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Phylogenetic status of endemic *Chionomys nivalis mirhanreini* in the Western Carpathians

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Abstract

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The Snow vole has a fragmented distribution restricted to the mountain and rocky regions from the south-western Europe to the Caucasus and the Middle East. Several subspecies have been described on the basis of dental characters. In this study we provide more detail insight into phylogenetic status of the Snow vole *Chionomys nivalis* occurring in the Western Carpathians compared to its populations within Eurasia. We analysed 588 bp fragment of mtDNA cytochrome *b* gene in samples collected in the two isolated populations within the High Tatras and Low Tatras Mountains. Our results indicate Western Carpathians as the long term isolated refugia for *C. nivalis* and support the subspecies status of *C. n. mirhanreini* in the Western Carpathians. The European populations are formed by two southern phylogenetic lineages – the first originates in Iberian Peninsula and the other expanding from Balkans to the Eastern Carpathians is also visible.

Keywords

haplotypes, post-glacial recolonization, phylogenetic lineage, Rodentia

Introduction

The European biota was strongly influenced by climatic fluctuations during the last two million years of Pleistocene (Castiglia et al., 2009), while the environmental changes in the last 20,000 years were probably the most dramatic for mammalian fauna. Pleistocene glaciatio affected the movement of several species (Moravcová, 2010). In the peak of glacial period, most species areas were fragmented into isolated glacial refugia. The European refugia were located in the Iberian, Apennine and the Balkan Peninsula and Great European Plain (Schmitt and Seitz, 2001).

The genus *Chionomys* is represented by only three species that exclusively inhabit mountainous regions of Europe, Asia Minor, and parts of Western Asia (Nadachowski, 1991) and these are: *Chionomys gud* (Satunin, 1909), *Chionomys roberti* (Thomas, 1906) and *Chionomys nivalis* (Martins, 1842). The first indisputably identified fossils of Snow vole, *C. nivalis*, are recorded from the island of Chios in Greece probably dates from the Middle Pleistocene (Storch, 1975) and their number is increasing in the upper Pleistocene (Nadachowski and Baryshnikov, 1991). Older fossils traditionally attributed to this species actually included *Microtus agrestis* and *Microtus oeconomus* (Nadachowski, 1991).

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Since then, in some areas Snow vole recognized gradual changes in the construction of teeth, while in other areas the development is not observed (Chaline and Graf, 1988). Climatic fluctuations in mid and late Pleistocene played an important role in changing the geographical distribution of the species (Janeau and Aulagnier, 1997). When most of the mountains were covered by ice, *C. nivalis* moved from mountains to adjacent lowland areas (Terzea, 1977). After the retreat of glaciers, the area of Snow vole has become fragmented into isolated populations, while in the smallest changed dramatically (for example, population in the Tatras can be considered as distinct species based on different dental morphology; Nadachowski, 1992). During Würmian glaciation, *C. nivalis* was widespread in Central and Western Europe especially during the coldest periods. At the end of the Würm suitable rocky habitats occurred only sporadically (Terzea, 1977). Recent distribution of Snow vole has relict character and it is occurring in the mountains of Europe, from southwest Europe in the Pyrenees and the Alps, through Southeast Europe to Turkey, Israel, Lebanon, Palestine, Syria, Iran, and South Caucasus to mount Kopet-Dagh (Corbet, 1978). Thus its distribution is fairly fragmented, mainly because of habitat requirements. Although *C. nivalis* is often found in mountainous conditions, this species is not physiologically suitable for cold environments (Bienkowski and Marszalek, 1974). Rather, it is suited for micro-environments with medium humidity in rocky habitats, specifically the stone and rubble piled boulders, cracks in the rocks, cliffs, cave entrances and slots in rock substrate (Luque-Larena et al., 2002), regardless of the altitude (Kryštufek and Kovačić, 1989).

In isolated populations of *C. nivalis* significant morphological divergence was identified (Amori, 1999) what resulted in large number of morphologically described subspecies. Corbet (1978) recognizes four subspecies, while Krapp (1982) distinguishes thirteen, Ellerman and Morrison-Scott (1966), similarly like the Kratochvíl (1981) indicate sixteen subtypes and Nadachowski (1991) in his most comprehensive review suggests eighteen of them. Somatic variability in European populations revealed an alpine branch (subtype) and branches including populations of Italy, Spain and France (Graf, 1982). Generally, the "North mountain" population group (Alps, Carpathians and Balkan Peninsula, but also the Caucasus and even Kopet-Dagh), is formed by *C. nivalis* and *C. mirhanreini*. Their territories were colonized relatively late, in most cases, after the retreat of glaciers (Janeau and Aulagnier, 1997). "Southern populations" group comprise *lebrunii*, *cedrorum* and *spiizenbergerae* which occupy lower altitudes and areas that have never been covered by glaciers. It is noteworthy that southern populations, except *C. n. hermonis* (southernmost population in the Lebanese mountains, which is classified as *C. n. nivalis*) are the most primitive. They are preserved primitive features characteristic of *C. nivalis* dates from the Middle Pleistocene. During cool periods, these populations were not forced to change their habitat contrary to mountain populations that emigrated. (Nadachowski, 1991).

Phylogeography of *C. nivalis* and its evolutionary history in relation to closely-related species was recently inferred using independent molecular markers (Yannic et al., 2012). However, this almost comprehensive study did not have included the northernmost population in the Western Carpathians where significant morphological differentiation was recorded in several traits (Luque-Larena et al., 2002). Therefore in this study, we attempt to elucidate phylogenetic status of Western Carpathians population of Snow vole based on analysis of mtDNA cytochrome *b* gene fragment.

Material and methods

Sampling

Within the Western Carpathian population we sequenced four samples from trapped individuals in Nízke Tatry Mts – Chopok (48°56′32.2″N, 19°35′15.5″E), Kráľova Hoľa (48°52′59″N, 20°08′21″E) and Tatry Mts – Roháče (49°12′26″N, 19°44′44″E) during 2002–2014. Tissue samples were stored in 96% ethanol. We also retrieved 35 sequences covering whole *C. nivalis* range from the GenBank (Table 1).

DNA extractions and amplifications

DNA was extracted from tissues using a QIAmp Tissue Extraction Kit (Qiagen), following the manufacturer's instructions. For the phylogenetic study, the mitochondrial cytochrome *b* gene was amplified using primers L14841 and H15915 (Kocher et al., 1989; Irwin et al., 1991). The PCR mix of 25 µl total volume contained 50–100 ng DNA, 1 × PCR buffer, 0.4 µM each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 0.5 U Taq polymerase (Qiagen). The PCR reaction was performed in an Eppendorf Thermal Cycler with the following steps: 95 °C for 4 min, 40 cycles at 94 °C for 30 s, 58 °C for 1 min and 72 °C for 2 min, and a final elongation step at 72 °C for 10 min (Yannic et al., 2012). PCR products were checked in 1.6% agarose gel. Products were analysed on ABI PRISM 3100 capillary DNA sequencer (Applied Biosystems).

Alignment and analyses of mtDNA sequences

The sequences were aligned using ClustalW (Larkin et al., 2007) and manually edited in BioEdit (Hall, 1999). The total length of the alignment was 558 bp.

Table 1. Genetic diversity indices based on mtDNA cytochrome *b* sequences for the Snow vole *Chionomys nivalis*. Number of analysed individuals (*n*), number of haplotypes (N*h*), number of segregating sites (*S*), nucleotide diversity (π) and haplotypes diversity (*Hd*) ± standard deviations

Population	Subspecies *C. nivalis*	*n*	N*h*	*S*	π	*Hd*
Western group		23	23	63	0.024 ± 0.013	1 ± 0.034
Alps & Apennines	*nivalis*	12	12	28	0.015 ± 0.009	1 ± 0.034
Western Carpathians	*mirhanreini*	3	3	4	0.005 ± 0.004	1 ± 0.272
Balkan	*aleco*, *wagneri*, *malyi*	4	4	16	0.014 ± 0.010	1 ± 0.177
Pyrenees, Sierra de Gredos	*abulensis*	4	4	14	0.014 ± 0.010	1 ± 0.177
Eastern group		12	12	45	0.031 ± 0.017	1 ± 0.034
Anti-Lebanon	*hermonis*	3	3	5	0.006 ± 0.005	1 ± 0.272
Taurus	*cedrorum*	2	2	1	0.002 ± 0.002	1 ± 0.500
Caucasus	*trialeticus*	3	3	5	0.005 ± 0.005	1 ± 0.272
Khorasan	*dementievi*	4	4	5	0.004 ± 0.003	1 ± 0.177

Intrapopulation genetic characteristics such as number of samples per haplotype *n*, number of haplotypes N*h*, nucleotide diversity π and haplotype diversity *Hd* were estimated in ARLEQUIN 3.1 (EXCOFFIER et al., 2005). We divided the populations into different groups based on their geographical ranges and environmental condition where they occur: a European group (Eastern Carpathians, Western Carpathians, Alps, Appenines, Dinarides, Baba, Rila) and an Asian group (Khorasan, Anti Lebanon, Saleh, West Toros, Caucasus). The matrix of pairwise genetic distances θ_{ST} (NEI and LI, 1979) with their corresponding *P* values was computed in ARLEQUIN 3.1 with 10,000 MCMC iterations (EXCOFFIER et al., 2005). To test the statistical significance of genetic differentiation between populations we used Fisher's exact test with 10,100 permutations. The AMOVA implemented in ARLEQUIN 3.1 (EXCOFFIER et al., 2005) was employed with 10,000 permutations.

Phylogenetic analyses were performed using neighbour-joining (NJ), maximum likelihood (ML), maximum parsimonious (MP) and Bayesian trees. Firstly the distance based NJ tree was constructed under Tajima-Nei distance model (TAJIMA and NEI, 1984) and Gamma distributed rate among sites. Gamma was set to one. The reliability of the NJ tree was assessed by 10,000 bootstrap replicates. A ML tree was constructed by implementing GTR+I model, inferred by the Nearest-Neighbour-Interchange heuristic method (NNI) with default set NJ/BioNJ initial tree and very strong branch swap filter. The phylogeny was tested by 10,000 bootstrap replicates. The topology of the tree was further investigated by Min-Mini Heuristic MP search method; search level three, max number of trees to retain was set to 10. A MP consensus tree was inferred from 10,000 bootstrap replicates in MEGA 5.2 (TAMURA et al., 2011). The substitution model GTR+I and model frequencies A = 0.3308, C = 0.2722, G = 0.1098, T = 0.2872 were selected in JMODELTEST (POSADA, 2008), based on the Akaike information criterion corrected for small sample sizes (AICc). Phylogenetic relationships between haplotypes were inferred by Bayesian inference using 1,000,000 the Markov Chain Monte Carlo (MCMC) starting from random tree and sampling every 1,000 generation (four chains, heating = 0.2) implemented in MRBAYES 3.1.2 (RONQUIST et al., 2011). Searches were performed using GTR model including proportion of invariant sites. The Bayesian 50% majority rule consensus tree was visualised in FigTree v1.4.2 (RAMBAUT, 2014). The most divergent *C. roberti* haplotypes were used to place a root in all phylogenetic trees. A haplotype distribution maps with interpolate values of nucleotide diversity and pairwise genetic distances (θ_{ST}) were constructed in ArcMap 10.2 (ESRI).

Results

Genetic diversity analyses

We analysed 588 bp long fragment of the mtDNA cytochrome *b* gene in 35 distinct haplotypes that were defined by 123 polymorphic sites, 97 were parsimony informative. In this set of analysed haplotypes, two were novel (GenBank Accession Numbers KX077599 and KX077600), obtained from sequences of four individuals sampled in Western Carpathians. Haplotypes were divided into two major groups: 1) the western group, 2) the eastern group (Table 1). Within 23 haplotypes of the western group we recorded 61 transitions and 6 transversion and 40 were parsimony informative. Within 12 haplotypes of the Eastern group we recorded 40 transitions and 7 transversions and 38 of them were parsimony informative. The western group included twelve haplotypes from Alps and Apennines, three haplotypes

from the Western Carpathians, four haplotypes from Balkan area and four haplotypes from the Pyrenees. The Eastern group included three haplotypes from Anti-Lebanon, two from Taurus and three from Caucasus. We also analysed four haplotypes of *C. n. dementievi* from the Khorasan Mountains.

The Western group showed lower nucleotide diversity compared to the Eastern group. The highest value of nucleotide diversity was recorded in the Alps and Apennines followed by Balkan area, Pyrenees and the considerably low value was recorded in the Western Carpathians as representatives of the Western group. Generally, within the Eastern population units restricted to different mountain ranges, we recorded low nucleotide diversity values similar to value recorded in the Western Carpathians (Table 1, Fig. 1).

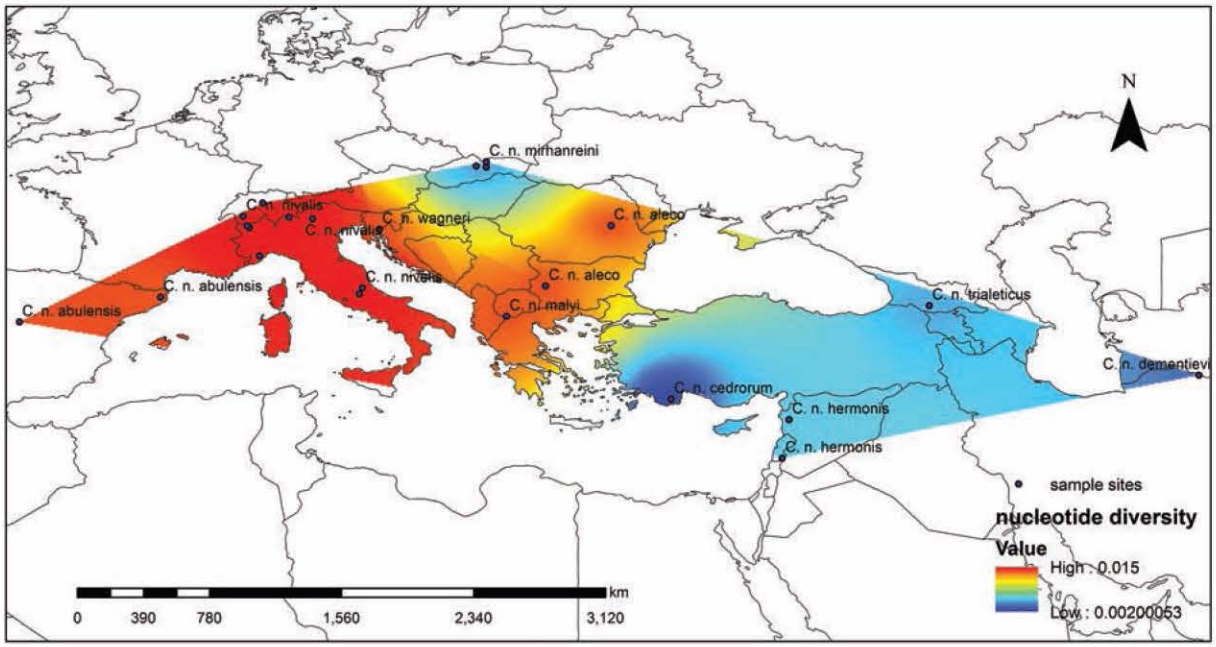

Fig.1. Map of interpolated nucleotide diversity (π) values among analysed subspecies.

Population differentiation

Interpolated θ_{ST} values clearly indicate lower genetic differentiation within the population on the West from the Western Carpathians than within the Eastern group (Fig. 2, Table 2). Genetic distances between pairs of populations (θ_{ST}) confirmed the highest differentiation of the Khorasan population (θ_{ST} = 0.80–0.93) where is assumed to be *C. n. dementievi*, very high differentiation of the Western Carpathian population from the other seven populations (θ_{ST} = 0.57–0.93) indicate the presence of *C. n. mirhanreini*. The lowest, differentiation was recorded between population of Alps, Apennines and Balkan population (θ_{ST} = 0.41). The genetic distance between the Western and Eastern group was θ_{ST} = 0.29. In the analysis of molecular variance (AMOVA) we calculated genetic variation among Western and Eastern group. The overall fixation index was 0.14 (P = 0.03) and 13.55% of the total genetic variation is due to differentiation between Western and Eastern group. Among populations within the Western and Eastern group the fixation index (F_{sc}) was 0.66 (P = 0.000) and 57.48% of total variation was due to differentiation among populations (Table 3).

Phylogenetic patterns

The evolutionary relationships examined in NJ, ML, MP and Bayesian trees had similar topology and only the topology of Bayesian inference is shown (Fig. 3). The phylogenetic reconstruction revealed strong support (99–100%) for dichotomy between *C. n. dementievi* and all other monophyletic subspecies. Poorly supported (<70% in all trees) were West (Pyrenees, Alps, Apennines, Western Carpathians, Eastern Carpathians, Rila, Baba, Dinarides) and East groups (Anti Lebanon, Saleh, Taurus, Caucasus). Within the Western group were well supported several geographic groups consistent with subspecies statuses of *C. nivalis*. Within the Western group additional sequences of *C. n. mirhanreini* created highly supported the Western Carpathians group (97–100%).

Discussion

Taxonomic status of the *Chionomys* genus was ambiguous for a long time (Nadachowski, 1991). Miller (1908) considered *Chionomys* as subgenus and

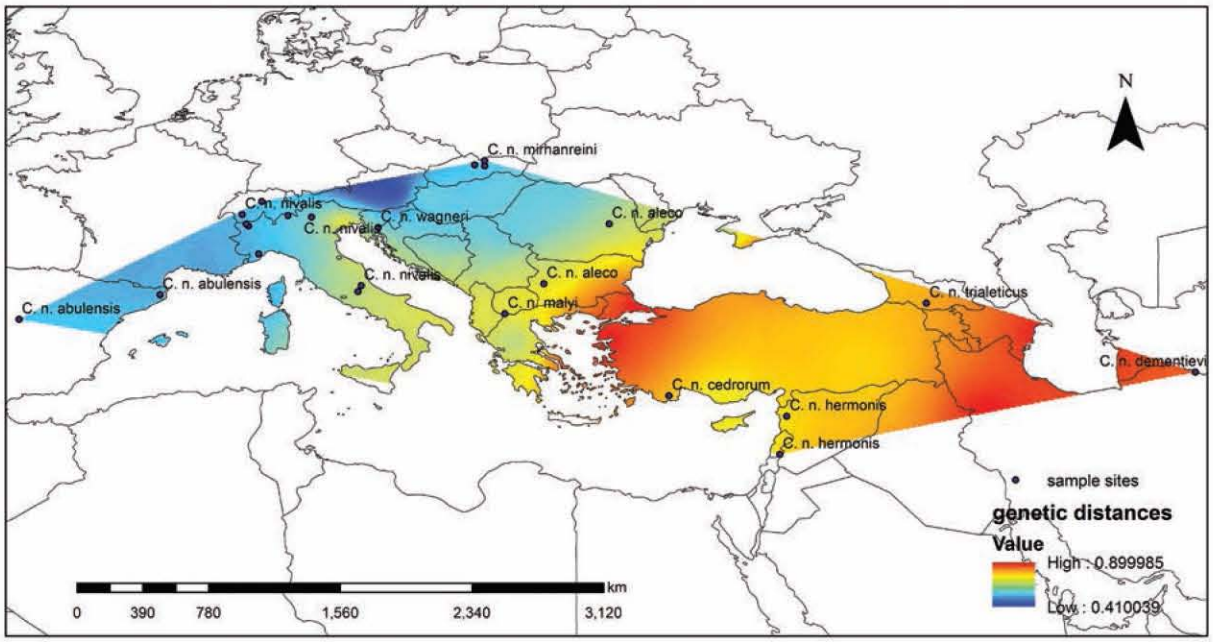

Fig. 2. Map of interpolated pairwisie genetic distances (θ_{ST}) among analysed subspecies. The red colour means high differentiation compared to low differentiation in blue.

Table 2. Pairwise genetic distances (θ_{ST}) among population groups of mtDNA haplotypes. *P*-values after 10,000 permutations are above the diagonal and θ_{ST} values are below the diagonal.

	Balkan	Alps & Apennines	Western Carpathians	Pyrenees	Anti Lebanon	Taurus	Caucasus	Khorasan
Balkan	0.000	0.001	0.029	0.029	0.029	0.063	0.029	0.027
Alps & Apennines	0.414	0.000	0.002	0.001	0.002	0.014	0.003	0.001
Western Carpathians	0.705	0.574	0.000	0.030	0.097	0.103	0.097	0.030
Pyrenees	0.520	0.495	0.730	0.000	0.029	0.070	0.031	0.026
Anti Lebanon	0.672	0.565	0.836	0.692	0.000	0.104	0.095	0.028
Taurus	0.663	0.594	0.904	0.684	0.735	0.000	0.099	0.063
Caucasus	0.680	0.621	0.878	0.713	0.773	0.780	0.000	0.027
Khorasan	0.799	0.747	0.926	0.818	0.898	0.924	0.907	0.000

many authors has accepted this view (Ellerman and Morrison-Scott, 1966; Corbet, 1978; Krapp, 1982). However, biochemical data support the hypothesis of separation of *Chionomys* from *Microtus* (Yannic et al., 2012). In addition, the genetic distance calculated between genera *Microtus* and *Chionomys* is even greater than between *Microtus* and *Arvicola* (Janeau and Aulagnier, 1997). Taxonomic studies based on morphological differences have led to the same conclusion (Gromov and Polyakov, 1992). Later, several other studies had tried using molecular markers to resolve the phylogenetic position of *Chionomys* in relation to the other species of *Microtus*. Based on cytochrome *b*, Jaarola et al. (2004) confirmed the genus *Chionomys* as distinct from *Microtus*. The mtDNA and Y-chromosomal variation suggest splitting of *Chionomys* into two monophyletic lineages: a group *nivalis* and a group *roberti/gud* (Yannic et al., 2012). Such phylogeny is also supported by data concerning dental morphology (Nadachowski, 1991) and differences in karyotypes (Zima and Král, 1984). As indicated Yannic et al. (2012), splitting occurred probably in the Early Pleistocene congruently with fossil data estimation (Nadachowski, 1991). It is generally acknowledged that *C. gud* and *C. roberti* which occur in the Middle East or the Caucasus diverged during the Middle Pleistocene (Bužan and Kryštufek, 2008). In contrast, *C. nivalis*, according to paleontological data from Holstein

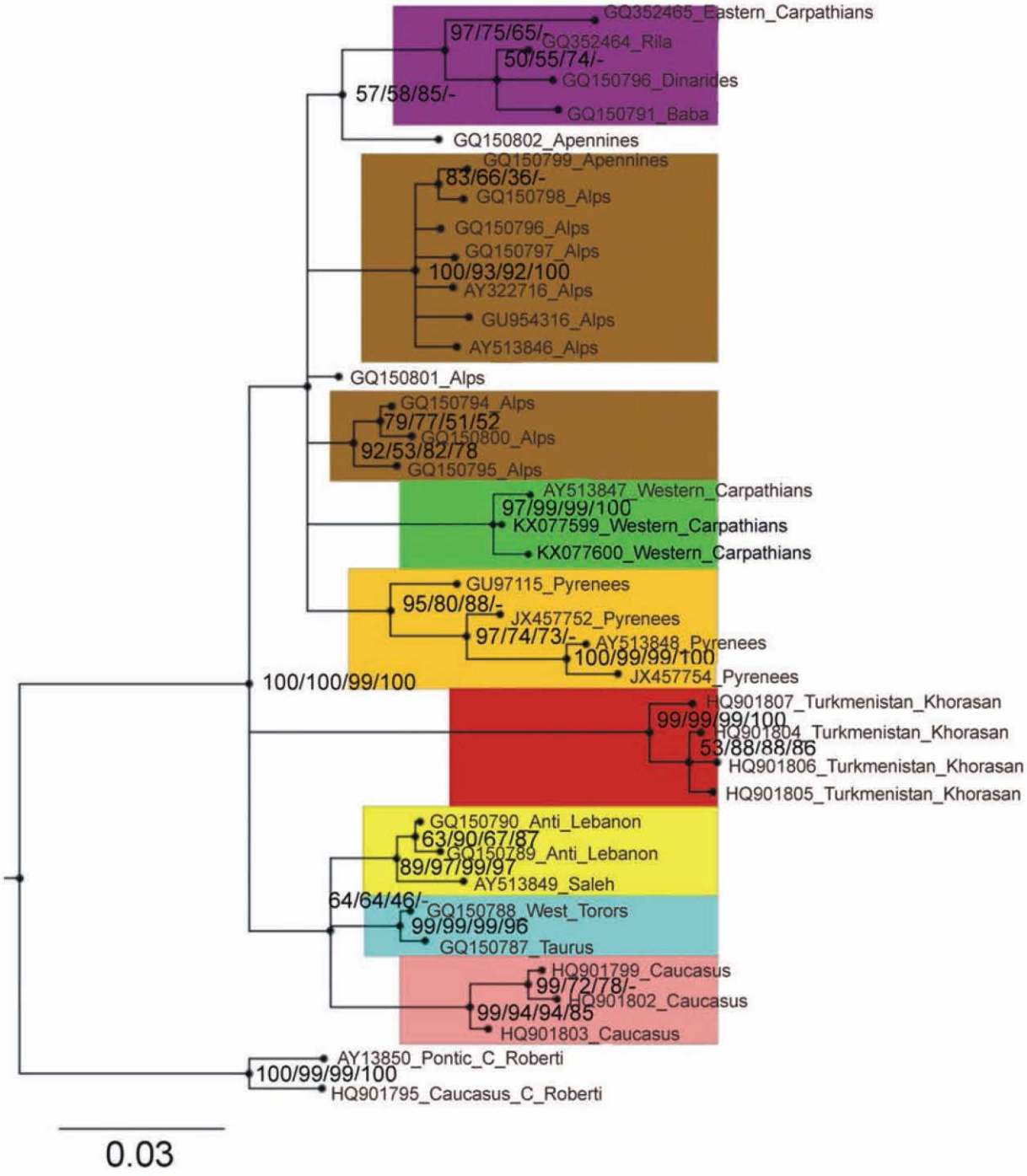

Fig. 3. Phylogenetic 50% majority rule consensus tree obtained with Bayesian inference (partitioned by haplotypes) with visualised main interior nodes of Bayesian posterior probabilities/ maximum-likelihood/ neighbour-joining/ maximum parsimony bootstrap support values. NJ, ML and MP trees reached the same topology Bayesian tree. The haplotypes are named according to their origin with GenBank Accession Numbers. Branch lengths are proportional to the number of substitutions per site (scale bar = 0.03 substitutions/ site). Subspecies information within visualised groups is presented in Table 1.

interglacial period had developed in the western mountain ranges in the Alps, the Carpathians, or the Pyrenees (Kowalski, 2001). While the eastern origin of *C. gud* and *C. roberti* was never questioned, western origin of *C. nivalis* remains still uncertain. The inclusion of the eastern subspecies *C. nivalis* was necessary to obtain a full picture of phylogeographic origin of *h. nivalis* (Yannic et al., 2012). Basic phylogenetic position of the eastern species *C. gud*, *C. roberti* and eastern subspecies of *C. nivalis* clearly establishes the Caucasus and the Middle East as the region of *Chionomys* origin (Yannic et al., 2012).

Table 3. Analysis of molecular variance of mtDNA haplotypes for the Western and the Eastern European group. The test of significance was assessed on 10,000 permutations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Statistics	*P*
Among groups	1	58.15	1.51 V_a	13.55	$F_{ct} = 0.14$	0.029
Among populations within groups	6	172.09	6.42 V_b	57.48	$F_{sc} = 0.66$	0.000
Within populations	27	87.33	3.23 V_c	28.97	$F_{st} = 0.71$	0.000
Total	34	317.57	11.17			

Based on our results, we suggest that *C. nivalis* evolved from ancestors *C. gud*, *C. n. dementievi* and *C. roberti*. Snow vole therefore would have broad and very fragmented distribution. Castiglia et al. (2009) indicate six different lineages, while four of them as allopatric, while the other two as sympatric in the Alps and the Apennines in contrast to previous scenario of two lineages based on allozymes only (Filippucci et al., 1991). Castiglia et al. (2009) also stressed the presence of one distinct haplotype in Tatra Mts which can provide proof of the existence of another glacial refugia in Central Europe, north of the main South European and the Middle East refugia. Phylogenetic reconstruction carried out by Bužan and Kryštufek (2008) suggests, that the easternmost subspecies *C. n. dementievi*, represents the oldest lineage of *C. nivalis* and this supports Eastern origin of the species. Level of nucleotide differentiation between *C. n. dementievi* and other subspecies of *C. nivalis* lies beneath the pragmatic limits of interspecies differentiation (>5%), as proposed Baker and Bradley (2006). Mitochondrial data thus provided no evidence to the recognition *C. n. dementievi* as a full species. Surprisingly low nucleotide diversity observed in *C. n. mirhareini* might indicate persistence of long term isolated population in the glacial refugia of the Western Carpathians. The Western Carpathians has been described as isolated glacial refugia for many species (Krascsenitsová et al., 2013; Zieliński et al., 2014; Klinga et al., 2015). Very low genetic differentiation between Alpine and the Western Carpathian populations indicate the origin of *C. n. mirhareini* in the Alps. Further work is needed to provide more detail information on dating of subspecies divergence.

Conclusions

Although the samples used in this study covered almost the whole species range of Snow vole in Europe, full reconstruction of its evolutionary history will need more complex species(-super) tree approach having more independent molecular markers. In this study we indicated impact of long term isolation due to climate change on genetic diversity of species.

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