as mutually monophyletic lineages. Keeping in mind that there is a great gap in the distribution of *P. nausithous* in East-Central Europe which is situated in the Pannonian lowland, our results suggest that these two monophyletic lineages are not only strongly differentiated from each other but they also have distinct distribution areas. As a consequence, the western and eastern populations of this species can be considered as different Evolutionary Significant Units (ESUs) (Moritz 1994; Crandall et al. 2000) and they probably belong to different subspecies as it was proposed by Rákósy et al. (2010). These conclusions were also supported by Ritter et al. (2013) who described a high level of differentiation between the Central- and East-European *P. nausithous* populations on the basis of mtDNA COI sequence and microsatellite data. They also found that the genetic composition of the Transylvanian populations is similar to that of the East-European and Asian populations. Nevertheless, they also detected strong differentiation between two genetic lineages in the West-European

Fig. 7 UPGMA dendrogram constructed on the basis of the Cavalli-Sforza and Edwards chord distances computed for the *M. nausithous* populations. Three *M. teleius* populations were used as outgroups (black letters). *NYDT* Western Transdanubia (light green letters), *TRY* Transylvania (blue letters), *BUK* Bukovina (red letters). Values at the nodes of the branches indicate bootstrap percentages from 3000 iterations



populations of *P. nausithous*. The genetic differentiation of the *P. nausithous* populations in the Carpathian Basin also showed a geographic pattern in the study by Bereczki et al. (2015). They concluded that the mtDNA sequence data together with the results of the microsatellite and allozyme studies reflect the biogeographical history of the species, since the differentiation of the large geographic regions coincides the disjunct distribution of *P. nausithous*.

Furthermore, our results (especially those of the PCA and the dendrogram) also indicate a clear but a lower level of differentiation between the two eastern regions (Transylvania and Bukovina). This suggests that their genetic composition is specific to a certain degree. Moreover, populations of these two regions use different host ant species (Tartally et al. 2008; 2019) implying a certain level of ecological differentiation between them. Funk et al. (2012) defined the functional conservation units (FCU) as groups of populations with specific genetic composition which contribute significantly to the genetic diversity of an ESU. Based on this definition, we can assume that the populations of Transylvania and Bukovina belong to two different FCUs within the eastern ESU of *P. nausithous* (probably within the *P. nausithous kijevenensis* subspecies).

Conclusions for future biology

Keeping in mind the significance of nature conservation nowadays, the study of genetic variation in natural populations seems to be an everlasting research field. While the aims of such research works are hardly changed, the methods to fulfil them are developing fast. New DNA techniques together with evolving statistical procedures provide more and more efficient tools to obtain deeper insight in the genetic composition and the past history of the species. Currently, the microsatellites are the most widespread markers in these studies. Nevertheless, a new technique, the analysis of SNPs (single nucleotide polymorphisms) via sequencing longer stretches of DNA in several individuals of the populations, is also expanding.

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Declarations

Conflict of interest The author declares no conflict of interest.

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