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# MOLECULAR APPROACHES IN THE STUDY OF BAT POPULATIONS: THE GREATER MOUSE-EARED BAT *MYOTIS MYOTIS* IN EASTERN EUROPE

**Sz. Bücs[1], Z. Nagy[2], S. Boldogh[3], O. Popescu[1]**

[1] Molecular Biology Centre, Institute for Interdisciplinary Experimental Research, Babeş Bolyai University, Treboniu Laurian Str. 42, 400271 Cluj-Napoca, ROMANIA, email: szilard_bux@yahoo.com, opopescu@biolog.ubbcluj.ro

[2] Foundation for School, Densusianu Str. 6/A, 400428 Cluj-Napoca, ROMANIA, email: bigze@mail.rmdsz.ro

[3] Aggtelek National Park, Tengerszem side 1., 3758 Jósvafő, HUNGARY, email: sandorboldogh@yahoo.com

## SUMMARY

In this study, we present preliminary data sets about population structure of five Greater Mouse-eared bat Myotis myotis colonies, located in Eastern Europe. With the use of standard molecular methods we estimated genetic variability, population relatedness, the effect of the Carpathians on gene flow, and tried to reconstruct postglacial colonization routes for this region and the whole Europe. We genotyped 100 individual bat samples for five nuclear microsatellite loci, and also sequenced the samples for the HVII hypervariable domain of the mtDNA control region and partially for the cytochrome b gene. Nuclear markers show high levels of differentiation in the region, suggesting different origins of populations and limited gene flow across the Carpathians. The results from the nuclear level are not in concordance with those from the mtDNA level, sequence analysis indicating the presence of Lesser Mouse-eared bat Myotis blythii specimens in our samples. Sequencing other markers (like the nuclear RAG2) and the addition of further colonies from the region will clarify the overall picture. Due to the fact that Romania is one of the few countries in Europe where the Greater mouse-eared bat Myotis myotis is abundant and has several colonies numbering many thousand individuals, our study, beside the conclusions from genetic data will have in the future direct applications in establishing

proper conservation and protection strategies for the target species.

## INTRODUCTION

With the number of endangered species rising every year, proper conservation efforts are becoming more and more actual. The development of PCR and routine DNA sequencing, as well as the use of universal PCR primers [1] gave molecular biology powerful tools to better understand the evolutionary processes of living taxa. Since the genetic architecture of natural populations depends on historical events (colonization, recolonization, isolation), as well as on current factors related to the biology and ecology of the species (breeding structure, life cycle, gene flow), the elucidation of these processes can have far ranging effects for conservation management. The two marker groups largely used in such studies are mitochondrial DNA (mtDNA) markers (usually the mtDNA control region, COI or cytochrome *b*) and nuclear markers (mainly microsatellite loci).

The Greater Mouse-eared bat *Myotis myotis* occurs in Central and Southern Europe, on the northern border of France, Belgium, Germany, and extends through Poland to the Baltic Sea coast [2]. The north-eastern distribution of the species is limited by the Eastern Carpathians, in Romania and the Ukraine. It is a common bat species in the Balkans and the Mediterranean region. It is a partial migrant, with distances between summer and winter roosts of about 100 km [3]. The IUCN classifies the species as not threatened [4] but in the Carpathian Basin, particularly Romania, the Greater mouse-eared bat is widely distributed [5], earning a common status in many regions.

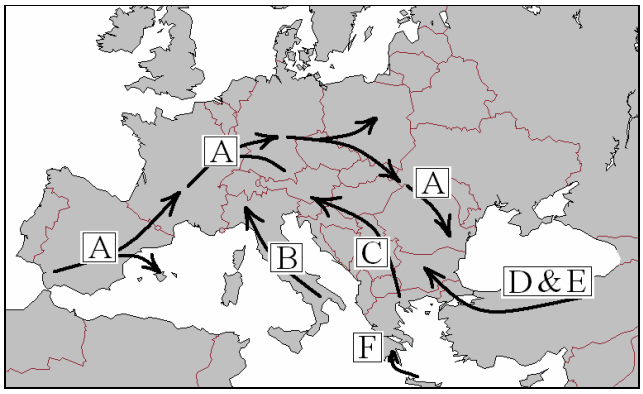


Figure 1. Postglacial colonization routes of *Myotis myotis* after the last glacial maximum [7]. Letters denote different lineages.

There have been several studies about genetic structure in Greater Mouse-eared bat

colonies. A large study from Central Europe evidenced the negative effect of the Alpine region on gene flow between colonies [6] whereas an other study suggest that the major recolonization processes for Greater Mouse-eared bats after the last glacial expansion started from the Iberian refugium [7], from where the species has recolonized most of Western and Central Europe (Figure 1). Another starting point would be Southern Italy, but the Alpine region, acting as barrier, ultimately stopped this expansion [7]. The colonies from the Balkan Peninsula are presented as being confined only to this region, but this fact was concluded only from three colonies, all originating from northern Greece. The only study from Eastern Europe, evaluating the genetic status of colonies located in the Carpathians, evidenced a high level of polymorphism at the nuclear level, suggesting the presence of a contact zone between European lineages in this region [8].

The present study analyzes five Greater Mouse-eared bat colonies from the Carpathian Region, both on the nuclear and mtDNA level. We test the hypothesis about the possible role of the Carpathians as barriers to gene-flow, estimate overall genetic diversity and try to reconstruct regional trends in the case of *M. myotis*. Beside the conclusions from genetic data, the study will have direct applications in establishing conservation and protection strategies for the target species in the Carpathian region, especially Romania and also in continental Europe.

## MATERIALS AND METHODS

In summer 2005, we sampled five Greater mouse-eared bat *Myotis myotis* maternity colonies (20-20 individuals) from five representative locations in the Carpathian region (Table I). Bats were captured, aged, sexed and measured (weight, forearm length). Skin samples were taken using sterile biopsy punches, according to a bat specific protocol [9]. The animals were set free within 30 minutes from their capture. Samples were stored in 96% ethanol until further use.

**Table I. Sample locations and colony characters used in the present study**

| Location | Roost | Geographical area | Elevation (m) | Individuals |
|---|---|---|---|---|
| Aştileu | Cave | Pădurea Craiului Mountains, Romania | 366 | 4000-5000 |
| Fusteica | Cave | Vîlcan Mountains, Romania | 437 | 2500-3000 |
| Voşlăbeni | Building | Gheorgheni Basin, Romania | 977 | 400-500 |
| Kelemér | Building | Borsod-Abaúj-Zemplén County, Hungary | 345 | 300-400 |
| Krynica | Building | Sadecki Beskid Mountains, Poland | 646 | 200-250 |

DNA extraction followed a standard phenol-chloroform method. The microsatellite survey involved the loci A13, C113, D9, E24 and G25 (Table II), characterized by Castella & Ruedi [10]. For every locus, the forward primer was fluorescent labeled with 6-FAM. Polymerase chain reactions were carried out in 25 µl reaction mixture, containing: 2.5 µl 10X termophylic PCR buffer, 1.8 µl 25 mM $MgCl_2$, 0.25 µl dNTP (20 mM each), 0.25 µl 100 pmol/µl reverse primer; 0.25 µl 100 pmol/µl forward primer, 0.25 µl *Taq* polymerase (5 units/µl) (Fermentas/Promega) and water to 25 µl. Reactions were carried out in a Mastercycler (Eppendorf) thermocycler, and followed the conditions given in [10], with necessary changes to facilitate individual PCR optimization for every locus. The general steps of PCR were: 3 min at 95ºC, followed by 30 cycles of 45 sec at 94ºC, 30 sec at primer annealing temperature, and 1 min at 72ºC, with one final extension of 10 min at 72ºC. PCR products were run on an ABI Prism© 310 Genetic Analyzer and sized with internal lane standard (GeneScan 500-ROX© and GeneScan 500-LIZ©) using GeneScan© Analysis version 3.7 [11].

At the mitochondrial level, we sequenced and aligned 291 bp from the HVII hypervariable domain of the mtDNA control region in 59 individual samples and 322 bp from the cytochrome *b* gene in 19 individual samples (including outgroups) using universal primers. Sequencing PCR programs were, as it follows: 2 min at 96ºC, 10 min at 96ºC, 15 sec at primer annealing temperature, 4 min at 60ºC, with a ramp of 1ºC/s in each step. Cycles were repeated 40 times. Isolated PCR products were run on a Beckman-Coulter CEQ 8000 DNA Sequencer. Raw sequences were analyzed and aligned with BioEdit version 7.0.1 [12].

Microsatellite variability was quantified with the number of alleles and Nei's genetic diversity ($D_a$). Loci with the greatest and lowest number of alleles were determined with mean numbers (M), as well as observed ($H_O$) and expected ($H_E$) heterozygosity for colonies and each locus. Departures from Hardy-Weinberg expectations and positive associations between loci (linkage disequilibrium) were also tested. We calculated pairwise differentiation indexes ($F_{ST}$) for comparisons across populations and also between hypothetical species. Since the use of whether $F_{ST}$ of $R_{ST}$ is still in debate [13, 14, 15], we used $F_{ST}$ in the case of comparison with the data from [6] and [7]. We used the software package ARLEQUIN 3.01 [16] for calculations at the microsatellite level.

**Table II. Properties of microsatellite loci [10] used in our study. Annealing temperatures are from [10], values in parentheses are those obtained after PCR optimalization**

| Locus | Repeat motif | Fragment size (bp) | Annealing temperature (°C) |
|---|---|---|---|
| A13 | $(TC)_5TT(TC)_{25}$ | 237 | 55 |
| C113 | $(ACC)_7$ | 101 | 60 |
| D9 | $(CT)_{29}$ | 148 | 60 (57) |
| E24 | $(TC)_{32}$ | 236 | 60 (57) |
| G25 | $(AGC)_{11}AAT(AGC)_4$ | 147 | 55 |

The relation between mtDNA haplotypes was determined by various tree building methods. We used as outgroups *M. punicus* from Morocco and *M. blythii oxignathus* from Crimeea and Armenia. We inferred the best fitting nucleotide substitution model with the program MODELTEST [17]. Both the Akaike Information Criterion (AIC) and the Hierarchical Likelihood Ratio Test gave the same results, selecting as the best model the HKY+G model. We calculated minimum evolution trees using MEGA 3.1 [18], and using several parameters (Kimura2, Tamura-Nei), but these all showed similar results. The maximum likelihood tree was estimated by the PHYML software [19]. The proportion of the invariable sites was set to zero and all other parameters were estimated from the data. The statistical support of the clades was determined by nonparametric bootstrap (1000 replicates). The Bayesian inference of the phylogeny was conducted with MrBayes [20], using flat priors. Parsimony analysis was conducted using simple heuristic search.

## RESULTS

During data analysis we obtained controversial results. At the nuclear level, microsatellites evidenced high and significant differentiation between colonies, but this does not reach the differentiation level of separate species. In case of mtDNA analysis however, 32 individuals out of 100 were classified as *Myotis blythii oxignathus*, the sibling species of *M. myotis*. With these facts, we could not compute population genetic analysis at the mtDNA level. Due to the facts that analyzed sequences are too short for strong conclusions and tree branches are not supported by great bootstrap values (see below), we classify mtDNA results as partial and inconclusive.

**1. Intracolonial genetic diversity.** In the case of 100 individual bat samples a total of 89 alleles were scored. No null alleles were scored, all loci are polymorphic. Significant associations between loci were rejected; they represent independent units of the genome. All colonies were in Hardy-Weinberg equilibrium, however some loci (A13, E24, G25) showed significant departures from the null hypothesis (no equilibrium), but not in all colonies. The

locus with the greatest number of alleles is E24 (28 alleles), whereas C113 is the least polymorphic (6 alleles). Loci with the greatest mean allele number and average observed heterozygosity in colonies are D9 ($M_A$=13.4, $H_O$=0.76) and E24 ($M_A$=15.6, $H_O$=0.63). The locus G25 was almost monomorphic (two alleles) in the colony from Voşlăbeni, with 19 homozygous and one heterozygous individual. A similar case is for the locus C113 in the colony from Krynica. The overall mean number of loci is high ($M_A$=9.56).

All colonies show high allele polymorphism and gene diversity. The colonies with the greatest number of alleles and mean number of alleles are the colony from Kelemér and Voşlăbeni (51 and 50 alleles, respectively M=10.2 and M=10). The colony with the lowest number of alleles and mean number of alleles is Krynica (43 alleles, respectively M=8.6). The greatest gene diversity was found in the colony from Aştileu ($D_a$=0.7861), the lowest in the colony from Krynica ($D_a$=0.6692). In contradiction with these data, the greatest observed heterozygosity was found in the colony from Krynica ($H_O$=0.70), and the lowest in the colonies from Kelemér ($H_O$=0.55) and Voşlăbeni (0.47). The overall gene diversity is high ($D_a$=0.7302) and the overall mean rate of heterozygotes is 0.616. In the comparison of roost types, both types (caves and buildings) show great diversity. There is no great difference between allele number (47.5 vs. 48) and mean allele number (9.5 vs. 9.6). However, in the case of the rate of heterozygotes (0.68 vs. 0.57) and average gene diversity (0.76 vs. 0.70), the caves show greater values, indicating that colonies from artificial roosts could be less diverse that colonies located in the stabile environment of caves.

Based on mtDNA sequences, individuals (hypothetically) classified as *M. myotis* have a mean number of alleles of 14.8, whereas *M. blythii oxignathus* individuals have on average 13.6 alleles. Loci with greatest number of alleles are the same as above. Gene diversity in *M. myotis* is 0.7748, and rate of heterozygotes of 0.65. *M. blythii oxignathus* individuals have similar gene diversities and rate of heterozygotes ($D_a$=0.7629, $H_O$=0.52).

**Table III. Pairwise $F_{ST}$-s and their significance values in the analyzed colonies**

| | Aştileu | Fusteica | Voşlăbeni | Kelemér | Krynica |
|---|---|---|---|---|---|
| **Aştileu** | 0.00000 (----) | ------ | ------ | ------- | ------- |
| **Fusteica** | 0.01529 (0.0810) | 0.00000 (----) | ------ | ------- | -------- |
| **Voşlăbeni** | 0.15200 (0.0000) | 0.10983 (0.0000) | 0.00000 (----) | -------- | -------- |
| **Kelemér** | 0.07634 (0.0000) | 0.04834 (0.0000) | 0.02071 (0.09009) | 0.00000 (----) | -------- |
| **Krynica** | 0.05094 (0.0000) | 0.03864 (0.0000) | 0.19197 (0.0000) | 0.11726 (0.0000) | 0.00000 (----) |

On the mtDNA level (both HVII hypervariable region and cytochrome *b*) results are only preliminary. There is only minor diversity between individuals, the most widespread (in more than 50% of individuals) haplotype being identical to the H1 haplotype from the study of Ruedi & Castella [7]. In case of the partial cytochrome *b* gene, the H1 haplotype is similarly found in many individuals, however it shows some discrepancies when control region and cytochrome *b* haplotypes are combined. Since not all sequences were obtained, we did not calculate average gene diversity for the mtDNA markers at the level of colonies.

**2. Population structure.** AMOVA (analysis of molecular variance) tests about the source of intra and intercolonial diversity concluded that only a small percent of variation is found between different populations (8.06%) whereas the great part of the variations is contributed to individuals in certain populations (77.24%). At the level of separate populations, we calculated pairwise $F_{ST}$-s (Table III). Almost every pair of colony has significant $F_{ST}$ values, except the colony pairs Aştileu-Fusteica and Kelemér-Voşlăbeni. The overall fixation index is high and significant ($F_{ST}$=0.08055, $p<0.0005$), suggesting great levels of differentiation between populations. For comparison, the colonies analyzed in [7] had a lower fixation index ($F_{ST}$=0.035, $p<0.001$). The low and significant overall $F_{IS}$ index ($F_{IS}$ =0.1599, $p<0.0005$) indicates low levels of inbreeding. At the population level, the colonies of Voşlăbeni and Kelemér have the highest $F_{IS}$ index, suggesting inbreeding. On the contrary, the colony from Krynica shows low levels of outbreeding ($F_{IS}$ = -0.04724). The greatest (and highly significant) pairwise $F_{ST}$ is found between the colonies from Voşlăbeni and Krynica ($F_{ST}$=0.19197), suggesting the greatest difference between them. Furthermore, the colony from Voşlăbeni significantly differs from Aştileu ($F_{ST}$=0.15200) and Fusteica ($F_{ST}$=0.10983). The greatest similarity if found between the pairs of colonies Fusteica-Aştileu and Voşlăbeni-Kelemér (Table III).

If we assume the presence of *M. blythii oxignathus* individuals in our samples case, and compare the two sub-groups of samples (*M. myotis* vs. *M. blythii oxignathus*), nuclear markers show half the differentiation as if one species were present ($F_{ST}$=0.035), but the difference is still significant ($p<0.005$). The great amount of diversity is harbored at the individual level (76.88%), whereas only a small part of the variation is attributable to the differences between populations (3.58%). Since the difference between two species cannot be smaller, than the difference between populations of the same species, we assume that only *M. myotis* individuals were analyzed.

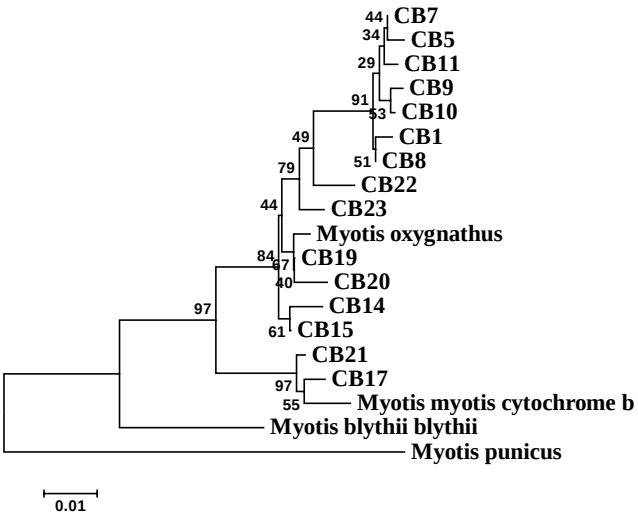


Figure 2. Minimum evolution tree inferred from 19 cytochrome *b* haplotypes of *M. myotis* and *M. blythii oxignathus*. Note the low bootstrap support of most of the branches.

At the mtDNA level we did not compute pairwise differences, but based on preliminary mtDNA sequences, the hypothetical *M. myotis* group has no (or small) differences at the nucleotide level, consequently at the interpopulational level, whereas the hypothetical *M. blythii oxignathus* group is highly divergent with phylogenetic trees mixing individuals from Romania with individuals from Poland and Armenia in several tree branches (Figure 2), making population structure hard to interpret.

**3. Phylogenetic analysis of mtDNA haplotypes and European lineages.** In case of the partial cytochrome *b* gene (322 bp), we found 62 variable sites (with outgroups) and 31 variable sites excluding outgroups. Alignment of 19 sequences (representing 19 haplotypes) yielded 260 constant and 27 parsimony informative sites. Excluding outgroups, parsimony informative site number decreased to 20. We inferred the minimum evolution tree using the Kimura2 substitution model for the 19 sequences, but branches show low bootstrap support (Figure 2). Other methods (Bayesian, maximum likelihood) yielded similar trees, also with low bootstrap support. Outgroups were clearly separated from our samples, supported with long branches. Inner branches of the tree were short, indicating minimum differences between samples.

For the mtDNA hypervariable region HVII, from a total of 292 bp sequenced for 59 individuals, there are 48 variable sites, of which 37 are parsimony informative. The minimum evolution tree (not shown), calculated using the Kimura2 and the Tajima-Nei

parameter, has low bootstrap support, with terminal branches being too short to denote any kind of difference. We also compared our data sets (only hypothetical *M. myotis* data) with *M. myotis* mtDNA sequences from other studies [7] and results indicate that on the European scale, there is little (or even no) variation in the sequences of *M. myotis*, all examined individuals being part of the H1 haplotype, presumably responsible for colonizing the whole Europe from the Iberian Peninsula [7]. Hypothetical *M. blythii oxignathus* from our data sets are part of the D and E *M. myotis* lineages analyzed in [7].

## DISCUSSION

Based on the analyzed 100 genotypes, the genetic status of Greater Mouse-eared *Myotis myotis* colonies of the Carpathian region is satisfactory, having high gene diversity and rate of heterozygotes. After the last glaciation and during range expansion, species should lose their genetic diversity along the way, making southern (source) colonies more diverse than new colonies [21, 22]. However other studies [23] showed that in case of 22 European tree species, greatest diversity is found at intermediate latitudes. Furthermore, in case of *M. myotis*, the Alpine region is evidenced as an admixture zone with elevated gene diversity [6]. Since colonies of the Carpathian region also show high values of diversity, we can assume the existence of another admixture zone located in this region, and a possible meeting point of different lineages.

Due to the fact that the rate of heterozygotes greatly affects gene diversity (being related to the amount of inbreeding), the most diverse colonies from the region are expected to show lowest levels of inbreeding. Overall $F_{IS}$ index shows a moderate value, suggesting inexistent (or low levels) of inbreeding. For the effect of roost type on genetic diversity, the equilibrium is slightly shifted in the direction of underground habitats, colonies located in caves having greater diversity and rate of heterozygosity. Since these habitats are more stable, offer constant climatic parameters and give the colony a relative protection from human disturbance, underground colonies are expected to have better conditions for life, and thus for the accumulation of diversity. As shown by ecological studies, roost characteristics affect colony size and reproductive success [24]. But since colonies located in caves are usually larger, the greater genetic diversity is not only attributable to roost characteristics. In our case, attic dwelling colonies have only a few hundred individuals, whereas cave dwelling colonies are composed of several thousand bats. The greatest gene diversity found in an attic dwelling colony (Kelemér, $D_A$=0.7674) slightly exceeds that of a cave dwelling colony (Fusteica, $D_A$=0.7497). Consequently, we cannot deduce roost characteristics solely on genetic basis. Many factors should be taken into consideration when assessing genetic status of bat colonies and roost optimality.

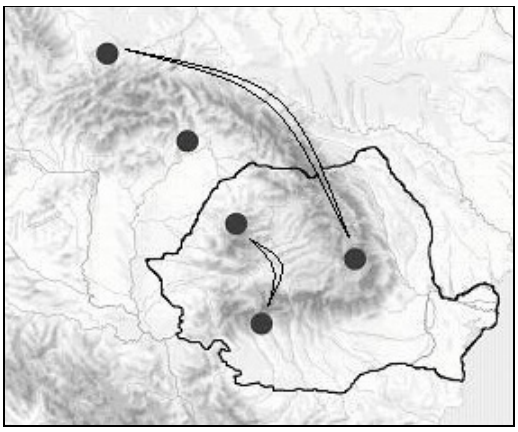

Figure 3. Divergence of colonies based on pairwise $F_{ST}$-s. Colonies are (from north to south): Krynica, Kelemér, Aştileu, Voşlăbeni, Fusteica. The long line represents the greatest, whereas the short line the smallest difference

Based on the great and significant value of the overall $F_{ST}$ we can predict highly diverged colonies in the Carpathian region. This partial isolation can be the result of great distances, a geographic barrier or lack of migration. Since microsatellites reflect the amount of current gene-flow, and females of the species are highly phylopatric, we predict that moderate levels of divergences between colonies are the result of male dispersal rate. The greatest geographical distance is between the pairs of colonies from Fusteica-Krynica and Voşlăbeni-Krynica (Figure 3). However, pairwise fixation indexes are highly different between these two pairs, the first one being negligible, and the latter high and significant. Similarly, the shortest geographical distance is between the colonies from Kelemér and Aştileu, but with a moderate $F_{ST}$. Based on these, we can not assess the effect of the Carpathians on intercolonial variance. Even if the Carpathian range is an effective barrier to gene-flow, divergence among colonies arises also with the effect of low male dispersal and female philopatry.

In population structure based on mtDNA sequences (*M. myotis* vs. *M. blythii oxignathus*), the overall fixation index (which is much smaller than the $F_{ST}$ for the previous scenario) suggests little differentiation. Based on these, we cannot exclude the possibility of two species being present. However, in estimating the role of the Gibraltar Strait as gene flow barrier [25], authors found that the overall $F_{ST}$ is 0.333. The populations from the African side of the Strait are since then recognized as a distinct species, *Myotis punicus*. If a similar scenario would be present in the Carpathians, than $F_{ST}$ indexes between hypothetical *M. myotis* and *M. blythii oxignathus* would be of similar value, but actually this is 10 times

smaller, indicating that we are dealing only with one species.

The mtDNA sequence analysis is in contradiction with the nuclear evidence, as the tree themselves created with different mtDNA markers (cytochrome *b* vs. control region). Solid evidence will come from other mtDNA (COI) or nuclear (RAG2) markers. The low clade credibility values on every phylogenetic tree are the effect of the analysis of short sequences. The sequencing of larger, more conservative markers will finalize the phylogeographic patterns in colonies from the Carpathian region. These partial results suggest that the entire European continent was recolonized after the last glacial expansion from the Iberian refugium, however in the present we are not aware of any colonization pattern that would follow this type. The three major recolonization patterns widely recognized (grasshopper, bear and hedgehog type) [22] always involve the presence of three lineages in Europe, originating from the three glacial refugia (Iberia, Italy and the Balkans) [21]. Clearly, the Alpine region prevented the expansion of the Italian *M. myotis* lineage into Central Europe [6, 7], but the assumption that the Rhodopes did similarly with Balkan lineages is premature.

## CONCLUSION

Due to the controversial evidence presented by mtDNA and nuclear makers, we were not able to infer continent wide trends in *M. myotis* population genetics, nor did we clarify postglacial colonization routes in Europe. Furthermore, due to the fact that inferred phylogenetic trees do not have high bootstrap supports and compared results (haplotypes) of cytochrome *b* and control region show dissimilarities, we can not draw solid conclusion or distinguish between the two possible scenarios (one or two species examined). In addition, based on the low differences on the nuclear level between hypothetical *M. myotis* and *M. blythii oxignathus*, we believe that our mtDNA results are to be reevaluated. The finalization of this study will yield in the elucidation of the correct phylogeographical pattern in the case of *M. myotis*, which could have direct applications in conservation efforts for already endangered bat species.

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