Final report of the research project OTKA K84071
Marginal and expanding populations of conservation biologically significant species in the Carpathian basin

Introducton: milestones of the project

Due to the nearly continuous research activity of our Conservation Genetics group from the 1990ths (OTKA ; MacMan, FaPa) we have both many conserved samples and long data sets on several target species used in this project. Therefore we could publish the results of most surveys within this periods in 24 peer-reviewed (IF=36.766) and 7 further communications, although some surveys will be closed and published in the nearest future. Two participants of the project (E. Juhász [Bátori] and J.P. Tóth) earned the Ph.D. and J. Bereczki received the Bolyai Research Fellowship from the HAS. Some results of the project have been presented in the 2014/4 number of the journal Magyar Tudomány, at the international workshop "When Phylogeny and Geography Meet Conservation" (Debrecen, 2014) and in the invited lecture of the Principal Researcher (Senckenberg Entomology Congress 2015, Frankfurt/Main).

We formulated the objectives of our research programme based on the recent advances of Quaternary research (e.g. Birks & Willis 2008; Stewart & Dalén 2008; Svenning et al. 2008; Magyari et al. 2013), the new knowledge on cryptic refugia, micro-refugia and the new view on glacials (e.g. Stewart & Lister 2001; Provan & Bennett 2008; Rull 2009; Stewart et al. 2010; Varga 2010), and the evolutionary and conservation biological significance of „leading edge vs. rear edge" populations (Hampe & Petit 2005; Petit et al. 2005; Bilton et al. 2006). The significance of "leading edge" vs. "rear edge" populations and their chances for survival have been surveyed also in connection with recent climatic change.

Based on these general considerations we have outlined a four-step research programme:

- *Step one*: Palaeo-ecological reconstruction and modelling of LGM ranges of species which have recent range edges in the Carpathian basin. Comparative phylogeography of faunal types with packing edges of distribution (see: questions 1-3).
  Publications: Tóth et al. 2012; Schmitt & Varga 2012; Pecsenye et al. 2015; Tóth et al. 2015 in prep.
- *Step two*: Reconstruction of coalescence time in species with genetically divergent populations and/or "phylogeographic rendezvous" with or without hybridisation in the Carpathian basin (see: questions 2-4).
  Publications: Tóth et al. 2014; Szabó & Vörös 2014; Bereczki et al. 2015.
- *Step three*: Reconstruction of the expansion vs. retreat, and of bottleneck vs. population growth periods of species in connection with the palaeo-environmental and recent climatic changes (see: questions 5-6).
  Publications: Bereczki et al. 2014; Pecsenye et al. 2014; 2015 subm..
- *Step four*: Prediction of their possible reactions of the species with range edges and/or hybrid zones in the Carpathian basin on climatic change scenarios. Which are the probable reactions of their marginal populations, and the chances for survival in a "traditional" vs "modern" cultural landscape?
  Publications: Tóth & Varga 2011; Bátori et al. 2012a,b; Szanyi et al. 2014; Végvári et al. 2014; Varga 2015.
- *Additional results*: we achieved considerable progress in the clarification of some long unsolved taxonomical questions in *Maculinea* (= *Phengaris*), *Melitaea* and *Euphydryas* species which also have significance in conservation of Evolutionarily Significant Units.
  Publications: Tóth & Varga 2011; Tóth et al. 2014; Bereczki et al. 2014, 2015; Tartally et al. 2014; Pecsenye et al. 2014, 2015.

Within this research programme we had to answer the following questions:

- *What are the structural and genetic characters of marginal populations of species which have northern-northwestern edges of distribution in the Carpathian basin (flightless vs. mobile species of Orthoptera e.g. Paracaloptenus caloptenoides, Stenobothrus eurasius)?*

  ► We found evident metapopulation network of populations in a flightless but mobile predatory bush-cricket, a partial network in a grasshopper with flightless females, and isolation of populations in a flightless grasshopper sp. High frequency of macropterous individuals was found in expanding bush-cricket species as possibly response of warming.
- *Are there any similar pattern in the genetic structure of marginal populations of allo- or parapatric sister taxa which expanded into the Carpathian basin from different directions, from different LGM refugia (Orthoptera: Pseudopodisma spp.; Lepidoptera: Parnassius mnemosyne, Euphydryas aurinia; Amphibia: Mesotriton alpestris ssp., Triturus dobrogicus-carnifex; Reptilia: Anguis fragilis-colchicus)?*

  ► More, mostly extra-Mediterranean and/or Carpathian core areas and multiple directions of re-colonisation was demonstrated in Lepidoptera: *Parnassius mnemosyne, Euphydryas aurinia*; Amphibia: *Mesotriton alpestris* ssp., *Triturus dobrogicus – T. carnifex*; Reptilia: *Anguis fragilis – A. colchicus.* Some marginal populations proved to be genetically diverse (*Parnassius mnemosyne, Aricia artaxerxes, Anguis* spp.). In other cases the level of genetic variation was rather moderate (*Maculinea* spp., *Euphydryas* spp.).
- *Are there any difference between the congeneric, but ecologically different species (Pseudopodisma spp., Euphydryas maturna – E. aurinia, Melitaea phoebe – M. ornata (= telona), sibling species of the Triturus cristatus-group, Anguis fragilis species group) in their genetic structure?*

  ► Our expectations were fulfilled, although they are similar to a certain degree in their reproductive strategies, they essentially differ in their ecological niche (incl. larval and adult behaviour). Essential differences were demonstrated in the „cuckoo vs. predatory" *Maculinea* species, and in the species pairs: *Melitaea phoebe – E. ornata*, *Euphydryas maturna – E. aurinia*, *Triturus* spp., *Anguis* spp.
- *What is the level and pattern of genetic differentiation between the partly synpatric/ syntopic and possibly hybridising sibling species (Melitaea phoebe – M. ornata (telona), Triturus dobrogicus – T. carnifex, Anguis fragilis – A. colchicus). Whether the genetic differentiation can be explained by the reproductive interference*?

  ► No general rule in the level of the genetic diversity was discovered. We could not find any genetic signal of hybridisation in the *Melitaea phoebe – M. ornata* pair, probably due to the strong food plant differentiation. No genetic signal of reproductive interference was observed. Different geographical patterns of variation were experienced in sibling species of *Triturus* and *Anguis*.
- *Are there significant differences in the genetic patterns between marginal populations of non-obligatory (Plebejus, Aricia) vs. obligatory myrmecophilous (Maculinea) lycaenid butterfly species? What population genetic factors (selection, genetic drift connected with bottle necks during life cycle, gene flow, etc.) can explain these differences?*

  ► High or very high level of genetic variation was found in non-obligatory (*Plebejus, Aricia*), but rather moderate (in some cases with geographical pattern: *M. alcon*) was found in obligatorily myrmecophilous *Maculinea* species. Serious bottlenecks were observed in some declining populations. Gene flow and similar level of *Wolbachia* infestation was demonstrated in different phenological forms of *Maculinea arion*.
- *Can we detect coherent trends of genetic differentiations in model species belonging to different major taxa (Orthoptera, Lepidoptera, Amphibia, Reptilia) which reflect on LGM refugia and migration directions?*

  ► We published a highly cited (over 30 citations in 2 years!) review paper on the extra-Mediterranean and Carpathian refugia.

We review the main results as follows according to the publications

Results connected with the Large Blue (*Maculinea*) species

One of the significant results is the taxonomic clarification of the excessively discussed and hitherto misinterpreted *Maculinea rebeli* Hirschke, 1905 together the discovery of the initial host plant *Gentianella rhaetica* and the host ant species *Myrmica sulcinodis*, in the region of the type locality. Our data confirm that *Ma. rebeli* is an ecotype of high altitudes, and not mere an individual form (oppositely to Kudrna & Fric 2013) but also not identical with the xerophilous ecotype using *Gentiana cruciata* as initial food plant and *Myrmica sabuleti, My. schencki*, etc. as host ant species.
More details in: Tartally et al. 2014. Zookeys 406: 25–40.

The morphological and phenological duality of some *Maculinea* species was repeatedly discussed but remained hitherto unsolved. In our first publication (Bereczki et al. 2011) we could not demonstrate any genetic differentiation of allozyme patterns in the phenologically differentiated populations of the Large Blue (*M. arion*). Therefore in our recent survey we used four character sets (mitochondrial sequences, allozymes, male genitalia and wing morphometrics). Comparative analyses of distance matrices, phylogenetic trees and ordination patterns have been applied. The genetic and morphometric patterns revealed by our studies were discordant. While we experienced a significant differentiation between the 'spring' and 'summer type' of *M. arion* in both wing and genital traits, the two phenological forms did not show any genetic differentiation on two mitochondrial loci and in allozymes.
Since we experienced clear geographical pattern hardly explained by climatic factors, we proposed that the wing traits involved in our study are probably determined genetically rather than environmentally. This pattern may also be in relation to the different host-ant use. At the same time, good agreement has been revealed between the outcome of the molecular studies and that of male genitalia morphometrics in numerous analyses. This suggests that selective pressures controlling genital structures are relatively homogeneous across taxa and the patterns of divergence in genital morphology may reflect overall genetic divergence rather than differential adaptive responses. Thus, quantitative traits of male genitalia may be good estimators of the divergence among populations and related species and generally considered reliable taxonomic characteristics for traditional systematic work at the species level.
Our molecular and morphometric analyses lead to discordant results, but we have to consider some basic facts. First, the diversity of the barcoding gene is proved to be reduced presumably due to *Wolbachia* infection. Second, the allozymes of *Maculinea* species are generally less polymorphic than those of other European lycaenid species. Therefore, it is possible that allozyme and mitochondrial DNA studies are not suitable for the detection of the divergence between the 'spring and summer *arion*'. Nevertheless, our morphometric studies have revealed significant differences between *M. a. arion* and *M. a. ligurica*. Our study clearly indicates that the combined use of different approaches and data sets is highly necessary to clarify systematic and evolutionary relationships among taxa despite the fact that molecular data often tend to receive more emphasisthan morphological ones. Although we did not find differences between the two forms of M. arion on the basis of molecular data, it is not at all unlikely that our markers are not suitable for the detection of the divergence between them. It is possible that all extant differences of the two forms are attributable to (1) differenthost-ant use, (2) incipient speciation, (3) cytoplasmatic incompatibility (CI) by *Wolbachia* or a combination of these factors. Further molecular and ecological studies are needed to elucidate the pattern of variation.
Further details in: Bereczki et al. 2014. *Journal of Zoological Systematics and Evolutionary Research* 52(1): 32-43.

The results of our surveys on *M. arion* have shown that further surveys needed on *Wolbachia* infection and the genetic diversity of *Maculinea* butterflies in the Carpathian Basin. The prevalence and the pattern of the infestation highly differ among *Maculinea* species. *M. alcon* and *M. arion* are infected in 100 %, each of these species with a single strain. Simultaneously, all studied individuals in the Polish and Italian populations of M. arion proved to be infected with one strain (Sielezniew et al. 2012; Patricelli et al. 2013). ; The *Wolbachia* 16S rRNA gene sequences were identical in all studied European populations in *M. alcon* and in *M. arion*, separately, but the two types of strains harboured by these species did not mix with each other in any particular case probably because there is cytoplasmic incompatibility between them. The infestation pattern, i.e. 100 % prevalence of a single strain each in *M. alcon* and *M. arion* refers to the perfect vertical transmission of *Wolbachia* in these hosts.

The effects of *Wolbachia* on these hosts' gene diversity seem to be variable in the different European regions. The Polish and Lithuanian populations of *M. alcon* have a complete lack of mitochondrial sequence variation. Moreover, barcoding gene sequences proved to be identical from Western Europe to Kazakhstan and Kyrgyzstan. The Polish populations of *M. arion* also have restricted mtDNA variation. However, mitochondrial haplotype diversity varies according to geographical regions in Italy. In both species, the nuclear elongation factor 1α characterized by low substitution rate showed higher variability than the mitochondrial sequences whose evolutionary speed is commonly known to be high. On the contrary, in the Carpathian Basin, nuclear variation based on 1680 bases from three different gene regions (GAPDH, MDH and wingless) was lower even than the restricted mitochondrial diversity, thus the nuclear data were uninformative to reconstruct the phylogeography of these species. In addition, neutrality tests did not result in significantly negative values in any of thesespecies. Therefore, we could not find evidence for *Wolbachia* induced selective sweeps which arose in the previous studies in spite of the presence of a single strain and the highly reduced mitochondrial variation. Additionally, it seems that not *Wolbachia* are responsible for the controversial patterns of mitochondrial and morphological variability of the different forms of *M. arion*. The results of the present study do not support the 'one barcode–two species' hypothesis. On the one hand, the allozymes do not show differentiation between the phenological forms. Although allozyme diversity of Maculinea species is generally low compared with other lycaenids (Schmitt et al. 2003; Schmitt and Hewitt 2004; Aagaard et al. 2002), it is suitable for species separation in this genus (Pecsenye et al. 2007).

The infection level of *M. nausithous* and *M. teleius* is much lower than in *M. alcon* and *M. arion*. Additionally, these hosts – even in a single population of *M. nausithous* – are infected with multiple strains belonging to supergroup A or B. This infestation pattern refers to incomplete vertical transmission of *Wolbachia* in these host species and extensive horizontal transfer in/between them. Although several potential horizontal transfer routes can exist in all *Maculinea* species, e.g. transfer by *Myrmica* host ants being in regular physical contact with caterpillars or transfer by parasitoids, the lifecycle of *M. teleius* and *M. nausithous* is closely related to each other in many respects. They can use the same host ants and even the same ant nest may harbour *M. teleius* and *M. nausithous* individuals since they often occur in the same habitat (Tartally 2008). Additionally, caterpillars can be parasitized by the same wasp species, e.g. *Neotypus melanocephalus* which infects the young larvae on the food plant. Namely, extensive horizontal transfer routes can exist to mediate *Wolbachia* in both species (and between them) which corresponds with the presumably incomplete vertical transmission. *M. teleius* and *M. nausithous* seem to be more resistant to *Wolbachia* than *M. alcon* and *M. arion* which is inferred from their much lower infection rate and the attempted invasion of multiple strains into these hosts. Probably different strains try to colonize these hosts and they could compete with each other.

Previously, cryptic speciation has been hypothesized for M. teleius and M. nausithous, based on deep mitochondrial split in each of these species. Ritter et al. (2013) tested the theory of cryptic speciation on a comprehensive sample across the Palaearctic ranges and revealed that deep mitochondrial divergence did not correspond with microsatellite data but was concordant with *Wolbachia* infection in both species. Haplotypes previously attributed to cryptic species were part of *Wolbachia*-infected clades thus deep intraspecific divergences found in DNA barcode studies coincide with specific infection patterns. In contrast with these results, in the Carpathian Basin, mitochondrial haplotypes of specimens infected with *Wolbachia* formed separate clades neither in *M. nausithous* nor in *M. teleius*. Although the genetic diversity was the highest in *M. nausithous*, the phylogenetic and allozyme patterns mainly reflect the biogeographical history of the species since the great part of the variability arises from the differentiation of the large geographic regions which coincides the disjunct distribution of *M. nausithous*. Namely, *M. nausithous* only occurs on the Western and the Eastern edges of the Carpathian Basin with a large distributional gap in the centre where the species is missing. The structured haplotype network reflects complex phylogeographical pattern in *M. nausithous*. Beside the various Asian haplotypes, the European populations are also divided to distinct haplogroups which suggest that the species survived during Pleistocene ice ages within different European glacial refugia. Therefore, it seems that biogeographical factors and Wolbachia infestation could shape together the genetic structure of *M. nausithous*. Simultaneously, the diversity of *M. teleius* was very low on the basis of all the studied genetic marker sets and neutrality tests did not lead to significantly negative results. The haplotype network that includes specimens from the whole Palearctic shows the higher diversity of the species in Eastern Asia. This elevated genetic variability refers to isolated refugial areas in this region owing to the complex topography.

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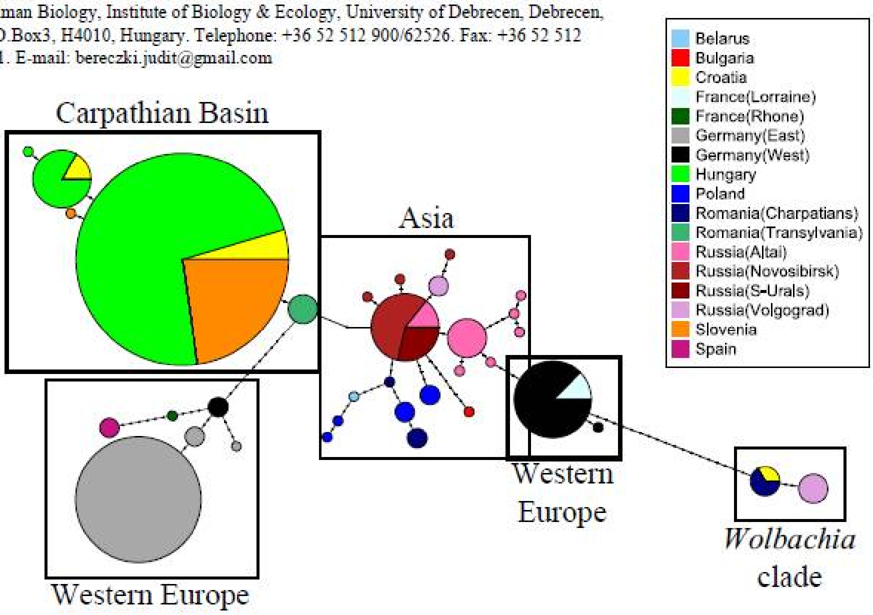


Based on our results, we cannot hold *Wolbachia* responsible for the highly reduced genetic diversity of the target species exclusively. Probably several factors shape together the level and pattern of genetic variability in *Maculinea* butterflies, e.g. biogeography and/or population dynamics. *Wolbachia* are only one of these factors but their importance is very high not only in terms of evolutionary but also conservation biology. A case study about North American Lycaeides butterfly species emphasizes that *Wolbachia* has to be considered before introducing individuals from one population to another because they may cause failure

of introduction attempts due to CI. Introducing individuals from a source population infected with *Wolbachia* to another population infected with another strain can destroy the population that we wanted to save originally. Therefore, we cannot work out and execute reintroduction action plans responsibly without considering *Wolbachia* infection.
Further details in: Bereczki et al. 2015. *Organisms Diversity & Evolution* 10.1007/s13127-015-0217-7.

The other focal point of conservation genetics and phylogeography of *Maculinea* species is the presence or lack of geographical pattern and its connection with the cuckoo or predatory life history. *Maculinea* species are known to have a relatively low genetic polymorphism due to successive bottlenecks in their life cycle. Our results partly fulfilled these expectations as we found a relatively low level of enzyme polymorphism in both species *M. alcon* and *M. teleius* compared to other European lycaenid butterflies studied. High level of polymorphism was detected in some *Polyommatus* species, in *Kretania sephirus, Aricia artaxerxes, A. agestis* and *Cupido minimus* (see: Pecsenye et al. 2005; 2007).
We hypothesised that a strong genetic drift due to the parasitic life cycle of the two *Maculinea* species induces high levels of differentiation among their populations. Although all *Maculinea* species are social parasites of different *Myrmica* species with a similarly specialised life cycle, we found significant differences between *M. alcon* and *M. teleius* in their genetic structure. These findings may be the consequences of the difference in their initial food plants and also in parasitisation mode of their host ants. The differences in the distribution of host plants and in the females' behaviour during ovipositon result in an uneven distribution of eggs in *M. alcon* populations compared to *M. teleius*. As a consequence, a stronger effect of drift is expected in *M. alcon* than in *M. teleius*. Thus, we can assume a lower level of variation combined with a higher level of differentiation in *M. alcon* populations.
We found exactly what was expected according to the above hypothesis: most parameters of polymorphism were significantly higher, while every index of differentiation was lower in *M. teleius* than in *M. alcon*. Nevertheless, more intense drift and consequently stronger differentiation in *M. alcon* alone cannot explain the differences we found between the two species in their genetic structure (e.g. regional differentiation as reflected by Nm values). Explicitly, strong differentiation among populations would generate a random structure and would spoil any specific (e.g. geographic or ecological) pattern of genetic differentiation. In contrast to this expectation, a clear regional differentiation was detected in *M. alcon* while it was less evident in *M. teleius*. The inconsistency between the expectations and the results especially in *M. alcon* indicated that differences between the two species in their genetic structure require other explanation than the variation in the distribution of eggs.
Further details: Pecsenye & Varga 2015. Conservation Genetics 08/2015; 16(4)DOI:10.1007/s10592-015-0713-5.

Results of surveys on non-obligatorily myrmecophilous Lycaenidae

High vs. declining genetic diversity in *Aricia artaxerxes*

The populations of the Carpathian endemic subspecies of the Brown Argus *A. artaxerxes issekutzi* proved to be extremely polymorphic. On average we found slightly than three alleles per locus (3.3) and a mean frequency of heterozygotes over 30 %. At the most polymorphic loci *Est*, *Pgi* and *Pgm*, resp., 17, 11 and 9 alleles were detected. This indicates far more genetic variation than that observed in other European lycaenid butterflies, e.g. in *Cupido minimus* (Habel and Schmitt 2009), *Polyommatus* spp. (Schmitt & Seitz 2001, 2002; Schmitt et al. 2002, 2003; De Keyser et al. 2012), and even in other populations of *Aricia* spp. (Aagaard et al. 2002; Habel and Schmitt 2009) but they are in agreement with those of our preliminary study on the Bükk and Karst populations of *A. artaxerxes issekutzi* (Pecsenye et al. 2007). There are several possibilities to explain the difference between the outcomes of the two surveys. It might be due to methodological differences or to the different biogeographical status of the populations. However, we also observed that rapid decline of population size gives rise to loss of genetic variation and may result in a reduction in fitness. It is, therefore, essential to detect past demographic changes in populations of endangered species.

Aricia artaxerxes occurs in two regions of Northern Hungary: Bükk Mts. and the Aggtelek Karst region. These are peripheric populations considering the total Eurasiatic range of the species and represented by the endemic Carpathian subspecies *A. artaxerxes issekutzi*. It prefers short-grass habitats, which are subject to succession. Due to climatic differences between the two regions succession is more rapid in the Bükk plateau resulting in the decline and isolation of *A. artaxerxes* populations there. In contrast, most populations have remained rather large and stable in the Karst region. This situation provided possibility to compare the genetic composition of stable and declining populations. *A. artaxerxes* was collected in four populations of the Bükk Mts and in ten in the Aggtelek Karst region in 2002 and 2005. We analysed 19 polymorphic allozyme loci in all samples. The results revealed an extremely high level of enzyme polymorphism. Most parameters of polymorphism proved to be significantly higher in the Karst region compared to the Bükk. The shape of the distribution of alleles among the frequency classes indicated the effect of bottleneck in three Bükk and one Karst population. Though heterozygote excess was only significant in one Bükk population a tendency was observed for higher values of standardised heterozygote excess in those populations that had a typical bottleneck distribution of alleles. The most obvious sign of bottleneck in our Brown Argus populations was obtained in the analysis of the distribution of alleles among the frequency classes. In a non-bottlenecked population we expect an L-shape distribution with most of the alleles occurring in the 0–0.1 frequency class. During bottleneck, however, rare alleles are prone to be lost from the population. As a consequence, the shape of distribution is shifted towards the intermediate frequency classes (Luikart et al. 1998). We found this pattern in three of the four Bükk populations and in a Karst one.

The results of the our study have definite conservation implications. One critical requirement to preserve the suitable habitat for this protected species in Northern Hungary is regular monitoring and management in order to maintain a proper vegetation structure for the species e.g. by traditional mowing. It is especially critical in the Bükk plateau, where succession is relatively fast and the rapidly growing scrubby and tall grass vegetation results in less and less proper habitat and consequently declining population size. In addition, it is also essential to facilitate migration among the habitat patches. Our results clearly demonstrated that those populations/subpopulations that compose a network of subpopulations with intense migration are able to maintain their genetic diversity in a long run (e.g. plateaus North of Jósvafő, Nagymező on the Bükk plateau), while the isolated small populations of the Bükk plateau have lost a sizeable amount of their variation.

High genetic polymorphism and geographical differentiation in *Kretania* (*Plebejus*) *sephirus*

Several groups of *K.* (*P.*) *sephirus* occur in Northern and Central Hungary with different combinations of substrate and food plant. Central Hungary: calcareous sand, food plant *Astragalus exscapus*; Sóshartyán: chalky sandstone, *A. exscapus*; Tokaj: volcanic rocks covered by loess, *A. dasyanthus*; Serbia (Deliblat): loessy sand, *A. dasyanthus*; Transylvania: calcareous clay, *A. exscapus, A. dasyanthus, A. monspessulanus.*

Earlier surveys (Pecsenye et al. 2007) have unravelled high level of genetic diversity and strong regional pattern. The results of molecular and morphometric survey of samples will be published in the next months.

Results connected with *Melitaeini* (Nymphalidae) species

The other main group of target species was formed by Melitaeini species, i.e. the *Melitaea athalia* group (*Mellicta* subg,), the *Melitaea phoebe* species group (*Cinclidia* subg.) and *Euphydryas*. A multi-level survey was performed, according to the aims and questions of the project: (i) the dependence of the morphological and genetic variability with environmental, and explicitly with climatic factors; (ii) the morphometry and genetic variability of closely related sympatric species, existence of Evolutionarily Significant Units; (iv) prediction of past and future distribution of these species by MaxEnt modelling; (v) morphometric and genetic backgrounds of subspeciation and speciation in allopatric taxa; and (vi) the phylogeny of the whole *Melitaea phoebe* species group.

In conservation genetics the existence of Evolutionarily Significant Units (ESU) is a crucial question in threatened or vulnerable species. It is of particular concern to determine whether subspecies or ecotypes of a species can be considered as separate ESUs. Some *Melitaea* subg. *Mellicta* species (*Mellicta aurelia, Mellicta britomartis*) are declining or scarce in Europe. The level of genetic differentiation and pattern of genetic variation were surveyed in three *Melitaea* (subg. *Mellicta*) species. Their habitat requirements and food plants partly overlap; accordingly they often co-occur in the same habitat. *M. aurelia* and *M. britomartis* have one brood per year in Hungary, while *M. athalia* has a monovoltine and a bivoltine ecotype. The purpose of the study was to estimate the number of genetically differentiated ESUs among these species. Enzyme polymorphism was studied using polyacrylamide gel electrophoresis. Structure of genetic variation was analysed by F-statistics, AMOVA, PCA and Bayesian clustering method. UPGMA dendrogram was constructed on the basis of Cavalli-Sforza and Edwards chord distances. The three species are clearly differentiated from each other in all statistical analyses. They are evidently different Evolutionary Significant Units. The two ecotypes of *M. athalia*, however, do not show any genetic differentiation.

Further details: Bátori et al. 2012. *Journal of Insect Conservation* 16(5): 647-656.

Patterns of genetic and morphometric differentiation were surveyed in *Melitaea* (*Mellicta*) *athalia* populations of the Carpathian Basin. It has two ecotypes in Hungary: with either one or two broods per year. It is of particular interest to reveal the main factors driving differentiation patterns in this species. Enzyme polymorphism, wing characters and male external genitalia were analysed using traditional morphometric methods. Statistical methods were optimized to compare morphological and genetic data. The results of genetic surveys revealed a clear regional pattern of differentiation in *M. athalia*. Moreover, the results of principal component analysis, Bayesian clustering and the dendrogram all suggested that the regions can be classified into two groups corresponding to the East or West zones of the Carpathian Basin. In contrast, differentiation between the two ecotypes was less expressed in the genetic variation of *M. athalia.* Results of the phenotypic variation also suggested a regional pattern for both sets of morphometric characters (wings and external genitalia). In sum, our analyses confirmed that both genetic and phenotypic variations of *M. athalia* exhibit a regional pattern rather than the differentiation between the two ecotypes.

Further details: Bátori et al. 2012. *Biological Journal of the Linnean Society* 107 (2): 398-413.

Distribution of *Melitaea ornata* (in earlier publications *M. telona*), a little known fritillary species was analysed on different time scales using MaxEnt software. Using the results of genitalia morphometry and the predicted potential refugia during the Last Glacial Maximum (LGM), we reconstructed the probable re-colonisation routes. We also predicted changes in the potential area for 2080. The present distribution fits well the known occurrence data except for the Iberian Peninsula and North-Africa where the species is missing. Based on our

predictions, temperate areas seem to be less suitable for the species. We proposed two hypotheses to explain this pattern: a less probable recent extinction from Iberia and a more supported historical explanation. Predicted distribution during the LGM mainly fits to widely accepted refugia: Europe was re-colonised from two main sources, from the Apennine peninsula and from the Balkans which was probably connected to the Anatolian refugia. Populations of the Levant and in the Elburs Mts. do not show any significant expansion. Further studies are necessary in the case of the predicted Central Asian refugia. Predictions for 2080 show a northward shift and some extinction events in the Mediterranean region. Core areas are identified which might have a potential for expansion including southern Russia, Hungary and possibly Provence in France. Predicted northward area shifts are only possible if the potential leading edge populations and habitats of the species can be preserved.
Further details: Tóth et al. 2012. *Journal of Insect Conservation* 17 (2): 245-255.

Based on former studies (Varga 2007; Tóth & Varga 2011) and co-operation with the molecular phylogenetic research group of the Turku University (Finland) we outlined the complete phylogeny of the *Melitaea phoebe* species group (subg. *Cinclidia*).
The genus *Melitaea* consists of about 80 species, divided into ten species groups, which are all restricted to the Palaearctic region. The *Melitaea phoebe* group was defined by Higgins based on morphological characters such as wing pattern and genital structures. The taxonomy of the *phoebe* species group has been poorly resolved and recent results suggest the need for a re-evaluation. In this study molecular sequences (5985 bp) including one mitochondrial (*COI*) and up to six nuclear (CAD, EF-1□□, GAPDH, MDH, RpS5 and wingless) gene regions from 38 specimens of the *Melitaea phoebe* species group *sensu* Higgins and some closely related taxa from the Palaearctic region were analysed. Based on the combined Bayesian tree and the shape of the processus posterior of the male genitalia, four main groups are recognised: (i) *collina* group, (ii) *arduinna* group, (iii) *aetherie* group and (iv) *phoebe* group. The status of *M. ornata*, *M. zagrosi* and *M. scotosia* as species is confirmed, and the results also indicate that *M. telona* (*s.s.*) from Israel is a separate species. The *collina* group (*M. collina*: Asia Minor, Syria; *M. consulis*: Iran, Shiraz region) was already recognised by Leneveu *et al.* (2009). Species in this group show very uniform morphology of the processus posterior (see: Fig. 1). *Melitaea arduinna* and *M. avinovi* belong to the *arduinna* group based on the very uniform wing pattern, the peculiar shape of the processus posterior and molecular data. Although *M. aetherie* has a very different wing pattern from the other two members of the *aetherie* group, some similarities are present in the shape of the processus posterior, namely the reduction of the middle process. All of the species of this group have a restricted distribution.

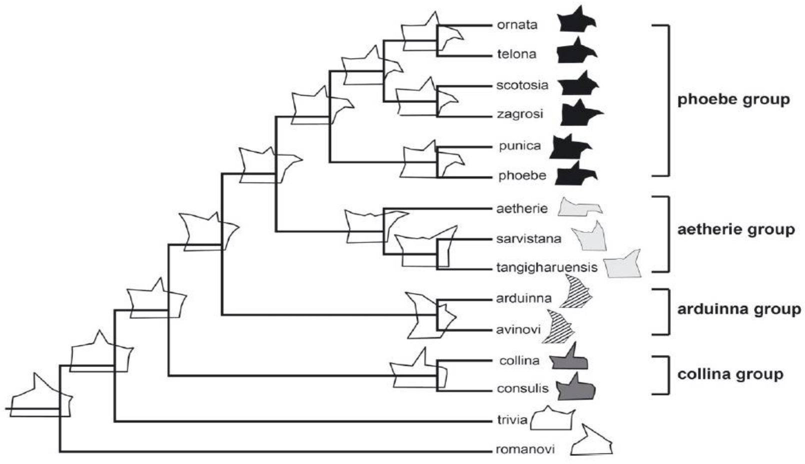


Fig. 1. Dendrogram based on the modelled morphology of processus posterior

In this study, the DNA-based phylogenetic hypothesis for species delimitation in the *M. phoebe* group agrees generally quite well with previous morphometric studies (Tóth & Varga, 2011), although some morphometrically close species seem to be more distant relatives of each other based on the phylogenetic reconstruction than was expected (Fig. 2). It has also become clear that the head colouration of the larvae in the close relatives of *M. phoebe* cannot be considered as a species-specific character because *M. ornata*, *M. telona* and *M. zagrosi* (G. Kuznetsov, personal communication) all have red head capsules. However, the black head capsule does not necessarily indicate a conspecific relationship, because *M. scotosia* larvae also have black head capsules. A black head capsule is known in *M. aetherie* and *M. arduinna*, suggesting that this may be the ancestral state. However, species in other groups of the *Didymaeformia* clade have orange-coloured head capsules, which may or may not be homologous to the brick red colour found in the *M. phoebe*-group. Therefore, the specific colouration of the head capsule cannot be considered as either a plesiomorphic or apomorphic trait, but as a possible parallelism, having evolved on multiple occasions from an originally polymorphic state with repeated loss of 'black' or 'red' alleles.

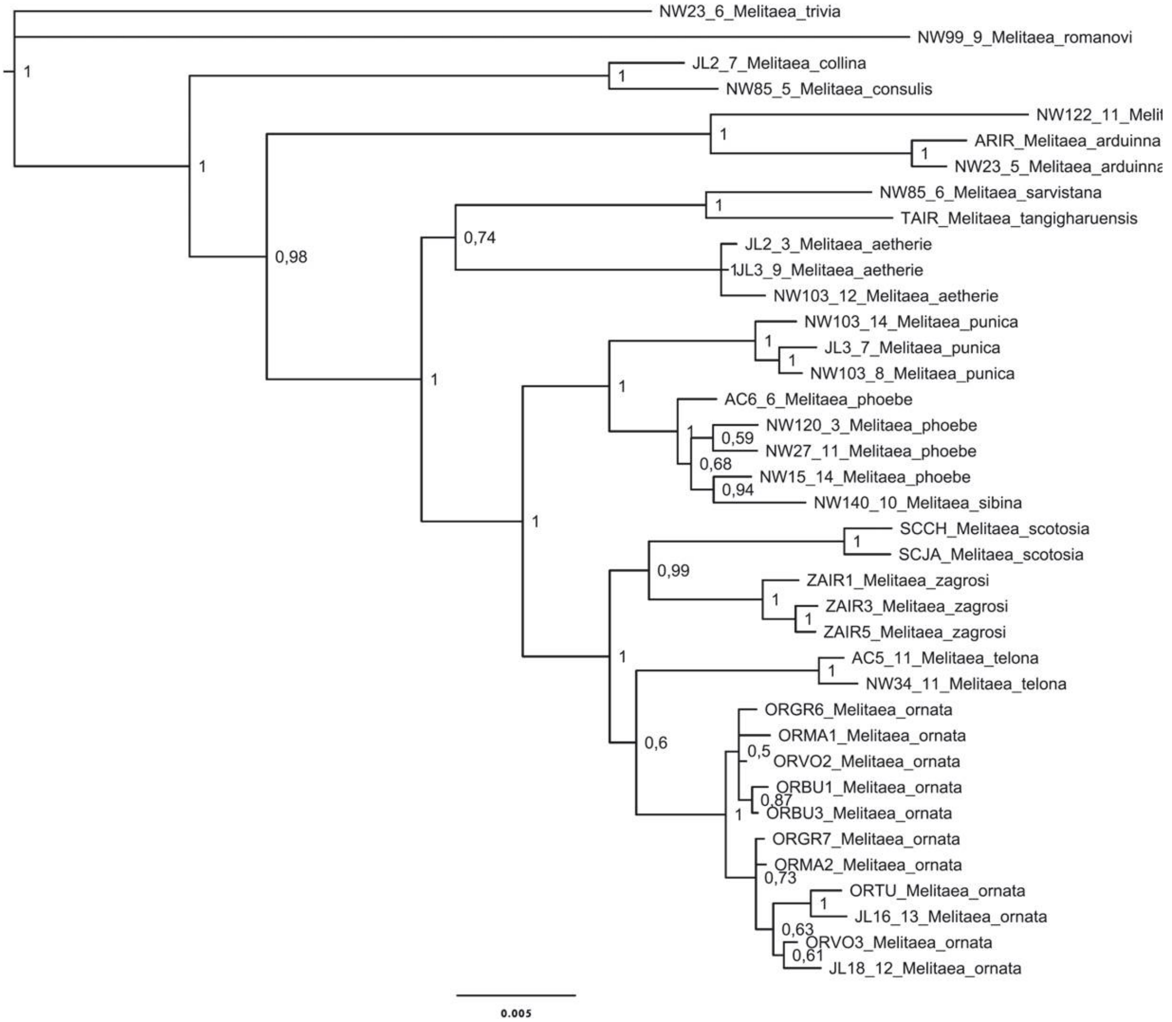


Fig. 2. Phylogenetic dendrogram based on one mitochondrial and six nuclear genes (Bayesian clustering)

Further details: Tóth & Varga 2011. *Zoologischer Anzeiger* 250 (2011) 258–268.; Tóth et al. 2014. *Systematic Entomology* 39(4): 749-757.

Results connected with the population structure and phylogeography of the Clouded Apollo (*Parnassius mnemosyne*)

P. mnemosyne populations of the Carpathian Basin proved to have a surprisingly high level of enzyme polymorphism with the average number of alleles amounting to 2.45 and the overall frequency of heterozygotes being 0.188. These results are in good agreement with those of Meglécz et al. (1997, 1998, 1999) who detected a similar number of alleles per locus (n_A=2.16) at 6 enzyme loci in the Hungarian populations. Clouded Apollo populations were also surveyed in southern France (Descimon & Napolitano 1993, Napolitano & Descimon 1994) where the level of enzyme polymorphism have been somewhat lower (H~0.13 and n_A~1.3) although 24 populations were surveyed at 23 enzyme loci. One possible explanation of the inconsistency between the results of the two sets of studies is the methodological differences between them. The actual gel and buffer systems used in electrophoresis can greatly influence the resolution of the enzyme pattern. Comparing the number of alleles at those loci that were investigated in all studies (e.g. *Mdh*, *Pgi* and *Pgm*) it turned out that using polyacrylamide gels we have identified far more alleles (*Mdh*: 7, *Pgi*:10 and *Pgm*: 18) than Napolitano & Descimon (1994) who used starch gels (*Mdh*: 4, *Pgi*: 3 and *Pgm*: 10).

Another similarity in all studies on enzyme polymorphism of *P. mnemosyne* populations was that they revealed a comparable level of differentiation among the populations. Napolitano & Descimon (1994) detected an F_{ST}=0.135 among the French populations, while Meglécz et al (1997, 1998, 1999) found an F_{ST}=0.109 among the Hungarian ones. In our study the general F_{ST} value was 0.138 at the 14 enzyme loci among the populations of the Carpathian Basin. These high fixation indices observed in parallel studies are in accordance with the dispersal ability of the species, which is low with the largest observed individual migratory distance being 1-2 km. The Clouded Apollo populations of the Carpathian Basin also exhibited a clear regional pattern of differentiation. In addition, significant isolation by distance was also detected in the Central range (NR + TDR), in the Eastern region together with the Northern range and also in the Eastern and Körös regions.

Nevertheless, the most significant result of our study was the genetic structure of Clouded Apollo populations of the Carpathian Basin inferred from the results of the Bayesian clustering analyses. The outcome of STRUCTURE was in accordance with the results of Gratton et al (2008) who found that the Pannonian/Carpathian samples harbour the ancestral mtDNA haplotypes of lineages EN and EE the two 'sublineages' of the Eastern lineage. The results of STRUCTURE assuming K=2 also suggested two genetic clusters in the populations of the Carpathian Basin: cluster 1 (K2Cl1) was characteristic for the Eastern populations of the Carpathian, while cluster 2 (K2Cl2) was predominant in the Northern and Transdanubian ranges (Central range). It thus appears that the distribution of the two clusters identified on the basis of the allozyme data is consistent with the EE and EN mtDNA sublineages of Gratton et al. (2008). Moreover, the isolation by distance structure of the populations of the Central range approved by the results of Mantel test suggested a gradual colonisation process along this mountain range; i.e. within the EN sublineage. Since colonisation is the result of successive founding events we expected a decreasing level of variation along this putative colonisation route. The results of correlation studies completely fulfilled our expectations: all parameters of polymorphism decreased significantly towards East. Though the correlation coefficients were not significant the tendency was evident in case of latitude as well: a slow decrease of variation was observed from South towards North.

The genetic structure of the Eastern populations was far less evident. After assuming the most probable number of genetic clusters (K=2) in the first series of the Bayesian analyses several questions remained unanswered: (i) Contrasting the cluster composition and the geographic range and position of the four genetic regions it seems odd that the populations of the easternmost region were actually the mixtures of clusters 1 and 2 (ii) Considering the putative colonisation rout of the EE sublineage suggested by Gratton et al. (2008) it is peculiar that the populations of the Körös region which have a central position in the Carpathian basin were principally comprised of cluster 1 while those of the Eastern region had a mixed genetic composition. These contradictions were partially resolved by assuming a less probable number of clusters (K=3) in the analysis of all 22 populations and by the outcome of the second series of Bayesian analyses where the two Eastern regions were only included. The results of both series of analyses indicated that the genetic composition of the Eastern region is actually the mixture of the Körös region and the Northern range. This was also supported by the Mantel tests where the populations of the Körös and BAC regions or those of the NR and the BAC region were included. The highly significant isolation by distance structure detected in both analyses also suggested gene flow among these regions. In addition, we were able to find certain alleles which showed a kind of introgression pattern mostly from the Körös to the Eastern region and to a much less extent from the Northern range to the Eastern region. At the same time, we also detected region specific alleles in the Eastern populations and their high number implied that the basic genetic structure of the Eastern region might have evolved a long time ago. Specifically, all 10 region specific alleles occurred sporadically in one or two populations and most of them were rare alleles. This pattern suggests that the region specific alleles in the Eastern region were probably the results of mutations occurring relatively recently in the present or some neighbouring populations.

In summary we can conclude that our results support those of Grattons' (2008) concerning the presence of two genetic lineages in the Clouded Apollo populations of the Carpathian basin. We can safely assume that the EN mtDNA lineage corresponds to cluster 2 at K=2 (K2Cl2) in the present study, which was characteristic for the Central range. At the same time we cannot be certain about the correspondence between the EE mtDNA lineage and cluster 1 at K=2 (K2Cl2) in our study. However, since the genetic composition of the samples from the Körös region was homogeneous and predominantly composed of cluster 1, it is highly probable that the EE lineage is equivalent with this cluster of our study. Nevertheless to make this question clear it is necessary to carry out a more intense sampling in the Southern part of the Great Plain of the Carpathian basin and over the East-Carpathians in Ukraine and also in Bukovina.

Our results have some conservation implications concerning the Clouded Apollo populations of the Carpathian basin. One important question in the short term protection of endangered species is the delineation of functional conservation units (FCU). In the Carpathian Basin we have recognized two well differentiated genetic clusters, which are likely to be the same as described by Gratton et al. (2008). Thus, we can safely assume that there are two FCUs of *P. mnemosyne* in the Carpathian basin implying that both units has to be preserved. The conservation of the species is especially critical in the Körös region, which only consists of 4 populations in a fairly restricted area. At the same time this region can be characterised by a specific genetic cluster (K2Cl) with 4 cluster specific alleles which do not occur in the other regions. In order to protect the *P. mnemosyne* populations of the Carpathian Basin forest management should maintain or restore structurally rich woodlands, which are the suitable habitats of the species both in the Northern and Transdanubian Mts. In addition, this type of forest management would be vital for many other woodland butterflies as well. Second, considering the low long distance dispersal ability of Clouded Apollo it is necessary to form/maintain ecological corridors between these forest patches in order to enhance migration among them. This would facilitate the development of a network of populations and would decrease their isolation, which is an essential condition to maintain their variation.

Further details: Pecsenye et al. 2015. to be submitted to *Journal of Insect Conservation*.

Results connected with range shifts and phenological changes in insects due to *climate change*

The glacial-interglacial cycles are connected with vertical range shifts in montane species. It implies a high risk of local or regional extinction caused by the recent warming of climate. The two closely related butterfly species *Erebia ottomana* Herrich-Schäffer, 1847 and *Erebia cassioides* (Reiner & Hohenwarth, 1792) show subalpine and alpine distribution settings, respectively. *Erebia ottomana* is found up to the treeline (1400–2400 m a.s.l.), whereas *E. cassioides* reaches much higher elevations (from about 1800 m a.s.l. in the Retezat Mountains, in Romania, to 2800 m a.s.l.). Thus, both species cover diverging climatic niches, and thus might also have been distributed differently during the cold glacial stages. Individuals of these two species were sampled over the mountain areas of the Balkan Peninsula and genetically analysed using allozyme electrophoresis. Additionally, we performed species distribution models (SDMs) to simulate the distribution patterns of both species in the past (i.e. during the Last Glacial Maximum and the Atlanticum). Our genetic data show contrasting structures, with comparatively low genetic differentiation but high genetic diversity found in *E. ottomana*, and with stronger genetic differentiation and a lower level of genetic diversity, including many endemic alleles, occurring restricted to single mountain massifs in *E. cassioides*. The SDMs support a downhill shift during glacial periods, especially for *E. ottomana*, with possible interconnection among mountain regions. We conclude that during the cold glacial phases, both species are assumed to shift downhill, but persisted at different elevations, with *E. ottomana* reaching the foothills and spreading over major parts of the Balkan Peninsula. In contrast, *E. cassioides* (the truly alpine species) survived in the foothills, but did not reach and spread over lowland areas. This more widespread distribution at the Balkan Peninsula of *E. ottomana* compared with *E. cassioides* is strongly supported by our distribution models. As a consequence, long-term geographic restriction to distinct mountain massifs in *E. cassioides* versus panmixia in *E. ottomana* produced two contrasting evolutionary scenarios.
Further details: Louy, D. et al., Varga, Z., Rödder, D. & Schmitt, T. 2014. *Biological Journal of the Linnean Society* 112:569-583.

Boreo-montane, glacial relict species as the strictly protected (Habitats Directive Annex II-IV) Violet Copper (*Lycaena helle*) are also focal species of nature conservation since they are affected by climate change due to fragmentation and habitat loss.
During the cold phases, the European lowlands north of the high mountain systems were covered by tundra-steppe habitats. Thus, suitable habitat for the Violet Copper butterfly *Lycaena helle* was possibly available over major parts of Central Europe. These areas might have fulfilled all habitat requirements of the species, like the presence of one of its food plants, i.e. *Polygonum viviparum*, but retreated in the wake of postglacial warming to small remnants. The genetic structure of the species studied using polymorphic microsatellites, allozymes and mitochondrial DNA sequences suggest a wide distribution during the cold phases of the past and relatively fast altitudinal and latitudinal range shifts during the postglacial warming, accompanied by strong population fragmentation of the remaining extant populations until today.
Based on three molecular markers, we derived the following biogeographic scenario for *L. helle*: (i) the species was widespread and represented an interconnected network of populations in the periglacial part of Europe during the cold phases mirrored by the lack of genetic differentiation of mitochondrial DNA sequences; (ii) postglacial northwards expansion to Fennoscandia as highlighted by microsatellite frequencies and allele elimination in the wake of this colonisation; (iii) extinction of *L. helle* over major parts of the Central

European lowlands; and (iv) escape to the higher elevations of mountain systems of Central Europe with subsequent distinct evolutionary processes on mountain archipelagos, as identified by allozymes, microsatellites and morphological data. These dynamics are further supported by results of climate and land-use modelling, supporting a large expansion of cool climatic conditions during the LGM (Habel et al. 2010) followed by strong retraction of the suitable climate niche. This is accompanied by the restriction of cool and moist conditions and respective habitat features to higher elevations over Central Europe and the more northern and north-eastern parts of the continent.
Further details: Habel et al. & Varga 2015. The molecular biogeography of the Violet Copper *Lycaena helle* – In: Habel et al. (ed.) Jewels In The Mist – A synopsis on the endangered Violet Copper butterfly *Lycaena helle.* Pensoft, Sofia-Moscow, ISBN 978-954-642-721-2.

Consequences of fragmentation of habitats and the maintaining of metapopulation networks was surveyed in two brachypterous Orthoptera species of Habitats Directive (*Pholidoptera transsylvanica*, *Paracaloptenus caloptenoides*) and two further target species affected by the climate change. All these surveys were conducted by non-invasive methods.
Fragmentation of habitats is a serious problem for many endangered species; a possible solution is the maintenance of landscape connectivity. Due to scarce sources, in managing and planning landscapes exact and quantitative priorities must be set. Application of mathematical tools, such as network analysis, can be useful help in these decisions. We illustrate the possibilities and results of this approach with a case study of endangered *Pholidoptera transsylvanica* bush-cricket population in the Aggtelek Karst, Northeast-Hungary, which inhabits 39 habitat patches connected with ecological corridors (Fig. 1).

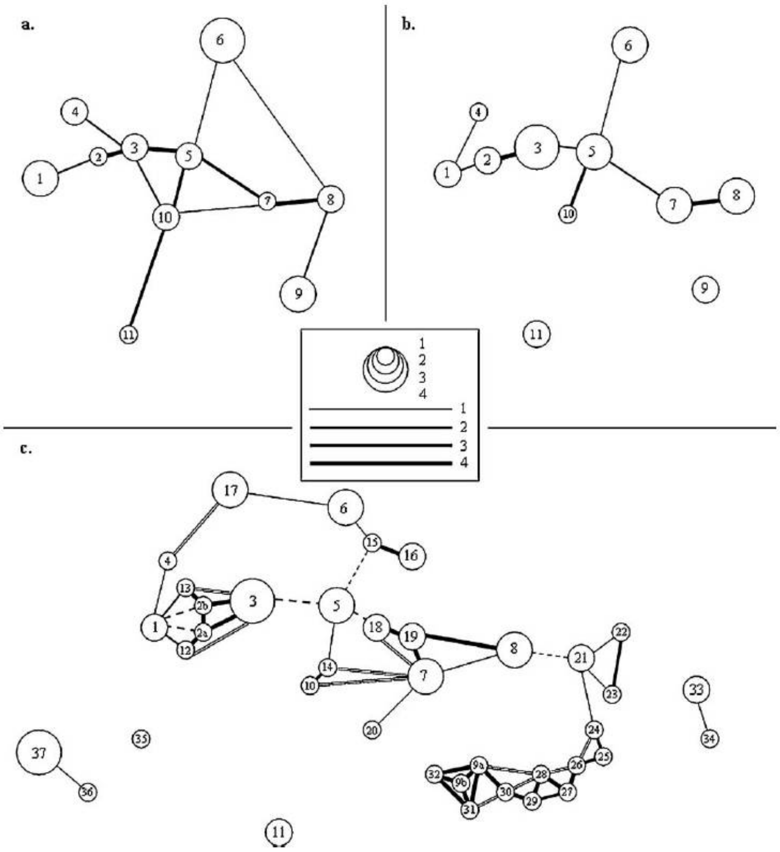
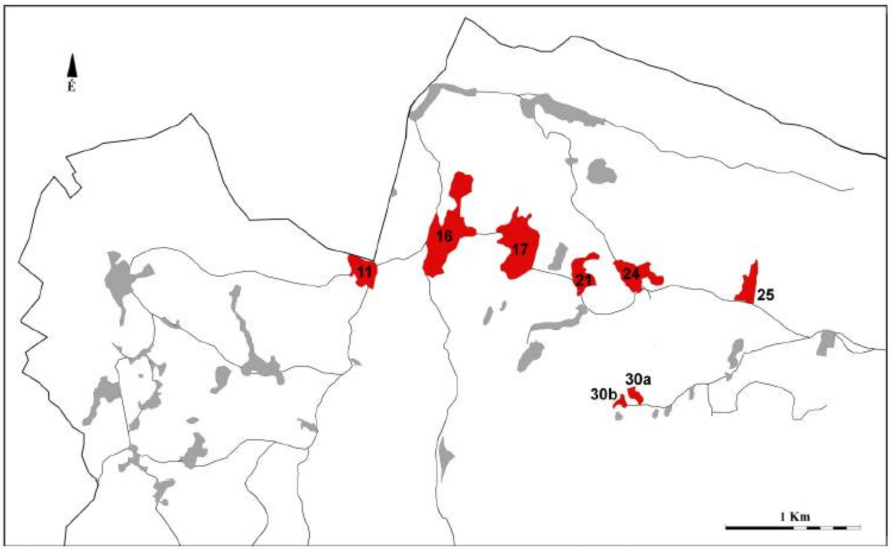


Fig. 1. Changes of habitat network between 2003 (a) followed by fragmentation (b) and discovery of further patches + reconstruction of corridors, 2007 (c)

A key issue in the long-term survival of this metapopulation is the maintenance of gene flow (by preserving the connectivity of the habitat network). We evaluated the landscape graph and our results are compared to earlier ones based on older methods. During the comparison, we used several network indices to set quantitative conservation preferences. In addition, we would like to draw attention to the need for constant monitoring (and possible treatments), because several changes (like secondary succession) have occurred during the years between the two studies, threatening landscape connectivity and long-term survival of certain species. A potential solution for preventing fragmentation is establishing new corridors or improving the existing ones: we estimated the possible effects of these changes. New corridors did not have major effect on the system; maintaining already functioning corridors is more effective. Further details: Benedek et al. & Varga 2011. *Ecological Indicators*: 11: 930–933.

In a further study we mapped the habitat patches of three Orthoptera species (*Pholidoptera transsylvanica, Paracaloptenus caloptenoides, Arcyptera fusca*) on the Aggtelek-karst, in 2010, inspecting 31 sampling sites. The registration of presence-absence and the population size estimation were carried out by counting along transects. In the predatory (scavanger) *P. transsylvanica* the nearly even values of sub-populations are characterized by strong regional dynamics. They expanded westwards, compared to previous data (2003; 2007). In the less mobile (herbivorous, flightless females) *A. fusca* nearly completely isolated sub-populations were found, without any connectivity among them (MRRS surveys). This species is declining because of partial abandoning of management of suitable grassland habitats. Furthermore, we did not found any connections among the sub-populations of the completely flightless, herbivorous *P. caloptenoides* either, due to the decline of short-grass swards. According to our results, the restoration of mowing and the establishing og grassy corridors are necessary to stabilise the metapopulation structures of these protected species.



Further details: Szanyi et al. & Varga 2013. *Állattani Közlemények* 98: 97-110.

We also studied the wing dimorphism in Roesel's bush cricket populations, mostly in the same area. A total of 410 individuals (77 macropterous and 333 brachypterous) were sampled in seven sites. We found a high frequency (≤ 53%) of macropterous individuals mostly in some meadows surrounded by forests. We measured the body weight, thorax weight, and abdomen weight in connection with macroptery vs brachyptery. We could find a positive

correlation between macroptery and thorax weight. It means that the macropterous individuals have more developed thoracal musculature and are able for active dispersal. The abdomen weight of females showed high individual variation; thus the supposed trade-off between macroptery and lower fertility of females was not supported.
Further details: Szanyi et al. & Varga 2014. *Estonian Journal of Ecology*, 63: 4-13.

We surveyed the **climatic responsiveness** of moths as a consequence of climate change in a review paper. Our dataset was collected over 20 years applying highly standardised collection methodology. The single collection site allowed a high-level replication of species-specific responsiveness of large number of noctuid taxa allowing multispecies analyses of 70 species after rigorous filtering. Third, we calculating multiple phenological estimates in order to avoid collection bias primarily affecting first emergence phenology.

Considering noctuid phenology, we detected no clear patterns in changes of emergence phenology, which might be related to the diversity in a number of life history traits of the studied species, as shown by recent investigations (Altermatt 2010). Additionally, complex trends of emergence phenology can also be a result of our finding that significant trends in climatic patterns were only found for June temperature. Our findings show that the degree of temporal shifts of various measures of emerging phenology was primarily related to hibernation stage, food type and migration strategy. Specifically, hibernation stage is the strongest predictor of climatic responsiveness measured as last capture dates and activity length in noctuid moths , similarly to the results of Diamond et al. (2011). This might indicate that noctuid moths overwintering as adults exhibited greater degrees of phenological shifts than species hibernating as larvae or pupae. This finding is in line with the results of a recent study reporting that species with more advanced overwintering stages have experienced relatively greater advances in their date of first appearance (Diamond et al. 2011).

However, the difference between the reactions of the bulk of the population and last active individuals to climatic trends seems to be different in our case. Since noctuids hibernating as adults showed no shifts in median emergence to earlier dates, the last individuals of these moths have prolonged capture dates over the study period in comparison to species hibernating in larval or pupal form. Prolonged autumn activity may allow longer time for replenishing fat reserves before hibernation which can increase survival chances and improve reproductive success. Moreover, all of these species are winter-active moths in the southern part of their distribution areas implying that noctuid taxa hibernating as imagos are forced to suspend activity in our climate and the prolonged autumn activities of the last emerging individuals might be the result of enhanced temporal plasticity of the timing of autumn emergence (László *et al*. 2000).

Food type was included in best supported models for climatic responsiveness measured as first capture dates, similarly to previous investigations: noctuids feeding on herbaceous plants advanced first capture dates to a greater degree than species connected to woody materials (Diamond et al. 2011, Altermatt 2010). Although a recent survey of British plants showed that annuals were more likely than perennials to advance phenology (Fitter & Fitter 2002), our results imply that moths feeding on herbaceous plants are able to track plant phenology faster than noctuids feeding on woody plants which evokes the following hypotheses. First, as herbaceous plants are shorter-lived on average than woody taxa, their herbivores are forced to synchronise phenology to a greater degree than moth larvae feeding on woody plants. Second, woody plants are on average larger than herbaceous species implying higher levels of phenological variance of plant parts consumed by herbivores in individual trees which allows for a wider range of phenological synchronisation. However, the complexity of climatic responses driven by trophic interactions is enhanced by the fact that the highly diverse family of Noctuidae is connected to a great variety of larval food plants. For instance, the majority of these species is connected to forested habitats and majority of spring- and autumn-active moths are linked to woody food plants. A large group of genera use a wide variety of food plants which are most often herbaceous (grasses for mostly monovoltine *Apamea*, and often Urticaceae for the phenologically more plastic Plusiinae), or partly herbaceous and woody (Acronictinae).

Migration strategy has emerged as a supported predictor of climatic responsiveness. Migrant noctuids have shifted first captures to later dates than resident moths which implies that migratory species arriving from southern regions may detect warming spring weather later than locally hibernating taxa. (Alerstam *et al.* 2011). Indeed, migrant moths need time to reach Central Europe and thus are outcompeted in advancements of climatic responsiveness measured as first capture dates by locally wintering moths hibernating as imagos.

Throughout our analyses we detected no phylogenetic relatedness on the subfamily level in climatic responsiveness of noctuid moths. As the existing politomies are largely unresolved implying that the absence of phylogenetic signal in climatic responsiveness of noctuid moths can be considered as the result of poor resolution of clades rather than missing phylogenetic relationships. However, we cannot exclude the possibility that climatic responsiveness is independent of phylogenetic relatedness in noctuid species as shown by a number of investigations showing that the advancement in spring phenology is not driven by common descent in a broad range of taxa (Végvári *et al.* 2010, Diamond *et al.* 2011). Further, the lack of phylogenetic signal in emergence phenology of the species in our dataset can also be an outcome of phenotypic plasticity which drives phenological patterns as a response to climatic variability, paralleling the findings of recent studies across a number of taxonomic levels (Hoffmann & Sgrò 2011). However, the resolution of current phylogenetic studies of noctuid moths does not allow us to draw more general conclusions on the existence of phylogenetic signal in climatic responsiveness of night-active lepidopterans. The similar results of Diamond et al. (2011) showing that phylogenetic relatedness among species predicted very little variation in butterflies' phenological responses imply that climatic responsiveness in lepidopterans can be treated as a species-specific reaction rather than driven by evolutionary relatedness (Altermatt 2010a).

Our study implies recommendations for lepidopteran conservation, as a reflection to current declining trends in a number of insect species on a global scale. We found that noctuid moths hibernating as adults are able to track climatic trends faster than species overwintering in less developed stages which suggest higher declining probabilities in these taxa, considering current climatic predictions (Stocker *et al.* 2013). Our result that resident moths are responding to ongoing climatic patterns to a greater degree than migratory taxa calls for more intensive surveys of migrating lepidopterans populations. In summary, we have demonstrated in a diverse set of noctuid moth species that temporal trends in emergence phenology (which is associated with a measure of temperature increase in the study area) is primarily related to hibernation stage, diet specialisation and migration strategy.

Further details: Végvári etal. & Varga 201

Results connected with phylogeography and conservation genetics of *Amphibia & Reptilia*

Due to successful sampling in 2012-13 the distribution and hybrid zones of the *Triturus cristatus* species group was successfully mapped. The results have been included in the Supplement of the New Atlas of Amphibians and Reptiles of Europe (Sillero et al., 2014a). In earlier publications the distribution of the newt genus *Triturus* was not resolved at the level of the species. The main reason for this was the lack of high quality distribution data from in and around the parapatric contact zones between species, where interspecific hybridization occurs. We were working extensively on *Triturus* and the (particularly genetic) data we have accumulated allow us to map the individual *Triturus* species at the appropriate scale. We here provide a database composed of distribution data for the individual species, at generally high resolution, particularly from in and around contact zones. Based on this database we produce maps at the 50 × 50 km UTM grid resolution as used in the new atlas and highlight those grid cells in which more than one *Triturus* species occurs.

Further details: Wiesltra B., Sillero N., **Vörös J.** & Arntzen J.W. (2014): Distribution of the crested and marbled newt species (Amphibia: Salamandridae: *Triturus*) – an addition to the New Atlas of Amphibians and Reptiles of Europe. *Amphibia-Reptilia* 35: 376-381.

Ichthyosaura alpestris genetic diversity and phylogeography in the Carpathian Basin

According to previous studies the Carpathian Basin has been colonized by two different *Ichthyosaura alpestris* lineages, one from the Alp region and a second one from the Balkan area. These two colonization routes meet in central-north Carpathians and likely form a secondary contact zone. The Hungarian planes might have played an important role as providing cryptic refugia for the species during the Pleistocene. The importance of the Carpathian basin however has not been studied yet, and the origin of the isolated populations in the low elevation mountain ridges remains unknown (Sotiropoulos et al 2007, Recuero et al 2014). Four different subspecies (*I. a. carpathicus, I. a. satoriensis, I. a. bükkiensis, I. a. bakonyiensis*) were described from the Carpathian Basin based on morphometrical characters (DELY, 1958, 1959, 1960a, 1960b, 1967), but distinctiveness of these subspecies were rejected. Analyzing the 16S subunit ribosomal and ND4 mitochondrial DNA genes the aim of our study was to infer the evolutionary history of *Ichthyosaura alpestris* within the Carpathian Basin, and to revise the inraspecific taxonomy of the species in the central-eastern European region.

Tissue samples were collected by tail clipping or swabbing from 24 living animals from 13 localities in 6 regions within the Carpathian during the breeding season of 2010-2012. (fig. 1). Methods of molecular analysis are described in details in our publication cited below. Phylogenetic relationships were inferred by constructing Bayesian inference (BI) phylograms in MrBayes 3.1 (Ronquist and Huelsenbeck, 2003).

Altogether 13 haplotypes were found in the sampled region (11 from own samples, two more from Recuero et al 2014). Newts from the Apuseni Mts. were closely related to the Southern Carpathian lineage, being part of the eastern (E2) clade. The North Hungarian Mountain populations (Mátra, Bükk and Zemplén Mts.) were very closely related, and they are embedded in the Northern Carpathian (C3) clade, with sharing haplotypes to samples from Poland and Romania. Bakony (five haplotypes) and Őrség (two haps) samples formed a distinct clade within the Western lineage.

Despite their geographic proximity, Alpine newt populations in the Carpathian Basin represent different mitochondrial lineages. Apuseni Mts. and North Hungarian Mountain populations are closely related to those inhabiting the Carpathians and the Alps, but the separate position of the Bakony and Őrség clade suggest that this population might have survived Pleistocene glaciations within the Carpathian Basin in a separate glacial refugium.

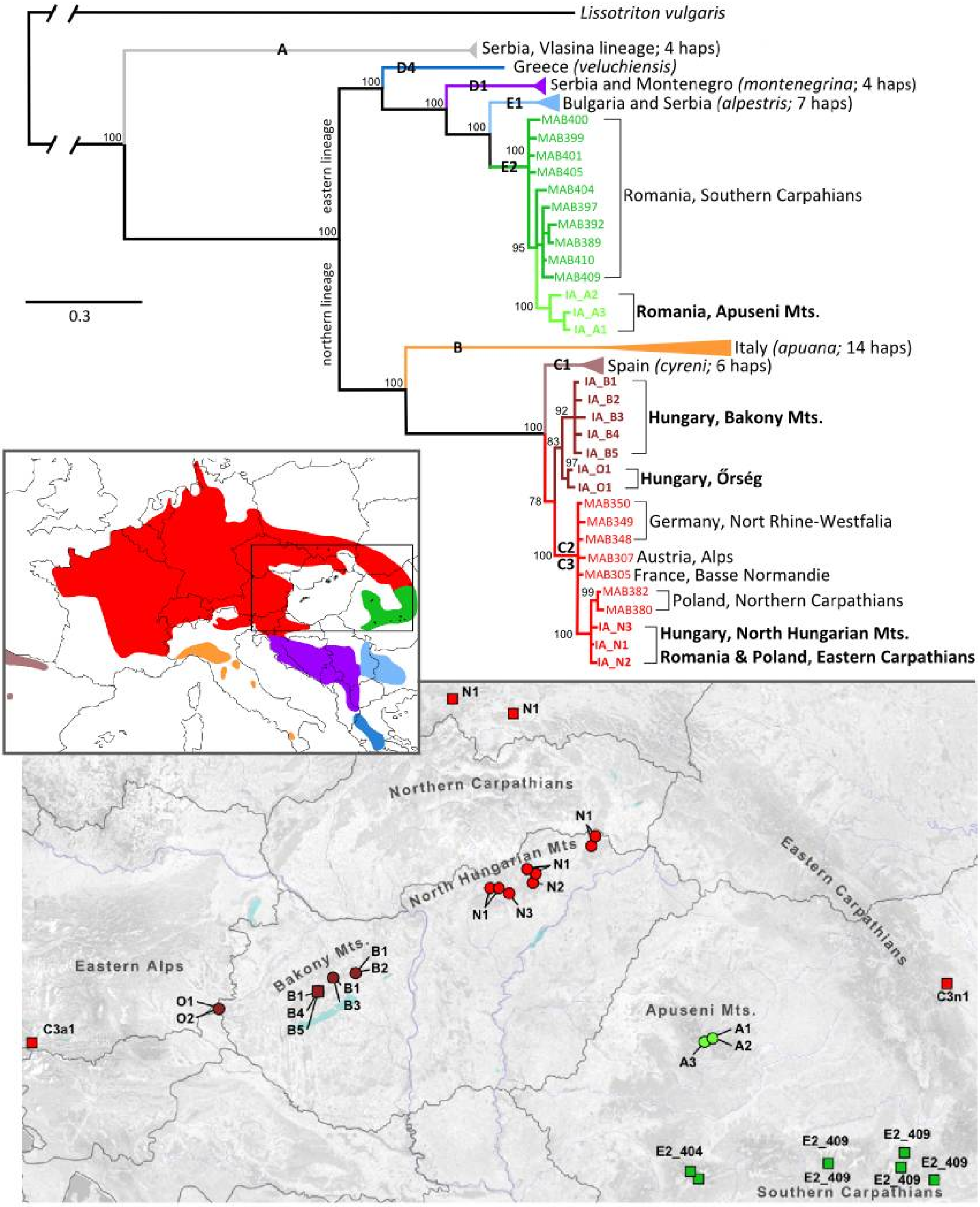


Fig1. Bayesian phylogram of the combined mtDNA haplotypes found in the Carpathian Basin (A). Distribution of I. alpestris in Europe (B) and sampling sites and haplotypes found in the Carpathian Basin (circles: our samples, squares: Recuero et al 2014 samples; C). Different colours refer to lineages found in Recuero et al. (2014). Sotiropoulos et al. (2007) lineages (A-E2) also marked on the clades. Bayesian posterior probabilities/ ML bootstrap support values are given at the nodes.

Further details: Vörös, J. & Szabó, K. 2014. Mitochondrial DNA diversity of the alpine newt, *Mesotriton alpestris*, in the Carpathian Basin. 17th SEH European Congress of Herpetology, Augusztus 22-27, Veszprém. Programme & Abstracts: 307

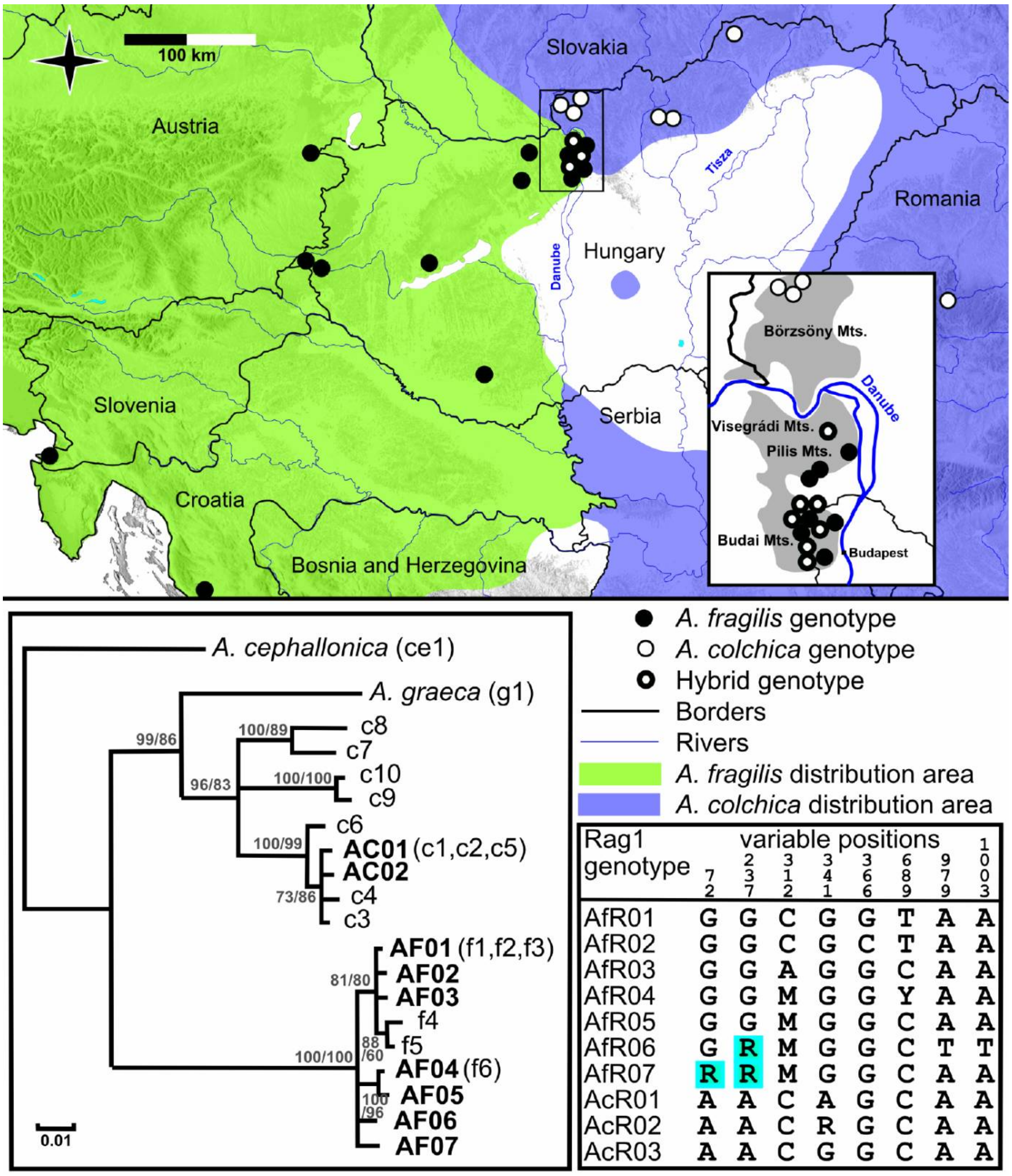
Two sister species of the *Anguis fragilis* species group occur in the Carpathian basin with a contact zone. They are widely distributed in the Carpathian Basin, occurring in most areas of Transdanubia, but inhabiting only undisturbed mountainous habitats east of the Danube (Northern Middle Range, fig. 1). As described by Dely (1972, 1974) based on morphological traits, both *A. fragilis* and *A. colchica* are present in the Carpathian Basin, their ranges are separated by the Danube River. Yet, in their contact zone in the Danube valley (Budai and Pilis Mountains) slow worms show an intermediate morphology, suggesting that the two taxa hybridize in this region. The aim of our study was to (1) discover the exact distribution of the two *Anguis* species in Hungary by identifying their mitochondrial lineages (2) to examine the hybridization between the two taxa in the Danube Valley contact area using single nuclear polymorphism in the Rag1 nuclear gene.

Altogether 40 tissue samples from living or road-killed animals were collected from eight regions of Hungary and from surrounding countries (Austria, Slovenia, Croatia, Romania, Spain), and 36 of them were used in this study (fig. 1). The Budai and Pilis Mountains in the Danube Valley were sampled more intensively, as Dely reported hybrid specimens from these regions (1972, 1974b). Tissue samples were preserved in 96% ethanol and kept at−20°C until processing. The molecular procedures are described in details in the publication below.

Sequences for the mitochondrial and nuclear gene regions were collapsed into haplotypes using Collapse 1.2 (Posada, 2011). These were aligned in ClustalW (Chenna et al., 2003) using the default settings for gap opening and extension penalties followed by limited manual correction of gap placement. For the ND2 mtDNA data set, standard diversity indices (haplotype diversity and nucleotide diversityπ) were calculated with DnaSP 5.0 (Rozas, 2009). Phylogenies were constructed using maximum likelihood (ML) and Bayesian inference (BI) methods. Sampled *Anguis* sequences were aligned and analysed together with previously published *A. fragilis* (f1-f6) and *A. colchica* (c1-c10) haplotypes (Gvoždík et al., 2010), using *A. graeca* g1 and *A. cephallonica* ce1 as outgroup species (fig. 1). The appropriate model of sequence evolution was estimated using the Akaike Information Criterion (AIC) using the jModelTest 0.1.1 software (Posada, 2008). ML analyses were performed in phyML 3.0 (Guindon and Gascuel, 2003). Bootstrap support values were calculated with 500 replicates. Bayesian infrence was performed using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) with two independent runs of Metropolis-coupled Markov chain Monte Carlo analyses, each with four incrementally heated Markov chains that were run for 1 million generations and sampled every 100 generations. Posterior probabilities were obtained from the 50% majority rule consensus tree.

There was a significant split in haplotypes parallel to the geographic distribution of slow worms. Samples collected west of the Danube (Western Hungary, Austria, Slovenia, Croatia, Spain) formed a distinct clade, together with haplotypes that were previously defined as *A. fragilis* (fig. 1). The other clade contained samples collected east of the Danube (North Hungary and Romania), grouping with *A. colchica* haplotypes from the NCBI database. In the *A. fragilis* clade, we detected 16 variable positions, defining seven haplotypes (AF01-AF07). In the *A. colchica* clade, our samples were less variable, with one single polymorpic position, defining two haplotyes (AC01 and AC02). Eigth variable positions were detected in the analyzed 1043 bp segment of the Rag1 gene, defining altogether 10 genotypes. Seven genotypes (AfR01-AfR07) were found in samples that belonged to the *A. fragilis* ND2 clade, another three genotypes (AcR01-AcR03) in samples of the *A. colchica* mitochondrial lineage. In seven animals, all collected in the putative contact zone (six in the Budai Mountains, one in the Pilis Mountains; fig. 1), and belonging to the *A. fragilis* mitochondrial clade, two Rag1 SNPs (on position 72 and 237, defining genotypes AfR06 and AfR07) contained alleles that were found in*A. colchica* but not in *A. fragilis* lineages outside of the hybrid zone (fig. 1).

Our molecular data confirmed the theory of Dely (1972) based on morphological traits that within the Carpathian Basin *A. fragilis* is distributed west while *A. colchica* east of the Danube. The hybrid zone of the two taxa seems to be located at the Pilis and Budai Mountains, again confirming the previous findings of Dely (1972). This hybrid zone lies at the meeting point of the two taxa with long independent evolutionary history recolonizing Europe from different glacial refugia. *Anguis fragilis* might have expanded to western Europe from a glacial refugium located in the north-western Balkans, while *A. colchica* populations survived the last glaciations in southern Caspian, Caucasian, and presumably Carpathian refugia, recolonizing Eastern and Northern Europe (Gvoždík et al., 2010).



Rag1 genotype	72	237	312	341	366	689	979	1003
AfR01	G	G	C	G	G	T	A	A
AfR02	G	G	C	G	C	T	A	A
AfR03	G	G	A	G	G	C	A	A
AfR04	G	G	M	G	G	Y	A	A
AfR05	G	G	M	G	G	C	A	A
AfR06	G	R	M	G	G	C	T	T
AfR07	R	R	M	G	G	C	A	A
AcR01	A	A	C	A	G	C	A	A
AcR02	A	A	C	R	G	C	A	A
AcR03	A	A	C	G	G	C	A	A

Fig. 1. Distribution of *Anguis* species in Central Europe, sampling sites, ND2 Bayesian phylogram and Rag1 genotype assignment of Hungarian *Anguis* samples. On the ND2 phylogram haplotypes found in samples of the present study are indicated as AF=*A. fragilis*, AC=*A. colchica*. Outgroup species (A. cephallonica and A. graeca) and other *A. fragilis*/colchica haplotypes (c1-c10, f1-f6) are retrieved from NCBI (Gvoždík et al., 2010). Bayesian posterior probabilities/ML bootstrap support values are given at the nodes. Shaded positions represent Rag1 diplotypes referring to hybrid origin.

Review of phylogeography of continental and steppic species

State of the Art: Extra-Mediterranean refugia – the exception or the rule?

As a synthesis of our surveys we elaborated the comparative aspects of phylogeographic patterns and processes connected with the very recent developments concerning the nested structures within refugia (‘*refugia within refugia*’, micro-refugia) and extra-Mediterranean (cryptic northern ‘*non-paradigmatic*’ refugia, e.g: Stewart & Lister 2001, Jaarola & Searle 2003, Kotlík et al. 2006, Birks & Willis 2008, Provan & Bennett 2008, Stewart & Dalén 2008). The phylogeographical analysis is intimately connected with the expansion and retreat of populations, with the problematic of the leading edge vs. rear edge and with population genetic processes at the edges of geographical ranges (e.g. Hampe & Petit 2005, Excoffier & Ray 2008). These theoretical considerations should be tested in connection with the recent results of historical and recent climatic changes (Schmitt & Varga 2009; Varga 2010).

A large number of recent publications have shown the importance of extra-Mediterranean refugia for temperate species and not only for alpine and arctic taxa. While the latter two groups may have frequently had wide ranges over this cold-continental zonobiome, the glacial range contractions of thermophilic temperate species, in most cases, must have led to smaller meso- or microclimatically favourable extra- or intrazonal areas within the extended periglacial belt. Many species with typical continental distributions might have had glacial distribution patterns with multiple extra-Mediterranean refugia, and were mostly not restricted to refuge areas in the eastern Palearctic, as often previously thought. Recent research showed that even thermophilic species, which were formerly thought of having been completely restricted to Mediterranean core areas, could in some cases survive in extra-Mediterranean refugia in addition to the typical Mediterranean areas. Such populations in many cases have an even higher genetic diversity and expansive power than populations restricted to the more southern “classical” refugia. These observations can be explained by two different factors, which might have acted in combination. The southern refugia of temperate species were often surrounded by extended cold-arid steppe areas, e.g. in the central part of the Balkan Peninsula and also in the Carpathian Basin (Varga 2010; Schmitt & Varga 2012; Magyari et al. 2013). Furthermore, the populations of the scattered extra-Mediterranean refugial pockets could expand and hybridise among each other during the milder interstadial phases of the Würm and also between the LGM and the younger Dryas period. It means that these extra-Mediterranean refuge populations have survived at the rear edge of the range during the ice ages, with all evolutionary consequences of this situation (Tóth et al. 2012; Pecsenye et al. 2015). These ice age rear edges became the leading edges of the postglacial northwards range expansions, thus strongly impacting the genetic constitution of Central and North Europe in many plant and animal species. In many cases, such populations have been characterised as localised subspecies of extended polytypic continental species, and they should be considered as evolutionarily significant units (ESUs) of high conservation priority (Pecsenye et al. 2014).

For all these reasons, the extra-Mediterranean refugia apparently represent an important biogeographical component of the western Palaearctic, *maybe nearly equivalent to the Mediterranean refugia further south*. Extra-Mediterranean refugia have been a common feature during, at least, the last ice age and thus they are paradigmatic. However, they are also represented by many individual patterns of particular biogeographical features so that each case shows at least some uniqueness. Although the principle of individual responses of the species to climatic oscillations between glacial and interglacial conditions was already postulated in this context (cf. Bhagwat & Willis 2008; Stewart et al. 2010; Varga 2010), it was demonstrated in this and an other review paper (Schmitt & Varga 2012; Kajtoch et al. 2015). During glaciations, ecosystems that exist today had been largely disintegrated and were represented by de novo ecosystems without close connection with the succeeding ones (e.g. the mammoth steppe with tundra, cold steppic and alpine elements). These ecosystems were locally intermingled with small forest refugia (i.e. the pockets of forests of Bhagwat & Willis 2008; Magyari et al. 2013).

Therefore, *no regular North–South shifts took place between glacial and interglacial conditions and vice versa* (as implicitly assumed in the biogeographical range paradigms). *Instead, a new sequence of ecosystems always had to be established, influenced by a combination of precipitation and temperature.* The newly evolved macro-biome of a continental cold steppe (that no longer exists) must have had characteristic macroecotones against the (glacially reduced) boreal forests and against the continental meadow steppes of temperate latitudes (Varga 2010; Tóth et al. 2012; Magyari et al. 2013; Feurdean et al. 2015). Although not existing under the recent climatic conditions, these macro-ecotones can be modelled based on the zonality of the cold-continental conditions of southern Siberia, northern Mongolia or even Yakutia. Here, many floral and faunal elements can be observed together on species-rich meadow steppes of these regions, species assemblages, which are partitioned to different habitats in eastern Europe like dry steppic grasslands, meadow steppes, damp meadows or even salt meadows (). The large number of macro-ecotones with their specific species assemblages is the background for the phenomenon of the evolution of so many species specific biogeographies, but hereby also for the paradigmatic patterns, i. e. the regular existence of micro-refugia. However, this is only a special case of the law of uniformity because even today such micro-refugia with peculiar mixtures of faunal and floral elements exist under analogous climatic conditions.

Publication: Schmitt & Varga 2012. *Frontiers of Zoology* (Authors equally contributed to the paper)

Steppic species – conclusions

The Principal Researcher was invited to participate as co-author in a review paper on steppic species, based on our phylogeographic surveys several times cited in recent publications. The conclusions presented below are based significantly on these surveys.

The phylogeography of species associated with European steppes and extrazonal, xeric grasslands is poorly understood. This paper summarizes the results of recent studies on the phylogeography and conservation genetics of animals (19 species of beetles, butterflies, reptiles and rodents) and flowering plants (17 species) of ‘steppic’ habitats in Eastern Central Europe. Most species show a similar phylogeographic pattern: relatively high genetic similarity within regional groups of populations and moderate-to-high genetic distinctiveness of populations from currently isolated regions located in the studied area. This distinctiveness of populations suggests a survival here during glacial maxima, including areas north of the Bohemian Massif – Carpathians arc. Steppic species generally do not follow the paradigmatic patterns known for temperate biota (south-north “contraction-expansion”), but to some extent are similar to those of arctic-alpine taxa. There are potentially three main groups within Eastern Central Europe that differ in their contemporary distribution pattern, which may reflect historical origin and expansion routes. Present diversity patterns of the studied steppic species suggest that assemblages of a unique genetic signature exist in each of the now isolated areas rich in steppic habitats. At least some of these areas probably act as present “interglacial refugia” for steppic species. This study strongly supports the need to protect steppic species throughout their entire ranges in the region, as the continuous destruction of steppic habitats in some areas may lead not only to the disappearance of local populations, but also to the extinction of unique evolutionary units.

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