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All quiet in the East: molecular analysis retrieves *Chionomys layi* as part of *C. nivalis*

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**Abstract.** Snow voles from Zard Kuh in Zagros Mts., Western Iran, were identified as distinct from the European snow vole *Chionomys nivalis* and described as a species new to science under the name *C. layi* Zykov, 2004. This description was missed in subsequent years by majority of authors dealing with the genus *Chionomys*, and taxonomic identity of the new species remains uncertain. In this study we conducted the first testing ever performed on taxonomic status of *C. layi*. We sequenced cytochrome b (1140 bp) and nuclear growth hormone receptor *ghr* (870 bp) in topotypes of *layi* and used them, together with all available haplotypes for snow voles, in phylogenetic reconstructions. Topology of phylogenetic trees accorded published results and topotypes of *layi* nested within reference samples of *C. nivalis*. They hold a supported sister position against sequences from the geographically nearby Caucasus and Alborz. Kimura-2 parameter metrics retrieved relatively low divergence (3.2 ± 0.5) between samples from the Zagros and the Alborz + Caucasus. We conclude that *layi* is synonymous with *C. nivalis*. Our results also show that the ancient refugium for the European snow vole in the Kopet Dagh was detached from the Hyrcanian refugium in the mesic mountains to the south of the Caspian Sea.

**Key words:** Hyrcanian refugium, Iran, Middle East, molecular taxonomy, phylogeography, Zagros Mts.

**Introduction**

The snow vole genus *Chionomys* consists of three species (Kryštufek 1999, Musser & Carleton 2005) and this taxonomic view remained remarkably stable ever since proposed by Ognev (1950) and Ellerman & Morrison-Scott (1951). While phylogenetic reconstructions utilizing molecular markers confirmed such a taxonomic arrangement (Yannic et al. 2012), they also challenged the taxonomic scope of the European snow vole *C. nivalis* on the eastern edge of its range. Bannikova et al. (2013) reported a highly divergent phylogenetic lineage from Aladağ in the Taurus Mts. of Turkey, and taxonomically linked it with the name *spitzenbergerae*. Arslan et al. (2016) showed however that cytochrome b (cytb) sequences of topotypes of *spitzenbergerae* (the type locality is Maden Köy, Ulukışla, Niğde, Turkey) cluster with *C. nivalis* haplotypes from the Taurus Mts. of south Turkey. Another candidate taxonomic name which could be linked with the snow voles from Aladağ is *C. layi*. The name was proposed for snow voles from Zard Kuh in Zagros Mts., Western Iran, which were originally classified as *C. nivalis* (Lay 1967) but were subsequently recognized as a species on its own right on the basis of morphological characteristics (Zykov 2004). The name *layi* however was missed in the influential compilation of the superfamily Muroidea by Musser & Carleton (2005) and therefore attracted very little attention. We provide in this study molecular evidence which removes ambiguities associated with the taxonomic status of *layi* by firmly nesting it in *C. nivalis*. Simultaneously we made available new sequences from north Iran which better define the geographic scope of phylogeographic lineages of *C. nivalis* in the Middle East.

**Material and Methods**

**Samples**

This study consists of seven snow voles collected in three regions of Iran (Table 1 and Fig. 1), two of...
which were topotypical with *C. layi*. All specimens are preserved as standard museum skins and skulls and deposited at the Museum of Vertebrate Zoology, Berkeley, U.S.A. (MVZ) and the Zoological Museum of Ferdowsi University of Mashhad, Iran (ZMFUM, Table 1). Taxonomic identification was facilitated by examination of traditional taxonomic characteristics (colour, external and cranial measurements, and enamel molar pattern) provided by Kryštufek & Vohralík (2005) and Zykov (2004). Cranial measurements were scored by Vernier calliper to the nearest 0.1 mm and the external measurements were deduced from specimen tags.

Cytb sequences of our voles were combined with 94 sequences downloaded from GenBank (supplementary information, Table S1) and belonging to all three currently recognized species of snow voles (Jaarola et al. 2004, Pfunder et al. 2004, Galewski et al. 2006, Bužan & Kryštufek 2008, Castiglia et al. 2009, Bannikova et al. 2010, 2013, Fink et al. 2010, Yannic et al. 2012, Barbosa et al. 2013, Arslan et al. 2016). Phylogenetic trees were rooted using three arvicoline species which are closely related to *Chionomys* (Zou et al. 2008, Bannikova et al. 2009): *Blanfordimys afghanus* (GenBank Accession Numbers EF599108-9), *B. juldaschi* (EF599112-3), and *Microtus fortis* (EU126809). To exclude possibility of introgression of alien mtDNA via hybridisation (Harrison & Larson 2014), we sequenced the nuclear gene for growth hormone receptor (*ghr*) in two topotypes of *C. layi* and two individuals from Binaloud Mts. All available *ghr* sequences from GenBank were used as supplementary data (*C. nivalis*: AM392378, JN244718-9, JX440339, JX440340, KP057338, *C. gud*: JN244708-14, *C. roberti*: JN244715-17; Galewski et al. 2006, Bannikova et al. 2013, Petrova et al. 2016). *Arvicola amphibius* (ZMFUM_4397), *Blanfordimys afghanus* (ZMFUM_1426), *Microtus pennsylavnicus* (AF540633, Adkins et al. 2003) and *Ellobius talpinus* (PMS_223) were used as putative close relations to rooting phylogenetic trees.

**Molecular analyses**

Nucleotide sequences were edited manually in CodonCode Aligner software (CodonCode Corp.), and aligned with the Clustal W (Thompson et al. 1997) algorithm using BioEdit 7.0.5 (Hall 1999). The best fit model of DNA substitution was chosen using jModeltest 0.1.1 (Posada 2008), based on the Akaike Information Figure 1. Records of snow voles *Chionomys* in Iran: 1 – Central Alborz Mts., 2 – Binaloud Mts., 3 – Zagros Mts. See Table 1 for further locality information. Range (shaded grey) is modified from Kryštufek & Amori (2008).

<table>
<thead>
<tr>
<th>No. on map</th>
<th>Sub species</th>
<th>Region/Locality</th>
<th>Coordinates</th>
<th>Specimen voucher</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>C. n. trialeticus</em></td>
<td>Northern Iran, Central Alborz Mts, Tar Lake</td>
<td>35°47′59.99′′ N 52°04′0.012′′ E</td>
<td>MVZ 198808*</td>
<td>KY761973</td>
</tr>
<tr>
<td>2</td>
<td><em>C. n. dementievi</em></td>
<td>North-eastern Iran, Binaloud Mts.</td>
<td>36°15′42.5′′ N 59°07′13.5′′ E</td>
<td>ZMFUM 5215</td>
<td>KY761972, KY761978</td>
</tr>
<tr>
<td>3</td>
<td><em>C. n. layi</em></td>
<td>Western Iran, Zard Kuh –Zagros Mts.</td>
<td>32°28′57.58′′ N 50°05′49.92′′ E</td>
<td>ZMFUM 5155</td>
<td>KY761974, KY761976</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ZMFUM KR02</td>
<td>KY761975, KY761977</td>
</tr>
</tbody>
</table>
Criterion (AIC). The General Time-Reversible (GTR) model with proportion of invariant sites ($I = 0.5502$) and gamma distribution of rates across sites ($\Gamma = 1.1005$) best fitted cyt$b$ dataset. The best fit model for ghr dataset was HKY + G ($G = 0.2193$). Pairwise genetic distances, both between species and within them, were estimated with Kimura-2 parameter model (K2P) using Mega v6 (Tamura et al. 2013).

Bayesian inference (BI) and Maximum Likelihood (ML) phylogenetic trees were constructed using selected substitution model. BI analysis was conducted in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003), using two simultaneous analyses with four Markov Chain Monte Carlo (MCMC) models which started from random trees and were run for 4 million iterations. The trees were sampled every 1000th generation after removing the first 10% of the trees as the burn-in stage. ML tree estimation was carried out using PAUP 4.0b10 (Swofford 2003) under 100 bootstrap pseudo-replicates with a ML heuristic tree search using 10 random additional sequence replicates. Nodal robustness for BI and ML analyses were assessed using Bayesian Posterior Probability (BPP) and Bootstrap values (BP) for BI and ML analyses, respectively. The values BPP > 0.95 and BP > 80 were assumed as good support for BI and ML analyses, respectively.

**Results**

**Taxonomic identity**

Comparison with published character states classified our specimens as snow voles (*Chionomys*). Any of them showed similarities with the Caucasian endemics *C. gud* and *C. roberti* but matched *C. nivalis-layi*. Zykov (2004) distinguished *C. layi* from *C. nivalis* by longer ears (20 mm), longer diastema (above 9.5 mm) and wider bullae (breadth over 7 mm). Morphological comparison with the data provided by Zykov (2004) showed broad similarity between the topotypes and the type series of

![Fig. 2](https://bioone.org/journals/Journal-of-Vertebrate-Biology) **Fig. 2.** (A) Bayesian inference tree reconstructed from cytochrome $b$ sequences of the genus *Chionomys* and rooted on *Microtus fortis*, *Blanfordimys afghanus* and *B. juldaschi*. (B) BI tree generated from the ghr sequences of the genus *Chionomys*. The numbers on the branches correspond to posterior probability values (BPP > 0.90) and bootstrap supports (BP > 70%). The triangles represent species and lineages of snow voles which are based entirely on published sequences. For haplotype designations see Table 1 and supplementary information (Table S1).

**Table 2.** K2P genetic distances between the geographical lineages of the European snow vole *Chionomys nivalis*. Within lineage distances are shown in diagonal.

<table>
<thead>
<tr>
<th>Lineage</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Kopet-Dagh, Binaloud</td>
<td>0.5 ± 0.1</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>2. Alborz, Caucasus</td>
<td>6.1 ± 0.9</td>
<td>1.7 ± 0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3. Zagros</td>
<td>6.0 ± 0.9</td>
<td>3.2 ± 0.5</td>
<td>0.4 ± 0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4. Turkey</td>
<td>5.7 ± 0.8</td>
<td>2.5 ± 0.4</td>
<td>3.1 ± 0.5</td>
<td>0.6 ± 0.2</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>5. Alps, Apennines, Tatra</td>
<td>5.7 ± 0.7</td>
<td>3.9 ± 0.5</td>
<td>4.0 ± 0.6</td>
<td>3.5 ± 0.5</td>
<td>1.6 ± 0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Spain</td>
<td>5.9 ± 0.8</td>
<td>4.2 ± 0.6</td>
<td>4.5 ± 0.7</td>
<td>3.6 ± 0.5</td>
<td>3.2 ± 0.5</td>
<td>1.3 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Israel, Syria</td>
<td>6.1 ± 0.9</td>
<td>2.9 ± 0.5</td>
<td>2.9 ± 0.5</td>
<td>1.8 ± 0.4</td>
<td>3.3 ± 0.6</td>
<td>3.8 ± 0.6</td>
<td>0.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>8. Balkans, Carpathians</td>
<td>5.8 ± 0.9</td>
<td>4.2 ± 0.6</td>
<td>4.2 ± 0.5</td>
<td>3.5 ± 0.5</td>
<td>2.9 ± 0.4</td>
<td>3.5 ± 0.5</td>
<td>3.8 ± 0.6</td>
<td>1.5 ± 0.2</td>
</tr>
</tbody>
</table>

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C. layi. Corresponding measurements in our topotypes of layi were (parenthesized is range for C. nivalis in Zykov 2004): length of ear 19 and 20 mm (12-18 mm), length of diastema 9.0 and 9.7 mm (7.5-9.3 mm), and breadth of bulla 7.0 and 7.4 mm (6.0-7.0 mm).

Molecular analyses
Altogether, five new cyt b haplotypes were found in our material generating 74 different snow vole cyt b haplotypes. Within the 1140 bp long sequences considered here, 193 polymorphic sites were found with a total of 267 mutations, 170 of which were parsimony informative. Our analysis of ghr retrieved three new sequences (one from Zard Kuh and two from Binaloud Mts.) generating 20 different ghr sequences. Within 870 bp long sequences, 25 and 20 sites were found to be polymorphic and parsimony informative, respectively. BI and ML trees of mitochondrial data showed similar topologies, therefore only the BI tree is shown (Fig. 2A). Branching pattern in both trees was congruent with published results (Bannikova et al. 2013, Arslan et al. 2016) therefore three species of snow voles emerged also in our reconstruction: C. nivalis, C. gud, and C. roberti. Sister position of C. nivalis against the clade of C. gud + C. roberti benefited strong BP support (100 %). Within C. nivalis the enigmatic sequence from Aladağ (JN244707, Bannikova et al. 2013) hold a supported (BP 95 %) basal position. The remaining haplotypes clustered into two strongly supported (BPP 1.00, BP 99 %) major lineages which contained also our new sequences from Iran. Four samples from Binaloud Mts. hold a supported (BPP 1.00, BP 99 %) sister position against the reference samples of C. n. dementievii from adjacent Kopet Dagh in Turkmenistan (Turkmenian lineage). The remaining haplotypes from Europe and Asia were in a single cluster (Euro-Asiatic lineage). Firstly, two strongly supported (BPP 1.00, BP 89 %) sublineages emerged in this lineage, the European and the Asiatic. Further substructuring within the latter retrieved two supported (BPP 0.98) clusters, the Caucasian and the Anatolian. The latter contained published samples from Turkey and the Levant coast in Syria and Israel. Our samples from Alborz (= Elburz) and Zagros clustered with the Caucasian haplotypes. This cluster however further substructured (BPP 0.98) and the two sequences from the Zagros were in a sister position against samples from Alborz + Caucasus. Phylogenetic analysis of ghr gene showed identical tree topology (Fig. 2B).

Putting aside the enigmatic haplotype from Aladağ, Turkey, K2P distances between European snow vole lineages varied between 1.8 ± 0.4 (Syria + Israel vs. Anatolia) and 5.7 ± 0.7-6.1 ± 0.9 (Turkmenian vs. the remaining lineages); median K2P was 3.8 %. Within-lineage distances were mainly below 1 %; higher values (1.3 ± 0.3-1.7 ± 0.3) evidently point on further substructuring, particularly so in the European lineages, and within the Caucasian lineage. Samples from Zard Kuh were separated from a cluster Alborz + Caucasus by K2P distance of 3.2 ± 0.5 (Table 2).

Discussion
In this study we conducted the first testing ever performed on taxonomic status of Chionomys layi which was described more than a decade ago (Zykov 2004) and attracted very little attention ever since. The two haplotypes obtained from the type locality in Zard Kuh (the Zagros Mts., Western Iran) nested with reference samples of C. nivalis and hold a supported sister position against sequences from the geographically nearby Caucasus and Alborz. K2P metrics in our results retrieved relatively low divergence from the remaining C. nivalis samples. We therefore synonymize C. layi Zykov, 2004, with C. nivalis (Martins, 1842). Since the divergence between layi and the sequences from the Caucasus and Alborz overlaps the distances among the major lineages occupying Europe, Levant coast, Anatolia, and Western Iran, layi may suit as a subspecific name for European snow voles occupying the Zagros Mts. Samples from Northern Iran clustered in accordance with their geographical position, either with the Caucasian lineage (Alborz) or with the Turkmenian lineage (Binaloud). Evidently, the ancient refugium for the European snow vole in the Kopet Dagh and the Binaloud Mts. was detached from the Hynarian refugium in the mesic mountains to the south of the Caspian Sea. On the other hand, snow voles from the Alborz are phyleogeographically remarkably similar to their conspecifics from the Caucasus. Therefore, although the Hynarian refugium harbours several small mammal endemics, most notably Microtus schelkovnikovi, Apodemus hyrcanicus, and Glis glis persicus (Naderi et al. 2013) it also shows historical links with the Caucasian refugium. Further sampling is unavoidable to identify the actual biodiversity pattern and retrieve the underlying process in this important refugial area.

Acknowledgements
We would like to thank Chris J. Conroy for providing one sequence (Voucher number; MVZ 198808). The first author is deeply thankful to Mojtaba Karami and Mehdi Dezhdnam who helped to collect C. n. layi at the type locality. This study was funded by the grants from Ferdowsi University of Mashhad (Project numbers: 2/37624) to A. M.
Literature


Lad D.M. 1967: A study of the mammals of Iran: resulting from the street expedition of 1962-63 (vol. 54). *Field Museum of Natural History.*


Supplementary online material

Table S1. Information on cytochrome *b* sequences of three species of snow voles (*Chionomys*) retrieved from GenBank: geographic origin of the samples and GenBank accession no. are provided. Subspecies names are reported only for *C. nivalis* (http://www.ibv.cz/folia_zoologetica/supplementarymaterials/mahomoudi_a_et_al_table_s1.docx).

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