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Phylogenetic position of *Chionomys gud* assessed from a complete cytochrome *b* gene

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A b s t r a c t . We present a phylogenetic analysis of snow voles by combining all published cytochrome *b* sequences of 47 species of *Microtus, Blanfordimys, Neodon* and *Chionomys* and a new sequence from *Chionomys gud*. By applying powerful, modern approaches to phylogenetic reconstruction such as maximum likelihood (ML) and Bayesian inference methods (BI), we provide new information on the relationships between *Chionomys* and *Microtus*. Both phylogenetic analysis methods showed that the genus *Microtus* is paraphyletic with respect to *Blanfordimys, Neodon* and *Microtus gregalis*. The BI topology recovered strong support for the monophyly of *Chionomys* + *Microtus gregalis*, while the monophyly of *Chionomys* was supported only by the ML analysis. The two *Chionomys* lineages (defined by molar morphology and karyological features), "nivalis" (*C. nivalis*) and "roberti" (*C. gud* and *C. roberti*), were strongly supported by cytochrome *b* analysis.

Key words: Arvicolidae, Bayesian method, *Microtus*, molecular phylogeny

Introduction

Three currently recognised species of snow voles inhabit the principal mountain ranges of Central and Southern Europe, the Caucasus, and the Near and Middle East (M u s s e r & C a r l e t o n 2005). Only the European snow vole *Chionomys nivalis* (Martins, 1842) is widespread; *Chionomys gud*, the Gudaur vole (Satunin, 1909), and *C. roberti*, Robert's vole, (Thomas, 1906) are endemic to the Caucasus and the adjacent Black Sea mountains of Turkey (S h e n b r o t & K r a s n o v 2005).

Although *Chionomys* was originally created as a subgenus of *Microtus* (M i l l e r 1908), cranial (P i e t s c h 1980), dental (N a d a c h o w s k i 1991), chromosomal (A g a d z h a n j a n & J a t s e n k o 1984, Z i m a & K r á l 1984), and genetic evidence (G r a f 1982, J a a r o l a et al. 2004) justify ranking it as a genus instead (M u s s e r & C a r l e t o n 2005). P a v l i n o v & R o s s o l i m o (1987, 1998) recognise two groups within *Chionomys*, the "*nivalis*" group (with *C. nivalis*) and the "*roberti*" group, with the remaining two species. This division is supported by dental morphology (N a d a c h o w s k i 1990, 1991) and differences in the fundamental number of chromosomal arms (Z i m a & K r á l 1984). Previous phylogenetic reconstructions of *Chionomys* have been biased by a lack of genetic data for *C. gud* (J a a r o l a et al. 2004). Given that comprehensive taxonomic sampling can vastly improve the accuracy of phylogenetic reconstruction (B u z a n et al. 2008), we provide the first cytochrome *b* (cyt *b*) sequence for *C. gud* in this paper. We based our study on the sequence analysis of the entire cyt *b* gene (1143 bp) because this gene evolves rapidly over the expected divergence times (J a a r o l a et al. 2004). Additionally, there are more sequences of cyt *b* in GenBank than there are of any

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other region of the arvicoline genome. By including all the available cyt *b* sequences for the arvicoline genera that are believed to be the closest relatives of *Chionomys* (Jaarola et al. 2004, Galewski et al. 2006), we minimize taxonomic sampling errors that might prevent a monophyletic assessment of certain lineages (Buzan et al. 2008) but also create problems for phylogenetic reconstruction through what is known as "long branch attraction" (Baldauf 2003).

The aim of this paper was to reconstruct the phylogeny of *Chionomys* by comparing cyt *b* sequences for all three species in the genus and by applying a powerful Bayesian approach to phylogenetic analysis. We tested two null hypotheses: (i) *Chionomys* is a monophyletic group and (ii) *C. nivalis* is sister to the *roberti* group. A broad taxonomic sampling allowed us to generalize phylogenetic relationships within *Microtus* in the wide sense. Thus, we also addressed the question of what the closest living relative of *Chionomys* is.

Material and Methods

Samples

The phylogenetic position of *Chionomys* was assessed using sequences from 49 species of *Microtus*, *Blanfordimys* and *Neodon* and two species of *Chionomys* (*C. nivalis* and *C. roberti*) downloaded from GenBank (Table 1). Additionally, we sequenced the cyt *b* gene for *Chionomys gud* from Çam Geçidi (Ardahan, Turkey). Given unresolved tribal affinities of *Chionomys* (Musser & Carleton 2005), we used the two arvicoline taxa that were proposed to be the closest possible relatives of the *Microtus* + *Chionomys* lineage as outgroups: *Lagurus lagurus* from the Lagurini tribe (Buzan et al. 2008) and *Myodes rutilus* from the Myoidini tribe (Mezhzherin et al. 1995, Conroy & Cook 2000).

DNA extraction, PCR amplification and sequencing

A 2 x 2 mm fragment of ethanol-preserved tissue was air-dried in sterile conditions to remove the ethanol. DNA was then extracted using a QIAamp® DNA Mini Kit (Qiagen).

Three overlapping cyt *b* fragments of 613bp, 320bp and 470bp were amplified from the *Chionomys gud* sample using the mammalian primers L14727-SP, H15348A-SP, H15915-SP (Jaarola & Searle 2002), L15162Marv, L15408Marv (Haynes et al. 2003) and H15497-SP (Jaarola et al. 2004). The alignment of these fragments yielded high quality sequence data for the entire cyt *b* gene (1143 bp).

DNA fragment amplification was performed using a 20 µL reaction containing 2.5 mM $MgCl_2$, 0.5 μM of forward and reverse primer, 0.2 mM of dNTPs and 1 unit of Fermentas *Taq* in the supplied ammonium buffer. Cycling conditions consisted of an initial stage of 95°C for 5 min followed by 40 cycles of denaturation (40 sec at 94°C), primer annealing (40 sec at 48°C) and extension (1 min at 72°C). Sequencing was performed on an ABI PRISM 3700 Genetic Analyzer using BigDye Terminators chemistry (Applied Biosystems).

Sequence analyses

The CodonCode Aligner program (version 1.63; CodonCodes Inc., Ewing et al. 1998) was used to align forward and reverse sequences. The resulting consensus sequences for each individual were aligned using ClustalW (version 4.0, Thompson et al. 1997)

Table 1. Species included in the phylogenetic analysis. Geographic origin of *Chionomys nivalis* samples: 1 – Italy, Trento, 2 – Slovakia, Prvé Roháčske pleso Lake, 3 – Spain, Queralbs, Girona, 4 – Syria, Saleh, As Suwayda, 5 – Spain, Sierra de Gredos, Hoyos del Espino. Palaearctic *Microtus* species are ascribed to a subgenus, based on Musser & Carleton (2005).

Species	Geographic range	Genbank reference number	Reference
Chionomys gud	Palaearctic	EU700087	This study
Chionomys nivalis 1	Palaearctic	AY513846	Jaarola et al. (2004)
Chionomys nivalis 2		AY513847	Jaarola et al. (2004)
Chionomys nivalis 3		AY513848	Jaarola et al. (2004)
Chionomys nivalis 4		AY513849	Jaarola et al. (2004)
Chionomys nivalis 5		AM392367	Galewski et al. (2006)
Chionomys roberti 1	Palaearctic	AY513850	Jaarola et al. (2004)
Chionomys roberti 2		AY513851	Jaarola et al. (2004)
Blanfordimys bucharensis	Palaearctic	AM392369	Galewski et al. (2006)
Neodon irene	Palaearctic	AM392370	Galewski et al. (2006)
Neodon juldaschi	Palaearctic	AY513808	Jaarola et al. (2004)
Microtus (Alexandromys) kikuchii	Palaearctic	AF163896	Conroy & Cook (2000)
Microtus (Alexandromys) middendorffii	Palaearctic	AF163898	Conroy & Cook (2000)
Microtus (Alexandromys) montebelli	Palaearctic	AF163900	Conroy & Cook (2000)
Microtus (Alexandromys) oeconomus	Holarctic	DQ452142	Brunhoff et al. (2006)
Microtus (Microtus) arvalis	Palaearctic	AY220766	Haynes et al. (2003)
Microtus (Microtus) agrestis	Palaearctic	AY167187	Jaarola & Searle (2002)
Microtus (Microtus) dogramacii	Palaearctic	AY513795	Jaarola et al. (2004)
Microtus(Microtus) guentheri	Palaearctic	AY513807	Jaarola et al. (2004)
Microtus (Microtus) kirgisorum	Palaearctic	AY513810	Jaarola et al. (2004)
Microtus (Microtus) levis	Palaearctic	AY513821	Jaarola et al. (2004)
Microtus (Microtus) socialis	Palaearctic	AY513831	Jaarola et al. (2004)
Microtus(Stenocranius) gregalis	Palaearctic	AY513803	Jaarola et al. (2004)
Microtus(Terricola) daghestanicus	Palaearctic	AY513792	Jaarola et al. (2004)
Microtus(Terricola) duodecimcostatus	Palaearctic	AY513797	Jaarola et al. (2004)
Microtus (Terricola) felteni	Palaearctic	AY513798	Jaarola et al. (2004)
Microtus (Terricola) gerbei	Palaearctic	AY513802	Jaarola et al. (2004)
Microtus (Terricola) liechtensteini	Palaearctic	AY513811	Jaarola et al. (2004)
Microtus (Terricola) lusitanicus	Palaearctic	AY513813	Jaarola et al. (2004)
Microtus (Terricola) majori	Palaearctic	DQ841704	Martínková et al. (2007)
Microtus (Terricola) multiplex	Palaearctic	AY513818	Jaarola et al. (2004)
Microtus (Terricola) pyrenaicus	Palaearctic	AJ717748	Tougard et al. (2008)
Microtus (Terricola) savii	Palaearctic	AY513828	Jaarola et al. (2004)
Microtus (Terricola) subterraneus	Palaearctic	AY513832	Jaarola et al. (2004)
Microtus (Terricola) tatricus	Palaearctic	DQ841702	Martínková et al. (2007)
Microtus (Terricola) thomasi	Palaearctic	AY513844	Jaarola et al. (2004)
Microtus abbreviatus	Nearctic	AF163890	Conroy & Cook (2000)
Microtus californicus	Nearctic	AF163891	Conroy & Cook (2000)
Microtus chrotorrhinus	Nearctic	AF163893	Conroy & Cook (2000)
Microtus guatemalensis	Nearctic	AF410262	Conroy et al. (2001)
Microtus longicaudus	Nearctic	AF187230	Conroy & Cook (2000)
Microtus mexicanus	Nearctic	AF163897	Conroy & Cook (2000)
Microtus miurus	Nearctic	AF163899	Conroy & Cook (2000)
Microtus oaxacensis	Nearctic	AF410260	Conroy et al. (2001)
Microtus ochrogaster	Nearctic	AF163901	Conroy & Cook (2000)
Microtus oregoni	Nearctic	AF163903	Conroy & Cook (2000)
Microtus pennsylvanicus	Nearctic	AF119279	Conroy & Cook (1999)
Microtus pinetorum	Nearctic	AF163904	Conroy & Cook (2000)
Microtus quasiater	Nearctic	AF410259	Conroy et al. (2001)
Microtus richardsoni	Nearctic	AF163905	Conroy & Cook (2000)
Microtus townsendii	Nearctic	AF163906	Conroy & Cook (2000)
Microtus umbrosus	Nearctic	AF410261	Conroy et al. (2001)
Microtus xanthognathus	Nearctic	AF163907	Conroy & Cook (2000)
Myodes rutilus	Holarctic	AF119274	Conroy & Cook (1999)
Lagurus lagurus	Palaearctic	AF429818	Dekonenko et al. (2003)

implemented in the MEGA package (version 4.0, Tamura et al. 2007) in combination with Bioedit (version 7.09, Hall 2004). Nucleotide and amino acid composition were analysed using MEGA. The total number of polymorphic site in each position was estimated with the DAMBE program (version 4.2.13, Xia 2000, Xia & Xie 2001).

Phylogenetic analysis

Phylogentic relationships were reconstructed using maximum likelihood (ML) methods and Bayesian inference (BI). The Akaike information criterion (AIC), hierarchical likelihood ratio test (HLRT) and Bayesian information criterion (BIC) implemented in the Modeltest 3.7 program (Posada & Crandall 1998) identified the most appropriate model of DNA substitution for the data. All three approaches selected the general time reversible model with a gamma-distributed shape parameter and the proportion of invariable sites (GTR+G+I).

The ML tree was constructed using the GTR+G+I substitution model, in which the parameters were estimated using PhyML software (version 2.4.5; Guindon & Gascuel 2003). Branch support in the ML tree was inferred using the non-parametric Shimodaira-Hasegawa-like procedure (SH-aLRT) provided by PhyML (Anisimova & Gascuel 2006). The SH-aLRT procedure is conservative; thus, we considered values of >90% as a cut-off for "good" support, and 80-90% was considered "moderate" support.

The MrBayes program (version 3.1, Huelsenbeck & Ronquist 2001) was used to apply a Bayesian approach to phylogenetic reconstruction (Mau et al. 1999, Rannala & Yang 1996, Yang & Rannala 1997). The GTR+G+I distribution model of DNA substitution was used with a Markov chain that started from a random tree with random branch lengths. Four Markov Chain Monte Carlo (MCMC) chains were run simultaneously for 2 million generations, with the resulting trees sampled every hundred generations (saving 200 000 trees). The first 20,000 trees were discarded as a conservative measure to avoid the possibility of including pre-burn-in, sub-optional trees. The remaining results were used to compute a 50% majority rule consensus tree. Bayesian posterior probabilities (BPP) assessed branch support of the BI tree. We considered BPP >0.95 as "good" and 0.90 - 0.95 as "moderate" support, in line with other authors.

Results

Sequence data

A total of 484 (42.3%) variable sites were observed across 49 species of *Chionomys*, *Microtus, Blanfordimys and Neodon*; of these, 407 were parsimony informative. The majority of polymorphic sites were at third positions (352, 72.7%), followed by first positions (109, 22.5%) and finally second positions (23, 4.8%). The average ratio of transitions/transversions was 2.5. Nucleotide composition was characterised by a deficit of guanines (12.9%), similar to that previously described in arvicolines (Conroy & Cook 2000, Jaarola et al. 2004) as well as other mammals (Irwin et al. 1991). A total of 383 variable sites were parsimony informative in *Microtus, Blanfordimys* and *Neodon*, while the number of parsimony informative sites in *Chionomys* was 152. The majority of polymorphic sites in *Chionomys* were at third positions (180, 84.1%), followed by first positions (31, 14.5%) and finally second positions (3, 1.4%).

Phylogenetic analyses

Phylogenetic relationships among 49 arvicoline species evaluated by the two different methods (ML and BI) gave very similar results. The gamma-distributed shape parameter (α) was 0.70 or 0.74 (with or without outgroup), and the proportion of invariable sites (I) was 0.54 in both analyses (i.e. with or without the outgroup). For the BI method, the topology of consensus trees, values of posterior probabilities and parameter estimates were highly similar in all four analyses.

Both algorithms resulted in two main clusters, one containing *Chionomys* + *Microtus gregalis,* and the other containing the remaining species of *Microtus* + *Blanfordimys* + *Neodon* (henceforth referred to as the main *Microtus* cluster). The monophyly of *Chionomys*, also evident from both trees, received good support (BPP=1.00, 96%) in both algorithms. In the genus *Chionomys*, both tree topologies confirmed the monophyly of *C. nivalis* (BPP = 1.00, 100%) vs. the *roberti* group. The monophyly of the *roberti* group and the sister position of *C. gud* and *C. roberti* were strongly supported (BPP = 1.00) only in the BI topology. The five *C. nivalis* haplotypes grouped into three geographic clusters. Two European snow voles from the Italian Alps and the Slovakian Carpathians formed one supported cluster. Another cluster contained the two haplotypes from Spain. The only animal available from Syria hold sister position to the European samples in ML topology (100%) (Fig. 1).

The total mean genetic divergence between *Chionomys* and *Microtus* (excluding *M. gregalis*) is 14.4% ±0.8. Between the two main lineages of *Chionomys,* the *nivalis* and the *roberti* groups, the mean divergence is 12.1% ±0.9.

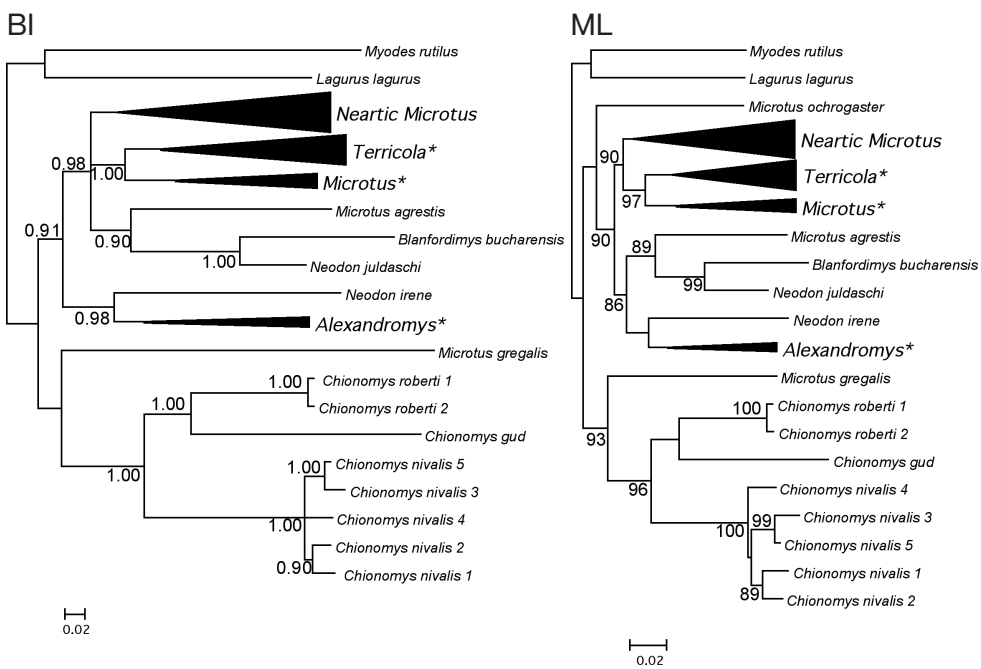

Fig. 1. Fifty percent majority rule consensus tree of 180,000 trees from a Bayesian analysis (BI) constructed using a GTR+G+I model of sequence evolution of cyt *b* and the maximum likelihood tree (ML). Included are the three species of *Chionomys* and 49 species of *Microtus, Blanfordimys* and *Neodon*. Trees are rooted with *Myodes rutilus* and *Lagurus lagurus* as outgroups. Numbers below branches represent posterior probability values >0.90 (BI) and ML support >80%. For species groups, see Table 1; note that Palaearctic subgenera *sensu* Musser & Carleton (2005) are indicated by an asterisk.

Topology of branches within the main *Microtus* cluster is largely in agreement with earlier studies (Jaarola et al. 2004, Galewski et al. 2006). However, we also noticed several discordances. Contrary to Conroy & Cook (2000) and Jaarola et al. (2004), we found no evidence for an early divergence of *Microtus* into the Palaearctic and Nearctic lineages. Additionally, the BI tree provides moderate support (BPP = 0.91) for a main cladogenetic event that separates *Alexandromys* + *Neodon irene* from the remaining species. In our analysis, the topology of the major branches was very similar in both trees; the only difference was an unresolved basal position of the Nearctic *Microtus ochrogaster* in the ML algorithm. Subgenera *Microtus* and *Terricola* were well supported as sisters within a monophyletic clade. Both algorithms placed *M. agrestis* as a sister species to *Blanfordimys* + *Neodon juldaschi*. However, this cluster, which received moderate support in the ML algorithm (89%) and BI (0.90), did not occupy a basal position within the main *Microtus* clade. Similar to Galewski et al. (2006), no support was recovered for the generic recognition of *Blanfordimys*, and *Neodon* was clearly polyphyletic. *Microtus gregalis*, the sole representative of the subgenus *Stenocranius* (Musser & Carleton 2005), emerged in our BI analysis as the closest relative to *Chionomys*.

Discussion

Our results suggest that *M. gregalis* and *Chionomys* may be sister taxa, a scenario that has not yet been proposed. Cranial and dental morphology (Gromov & Polyakov 1992) and karyology (Zima & Král 1984) of *M. gregalis* do not support a close relationship between it and *Chionomys*. Jaarola et al. (2004) stressed that *M. gregalis* is by far the most divergent species of *Microtus* in the cyt *b* data set and that its position relative to *Chionomys* is also unclear. The *Microtus* polytomy, which is probably due to the instability of *M. gregalis*, should be resolved by additional information from nuclear markers (cf. Jaarola et al. 2004). Similar to Conroy & Cook (2000) and Jaarola et al. (2004), we found no support for a close relationship of *M. gregalis* with *M. miurus*, and *M. abbreviatus*, the two Nearctic voles that were ascribed to *Stenocranius* by some authors (cf. Musser & Carleton 2005). Our results contradict those of Mezhzherin et al. (1993), who placed *M. gregalis* near the subgenus *Alexandromys*. Also note that the monophyly of *Alexandromys* benefited strong support (BPP = 0.98) only in the BI topology in our analysis. Consequently, the evidence available thus far supports defining *M. gregalis* as the sole member of *Stenocranius*. Whether *Stenocranius* is a subgenus of *Microtus*, a taxonomic solution not supported by our results, or an independent genus needs further testing using independent gene markers.

There is great deal of controversy regarding the ancestor of *Chionomys* as well as its closest living relatives. Allozyme (Mezhzherin et al. 1995) and fossil evidence (Kretzoi 1969, Chaline 1987) suggest a common ancestry of *Chionomys* with a predominantly rhizodont *Myodes*. Contrary to this, molecular evidence has placed *Chionomys* as nested within a monophyletic clade containing *Microtus, Neodon* and *Blanfordimys* (Jaarola et al. 2004, Galewski et al. 2006 and Buzan et al. 2008). In the opinion by Nadachowski (1991), who stressed the enigmatic ancestry of *Chionomys*, this genus most likely originated from *Allophaiomys* during the Lower Biharian (<2 Mya). The oldest *Chionomys* fossil was reported from the Lower Pleistocene in the Caucasus (Vereshchagin 1967); however, allozyme evidence suggests that the

genus is of older origin (>2.4 Mya; Chaline & Graf 1988). We avoided applying a molecular clock in our genetic distance estimates. Arvicoline cyt *b* sequences display highly saturated codon positions that are probably a consequence of the fast evolutionary rate of the muroid genome (Galewski et al. 2006). Nuclear genes, on the other hand, evolve rapidly enough to accumulate synapomorphies but slow enough to avoid substitutional saturation. Consequently, nuclear markers are more suitable for molecular clock estimates because of the presence of highly informative sites in third codon positions.

Molecular evidence confirmed the traditional division of *Chionomys* into two monophyletic lineages, the *nivalis* and *roberti* groups (Musser & Carleton 2005). The former is characterised by a simple, primitive molar pattern (Nadachowski 1991), a non-ossified distal baculum (Kryštufek & Vohralík 2004) and the acrocentric condition of the smallest pair of autosomal choromosomes (Agadzhanjan & Jatsenko 1984). In the *roberti* group, the molar pattern is complex with the distal baculum being more ossified (particularly in *C. gud*), while the smallest acrocentric chromosome pair is bi-armed. Nadachowski (1991) suggests an early split between the two *Chionomys* groups in the Lower Pleistocene, with subsequent divergence between *C. gud* and *C. roberti* during the Middle Pleistocene. Further geographic structuring in *C. nivalis* is less clear, and our results contradict allozymic evidence of Filippucci et al. (1991) who found genetic distances between the Israeli and European samples within the range observed among congeneric mammalian species. Our conclusions are based on a limited number samples, though, and geographic structuring in *C. nivalis* therefore remains a topic for future research.

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