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Taxonomic identity of *Chionomys nivalis spitzenbergerae* (Mammalia: Rodentia)

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The most genetically divergent populations of the European snow vole *Chionomys nivalis* occupy the extreme eastern range of the Black Sea and the Caspian Sea. It was recently suggested that subspecies *C. n. spitzenbergerae* from the Central Taurus Mountains (Turkey) represents a highly divergent lineage of *C. nivalis* from the Aladağ Range which induced us to address its status by examining topotype specimens. Two females karyotyped displayed 54 mitotic chromosomes of 25 acrocentric autosomal pairs. One of the smallest autosomal pairs was heteromorphic in both specimens, consisting of a submetacentric and an acrocentric chromosomes (NFa = 53). Cytochrome *b* sequences however unambiguously clustered both individuals with reference sequences from *C. nivalis* from Turkey. We conclude that a deviant haplotype from the Aladağ Range does not represent *C. n. spitzenbergerae.* Its taxonomic identity was not resolved in our study. Further attention should be devoted to snow voles from Central Anatolia and Western Iran, which are characterized by cranial peculiarities.

Keywords: Arvicolinae; cytochrome *b;* karyotype; molecular systematics; Turkey

Introduction

Of the three species of snow voles (genus *Chionomys*) currently recognised (Kryštufek & Vohralík, 2005), the European Snow Vole *Chionomys nivalis* has the most extensive range, covering the mountain regions of southern Europe and south-western Asia. Due to its narrow habitat requirements the species is restricted to fractured, rocky substrate, therefore the range is naturally fragmented into "continental archipelagos". The isolation of population fragments accelerated the selection for restricted local conditions and facilitated divergence in allopatry. As a result the interpopulation diversity in the European Snow Vole was categorized into about 20 traditional subspecies which differ in colour, size, relative length of tail, cranial proportions and enamel pattern of molars (Kryštufek, Klenovšek, Amori, & Janžekovič, 2015). On the other hand phylogenetic analyses retrieved at least eight allopatric lineages (Yannic, Burri, Malikov, & Vogel, 2012; Bannikova, Sighazeva, Malikov, Golenischev, & Dzuev, 2013). The most divergent populations occupy the extreme eastern range of the Black Sea and the Caspian Sea in Turkmenistan (Yannic et al., 2012) and in Iran (Zykov, 2004). From the Aladağ Range in the Central Taurus (Toros) Mountains of Turkey, Bannikova et al. (2013) reported a highly deviant snow vole haplotype which was tentatively classified as a

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subspecies *spitzenbergerae*. The K2P genetic distance between this individual and all the remaining *C. nivalis* haplotypes is indicative of cryptic species diversity. When discovered, *C. n. spitzenbergerae* was first classified as *C. gud* (Spitzenberger, 1971; Storch, 1988) and only subsequently recognised as a highly morphologically distinctive subspecies of the European Snow Vole (Nadachowski, 1990). Disentangling the identity of *spitzenbergerae* may therefore be relevant for an accurate taxonomic setting of the genus *Chionomys*. Our aim in this contribution was twofold: (i) to provide chromosomal and molecular evidence which will unambiguously define *spitzenbergerae*, a step not undertaken so far, and (ii) to compare the morphology of topotypical material of *spitzenbergerae* with the specimen reported by Bannikova et al. (2013).

Material

Our study is based on the examination of the following material of *C. nivalis*: **(1)** Two individuals collected in the summer of 2015 at Maden Köy, Ulukışla, Niğde, Turkey (37°27'N, 34°37'E), i.e. at the type locality for *C. n. spitzenbergerae*; museum vouchers (skins and skulls) are deposited in the Department of Biology, Faculty of Science, Selçuk University, Konya, Turkey (ZMSU404, ZMSU405). These specimens were also karyotyped and used in phylogenetic analysis. – **(2)** A museum voucher (an adult female) in the Zoological Institute and Zoological Museum, Russian Academy of Sciences, St. Petersburg, Russia (ZIN 98639), collected by A. A. Stekolnikov on 1 May 2009 at Aladağ Range, 3.5 km N from Mt. Karanfil, Turkey (37°36.504'N, 35°00.274'E, altitude of 1709 m); this individual yielded a highly divergent sequence reported by Bannikova et al. (2013) and was tentatively classified as *spitzenbergerae*.

We compared the above vouchers with extensive museum material of snow voles from Turkey and adjacent parts of Georgia, Lebanon, Israel and Iran. Vouchers are deposited in the following collections: Department of Zoology, Charles University, Prague, Czech Republic; Field Museum of Natural History, Chicago, USA; Collection of Prof. Dr. Hans M. Steiner, Vienna, Austria; Forschungsinstitut und Natur-Museum Senckenberg, Frankfurt a. M., Germany; Natural History Museum London, London, UK (BMNH); Natural History Museum of Slovenia, Ljubljana, Slovenia; Naturhistorisches Museum Wien, Vienna, Austria (NMW); United States National Museum of Natural, History, Washington, D.C., USA; Zoological Museum, Russian Academy of Sciences, St. Petersburg, Russia (ZIN).

Among museum vouchers, the type series of *C. n. spitzenbergerae* was of particular relevance to our study. This sample consists of the type (NMW 13271) and three paratypes (NMW 13290, 13291, 13292). We examined another topotypical specimen (adult female, NMW 13289) which was not reported in Nadachowski (1990). In addition we examined a further two types based on Turkish snow voles, *C. n. pontius* (Miller, 1908) (BMNH 5.10.4.53) and *C. n. cedrorum* (Spitzenberger, 1973) (Felten, Spitzenberger, & Storch, 1973) (NMW 20478).

Methods

Morphology. We focused on the enamel tooth pattern which Nadachowski (1990) reported to be diagnostic for *spitzenbergerae*. Observations were made under a dissecting microscope. Abbreviations used for 1st lower and 3rd upper molar are M_1 and M^3, respectively. Terminology of the molar elements follows Kryštufek & Vohralík (2005). Cranial measurements were made using a vernier caliper adjusted to 0.1 mm.

Karyotypes. Chromosome preparations were obtained from the two topotypes following a slightly modified standard technique of direct colchicine/hypotonic treatment of bone marrow (Ford & Hamerton, 1956). At least 20 well-spread metaphase plates were analysed per individual animal. In addition to a diploid number (2n), we also estimated the fundamental number of chromosomal arms for the entirety of mitotic chromosomes (NF), and separately for the autosomal pairs (NFa).

Cytochrome-*b* sequences. We analysed cytochrome *b* (cyt *b*) sequences in two topotypes of *C. n. spitzenbergerae*. Total genomic DNA was obtained from muscle preserved in 80% ethanol using the Qiagen method (DNeasy tissue kit, Qiagen, Hilden, Germany). Double stranded DNA ampli-

fications of mitochondrial cyt *b* were performed with primers L14727-SP and H-15915-SP (Jaarola & Searle, 2002). PCR products were checked on a 1% agarose electrophoresis gel and visualized with ethidium bromide staining to verify PCR quality. Amplified products were purified using QIA quick PCR purification Kit (Qiagen) following the manufacturer's instructions and commercially sequenced with same primers used for amplification using dye-labelled dideoxy terminator cycle sequencing with Big Dye V.3.1 (Applied Biosystems, Inc.).

For the phylogenetic analysis, a further 92 sequences belonging to all three species of *Chionomys* (*C. gud* and *C. roberti* in addition to *C. nivalis*) were downloaded from GenBank (Pfunder, Holzgang, & Frey, 2004; Galewski et al., 2006; Castiglia, Annesi, Kryštufek, Filippucci, & Amori, 2009; Bannikova et al., 2010, 2013; Fink, Fischer, Excoffier, & Heckel, 2010; Yannic et al., 2012). The sequences were checked for the absence of stop-codons and chimeric sequences.

Nucleotide, amino acid composition and genetic distances were analysed assuming a Kimura-2 parameter model (K2P) with 10^4 bootstraps in the MEGA v6 program (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The most appropriate models of DNA substitution for the data were identified using jModeltest 0.1.1 (Posada, 2008), based on the Akaike Information Criterion (AIC). The General Time-Reversible (GTR) model with proportion of invariant sites (I= 0.5619) and gamma distribution of rates across sites (G = 1.1229) best fit our dataset.

Bayesian inference (BI) and Maximum Likelihood (ML) phylogenetic trees were constructed using a selected substitution model. BI analysis was conducted using 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 1000^{th} generation after removing the first 10% of the trees as the burn-in stage. ML tree estimations were 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. We considered BPP >0.95 as "good" and BPP = 0.90–0.95 as "moderate" support in line with other authors. For branch support in the ML tree we accepted BP > 90% as "good" support, and BP = 80–90% as "moderate" support.

Trees were rooted with *Microtus fortis* (KJ081954; Gao et al., unpublished), *M. pennsylvanicus* (KC473494; Hope, Waltari, Payer, Cook, & Talbot, 2013), and two sequences each of *Blanfordimys afghanus* (EF599108, EF599109) and *B. juldaschi* (EF599112, EF599113; Bannikova et al., 2009).

Results and Discussion

Cytochrome-b sequence. – Altogether, one new haplotype was found in our material generating 65 different snow vole cyt *b* haplotypes. Within the 1140-bp long sequences considered here, 167 polymorphic sites were found with a total of 206 mutations, 142 of which were parsimony informative.

Both phylogenetic trees (BI and ML) yielded congruent topologies, hence only the BI tree is shown in Figure 1. Tree topology and branching pattern were concordant with previously published results (Bannikova et al., 2013). Therefore, all *C. nivalis* haplotypes clustered into a monophyletic lineage which holds a highly supported sister position against *C. gud* and *C. roberti*. Within *C. nivalis* the haplotype ZIN 98639 from the Aladağ Range was the most divergent, followed by the lineage from Turkmenistan. The majority of snow vole haplotypes were in two moderately supported clusters which showed strong geographic associations. Our new haplotypes of *spitzenbergerae* (ZMSU 404 and 405 in Figure 1) were in the Asiatic cluster. Although the branching of the Asiatic lineage benefited poor support, the taxonomic identity of *spitzenbergerae* implies no doubt. Therefore, *spitzenbergerae* was part of *C. nivalis* and showed no associations with the haplotype from the Aladağ Range, although the localities are only about 35 km apart.

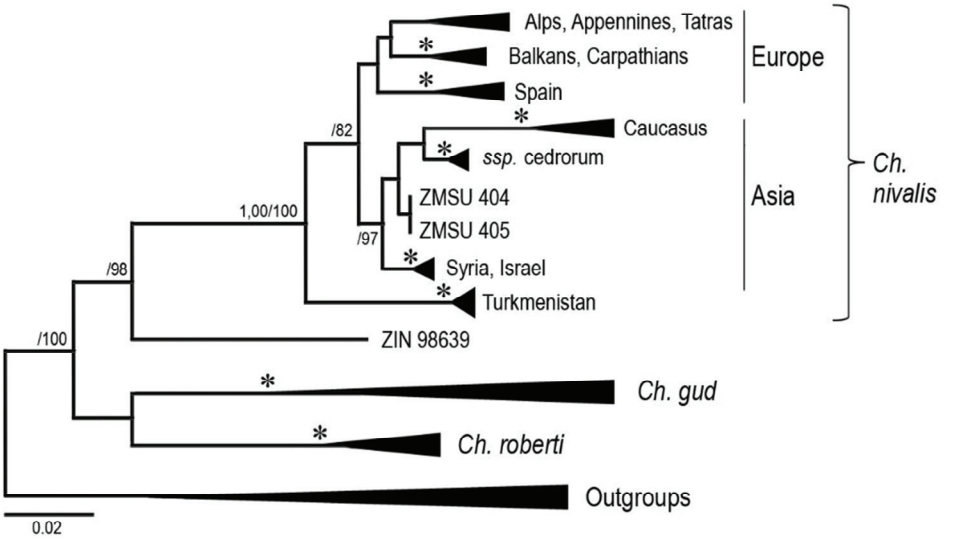

Figure 1. Bayesian inference tree reconstructed from cyt *b* sequences of snow voles and rooted with *Microtus fortis*, *M. pennsylvanicus*, *Blanfordimys afghanus*, and *B. juldaschi.* The numbers on the branches correspond to posterior probability values (BPP > 0.90) and bootstrap supports (BP > 80%). Asterisks indicate significant supports for branches which are not further discussed in our study. The triangles represent species and lineages of snow voles which are based entirely on published haplotypes.

Karyotypes. – Both ZMSU individuals were females displaying 54 mitotic chromosomes. The karyotype included 25 acrocentric autosomal pairs of diminishing size. One of the smallest autosomes (tentatively indicated as pair 25 in Figure 2) was heteromorphic in both studied specimens, i.e. it consisted of a submetacentric and an acrocentric chromosomes (NFa = 53). The X chromosome was a large submetacentric (NF = 57) (Figure 2). The heteromorphic pair is of interest because in *Chionomys* the smallest autosomal pair is acrocentric in *C. nivalis* and submetacentric in *C. gud* (Arslan & Zima, 2014). The heteromorphy observed therefore bears a superficial relation to the earlier classification of *spitzenbergerae* as *C. gud* (Spitzenberger, 1971; Storch, 1988) and also blurs the taxonomic relevancy of chromosomal evidence. However, as shown in the above phylogenetic analysis, *spitzenbergerae* is firmly nested within *C. nivalis*, showing no proximity with *C. gud*.

The conventional karyotype of *C. nivalis* is stable among the subspecies described from Turkey (i.e. *olympius, pontius*, and *cedrorum*; Arslan & Zima, 2014) and across the entire species' range (Zima & Král, 1984; Sablina, Radzhabli, Malikov, Meyer, & Kuliev, 1988). Heteromorphic chromosomes have thus far not been reported in *C. nivalis* or in the genus *Chionomys*. The phenomenon is also rare in other arvicolines. Among the voles occupying Turkey, heteromorphy was reported only in *Microtus obscurus* (Yorulmaz Zima, Arslan, & Kankiliç, 2013; Arslan & Zima, 2014).

Morphology. – Both topotypes of *spitzenbergerae* displayed light straw grey (drab) dorsal pelage, a common colouration of the European snow voles occupying the Taurus

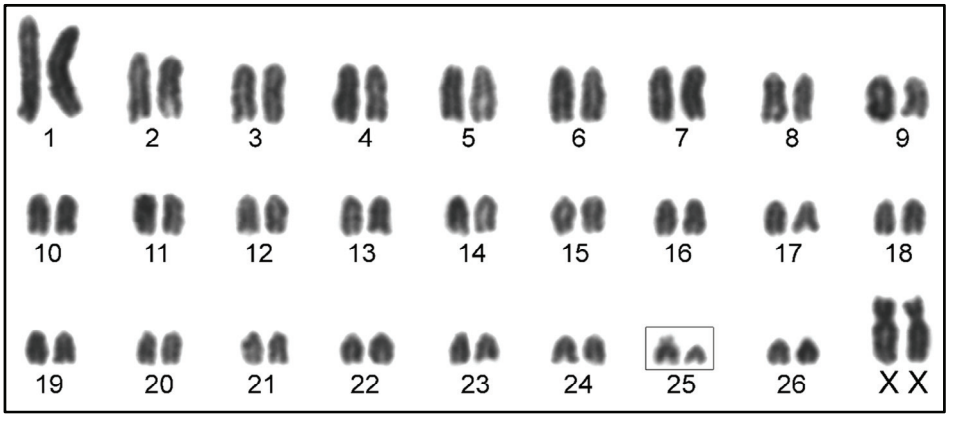

Figure 2. Standard karyotype of *C. n. spitzenbergerae* from Maden Köyü, Ulukışla. The heteromorphic pair is framed.

Mountains (Kryštufek & Vohralík, 2005). The anterior cap (AC) of M_1 was arrow-shaped and not divided by the antero-lingual re-entrant angle (LRA4); dental field of AC was also isolated from the triangle T5 (i.e. the nivalid morphotype). The M^3 showed four salient angles on the lingual side however, the posterior angle LSA5 was ill defined. The ZIN 98639 voucher showed M_1 with a deep LRA4 and confluent dental fields of the AC and T5 (the nivalid-ratticeps morphotype). The M^3 was of simple structure with three salient angles lingually. Although the nivalid-ratticeps morphotype was reported as characteristic for *spitzenbergerae* (Nadachowski, 1990) it is widespread in the Taurus Mountains. Regional differentiation within this mountain range is evident from a higher frequency of complex M^3 in *spitzenbergerae* as compared to a lower frequency in *C. n. cedrorum* occupying the Taurus Mts. further west.

The ZIN 98639 voucher shows similarities with the Central Anatolian populations of snow voles which are most evident in the wider braincase (14.8 mm in ZIN 98639 as compared to 14.5–15.2 mm in voles from Central Anatolia); the breadth of the braincase in Anatolian snow voles occupying the Black Sea Mountains and the Taurus Mountains is at most 14.5 mm (Kryštufek, 1999; Kryštufek & Vohralík, 2005). A broad braincase is also typical for *C. layi* (14.3 and 15.6 mm in two specimens from the type series; Zykov, 2004), which is a little known taxon of snow voles from Kuh Range, the Zagros Mountains of western Iran.

Molecular markers have thus far not been studied in snow voles from Central Anatolian and the Zagros Mountains. The karyotype of Elazığ snow voles, which are characterised by a wide skull (Kryštufek, 1999) does not deviate from the characters reported for other *C. nivalis* samples (Arslan & Zima, 2014). An imminent task for further studies would therefore be screening nucleotide sequences in snow voles from Central Anatolia and western Iran. Until this is done, the taxonomic identity of the lineage from the Aladağ Range remains unresolved.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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