The origin and phylogenetic relationships of *Microtus bavaricus* based on karyotype and mitochondrial DNA sequences

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A b s t r a c t . Geographic isolation of small populations in refugia during late Pleistocene glaciations resulted in population differentiation that in some cases lead to speciation. We report the karyotype of *Microtus bavaricus*, an evolutionary young and threatened rodent endemic to the Alps. Our results show that the karyotype of *M. bavaricus* is almost identical to that of *M. liechtensteini* (2N = 46, NF = 54). A close relationship between the two species was also supported by phylogenetic analysis of complete mitochondrial DNA sequences for the cytochrome *b* gene. The cytochrome *b* divergence between *Microtus bavaricus* and *M. liechtensteini* was 1.7 %, the lowest estimate observed among the 14 currently recognised species of Eurasian pine voles (subgenus *Terricola*).

Introduction

Vole species of the genus *Microtus* (Arvicolinae, Rodentia) differ considerably in age and various evolutionary stages of speciation can be observed within the genus. The Eurasian pine voles, subgenus *Terricola*, include species groups that are especially suitable for analysis of recent divergence events (J a a r o l a et al. 2004). Geographic isolation of small populations in refugia during the late Pleistocene glaciations could have served as "speciation traps" for several of these young taxa, thus promoting speciation (C h a l i n e 1987, M a r t í n k o v á & D u d i c h 2003).

The Bavarian pine vole, *Microtus bavaricus* (König, 1962), is an endemic species of the Alps with an extremely restricted range and rather enigmatic phylogenetic relationships. In fact, its distribution area covers only six known localities in the Innsbruck Alps in Bavaria, Germany (*terra typica* at Garmisch-Partenkirchen), and northern Tyrol in Austria (K ö n i g 1982, S p i t z e n b e r g e r 2002, C a r l e t o n & M u s s e r 2005). Because of the species' distributional pattern, it was suggested that *M. bavaricus* survived the last glacial period in a refugium situated in the northern Alps (K r a t o c h v í l 1970,

S p i t z e n b e r g e r 2002). Since the original morphological description by K ö n i g (1962), affinities of *M. bavaricus* to the two other lineages of pine voles endemic to the Alps and some nearby mountain ranges, *M. multiplex* and *M. liechtensteini*, have been indicated (K r a t o c h v í l 1970, S p i t z e n b e r g e r 2002) and confirmed by both morphological (S p i t z e n b e r g e r et al. 2000) and molecular genetic analysis (H a r i n g et al. 2000). This group, the *M. multiplex* complex, including *M. multiplex*, *M. liechtensteini* and *M. bavaricus*, is characterised by low morphological divergence and *M. multiplex* and *M. liechtensteini* were occasionally considered to be conspecific (K r a p p 1982). This opinion found particular support in a finding of a single natural hybrid in the area of parapatric contact between the two taxa (S t o r c h & W i n k i n g 1977). The F1-hybrid from Calliano, Trento province in Italy showed karyotype characteristics of both parental species and had an intermediate diploid number of chromosomes (2N = 47).

M. multiplex and *M. liechtensteini* can be distinguished by their parapatric distribution patterns and differences in karyotype: *M. multiplex* is distributed in the western parts of the Alps and certain adjacent mountain ranges (eastern margins of Massif Central, northern Apennines), whereas *M. liechtensteini* occurs in the eastern and north-eastern Alps and the western Dinaric mountains (M i t c h e l l – J o n e s et al. 1999). The diploid number of chromosomes differs between the two taxa (2N = 48 in *M. multiplex*, 2N = 46 in *M. liechtensteini*) and the two karyotypes can also be distinguished also by other details in the morphology of individual chromosomes (Z i m a & K r á l 1984). However, the molecular divergence between *M. multiplex* and *M. liechtensteini* falls within the 4–8 % cytochrome *b* range that includes both inter- and intra-specific divergence in *Microtus* (J a a r o l a et al. 2004).

The aim of the present paper is to report on the hitherto unknown karyotype of *M. bavaricus*. We have also examined sequences of the mitochondrial cytochrome *b* gene for this species, in order to estimate the taxonomic position and phylogenetic relationships of *M. bavaricus* to other species of pine voles (subgenus *Terricola*). Analysis of cytochrome *b* sequences enabled us to utilize the extensive data set of publicly available sequences of closely related *Terricola* species.

Material and Methods

C h r o m o s o m e s

The karyotype was studied in a male of *M. bavaricus* collected in the Rofangebirge in northern Tyrol, Austria. It was collected on 3 August, 2004 by Simon Engelberger northwest of Steinberg/Rofan in an open spruce forest near a small brook, a tributary to the Ampelsbach River (47° 32' N, 11° 45' E, 1100 m a.s.l.). The voucher skin and skull (NMW65362) are stored in the Mammal Collection of the Natural History Museum in Vienna. Mitotic chromosomes were prepared from bone marrow cells obtained from short-term culture, using the standard technique with hypotonic treatment and fixation in a mixture of ethanol and acetic acid. The karyotype was then analyzed by conventional Giemsa staining.

C y t o c h r o m e *b* s e q u e n c e s

Complete or partial sequences of the mitochondrial gene for cytochrome *b* were obtained for 14 individuals of *Microtus bavaricus, M. tatricus, M. majori* and *M. liechtensteini*

Table 1. Individuals voucher numbers, sample localities, cytochrome *b* haplotypes and GenBank accession numbers for sequenced specimens of *Microtus*, subgenus *Terricola*.

Species	Voucher No.	Locality	Haplotype	Accession No.
M. bavaricus	NMW65362	Steinberg am Rofan, Northern Tyrol, Austria	*bavaricus 1*	DQ841693
	NMW8072	Garmisch-Partenkirchen, Bavaria, Germany	*bavaricus 2*	DQ841694
	NMW26592	Steinberg am Rofan, Northern Tyrol, Austria	*bavaricus 3*	DQ841695
M. tatricus	NM-179	Prvé Roháčske pleso Lake, Western Tatra Mts, Slovakia	*tatricus 4*	DQ841696
	NM-546	Prvé Roháčske pleso Lake, Western Tatra Mts, Slovakia	*tatricus 5*	DQ841697
	NM-182	Prvé Roháčske pleso Lake, Western Tatra Mts, Slovakia	*tatricus 6*	DQ841698
	NM-194	Rakytovská dolina Valley, Velká Fatra Mts, Slovakia	*tatricus 7*	DQ841699
	NM-195	Rakytovská dolina Valley, Velká Fatra Mts, Slovakia	*tatricus 7*	DQ841699
	NM-202	Dolný Harmanec, Velká Fatra Mts, Slovakia	*tatricus 8*	DQ841700
	NM-76	Tretie Roháčske pleso Lake, Western Tatra Mts, Slovakia	*tatricus 9*	DQ841701
	NM-84	Smutná dolina Valley, Western Tatra Mts, Slovakia	*tatricus 10*	DQ841702
M. majori	TU601	Damar, Turkey	*majori 2*	DQ841703
	MM388	Hopa, Turkey	*majori 3*	DQ841704
M. liechtensteini	CR43	Croatia	*liechtensteini 2*	EF379100

NMW – National Museum, Vienna, Austria; NM – collection of N. M a r t í n k o v á ; TU, MM – collection of M. M a c h o l á n ; CR – collection of Heikki H e n t t o n e n .

(Table 1). Additional sequences were downloaded from GenBank (Accession Numbers in Fig. 2; J a a r o l a et al. 2004, G a l e w s k i et al. 2006, T o u g a r d et al., in press). The haplotype names used here are identical to the original references except for *brachycercus 1–3* (*cf.* C a r l e t o n & M u s s e r 2005) that were named *savii 1–3* in J a a r o l a et al. (2004).

Primers used for amplification and sequencing of the cytochrome *b* gene, PCR and sequencing conditions are described in detail in J a a r o l a et al. (2004). Sequences were completed in Sequencher 4.2 (GeneCodes) and manually aligned in BioEdit 5.0 (H a l l 1999).

Sequence composition and nucleotide diversity (π) were calculated in DnaSP 4.1 (R o z a s et al. 2003). Total (Dxy) and net (Da) divergence was estimated in MEGA 3.1 (K u m a r et al. 2004) using Kimura 2-parameter distances (K i m u r a 1980). Substitution model was assessed in ModelTest 3.7 (P o s a d a & C r a n d a l l 1998) using the Akaike Information Criterion. The model selected, GRT+I+Γ (T a v a r é 1986; I = 0.59, α = 1.5008), was used to estimate a maximum likelihood (ML) phylogenetic tree in PAUP* 4.0b10 (S w o f f o r d 2003). Bootstrap support of taxonomic units was calculated from 10,000 neighbour-joining (NJ) and 100 maximum parsimony (MP) parametric replicates. Bayesian posterior probabilities were estimated in MrBayes 3.1 (R o n q u i s t & H u e l s e n b e c k 2003) from 2,000,000 generations sampled every 1000th generation excluding a burn-in of 200,000 steps.

Results

C h r o m o s o m e s

The male diploid complement of *M. bavaricus* contains 46 chromosomes (Fig. 1). The largest pair consists of two subtelocentric chromosomes with tiny, but clearly visible short arms. The second largest chromosome is an odd submetacentric. There are three meta- or submetacentric chromosomes of medium size. The other chromosomes are acrocentric, and their size decreases gradually. The largest acrocentric pair approximately equals in size to the larger arm of the odd submetacentric chromosome. The number of chromosomal arms is therefore 54, however, additional small short arms are apparent in at least two pairs of acrocentric chromosomes. The odd large submetacentric element can be identified as the X chromosome. The Y chromosome is one of the three elements with the meta- or submetacentric position of the centromere. In most metaphases, one of these chromosomes was slightly larger than the other two, and this could be considered as the Y chromosome.

C y t o c h r o m e *b*

Altogether 11 complete cytochrome *b* haplotypes (1143 base pairs; bp) were submitted to public databases (GenBank Accession Numbers: DQ841693-DQ841704, EF379100). Additionally, two *M. bavaricus* individuals yielded partial cytochrome *b* sequences, of 1052 (DQ841695) and 650 bp (DQ841694). Phylogenetic analyses were based on the complete cytochrome *b* alignment of 38 haplotypes of European endemic *Terricola* species, including one available complete *M. bavaricus* sequence, and 11 haplotypes of *Terricola* species with distribution areas covering Europe and Asia Minor. Within- and between-species divergences were estimated from all available *Terricola* sequences using a 650 bp alignment of 54
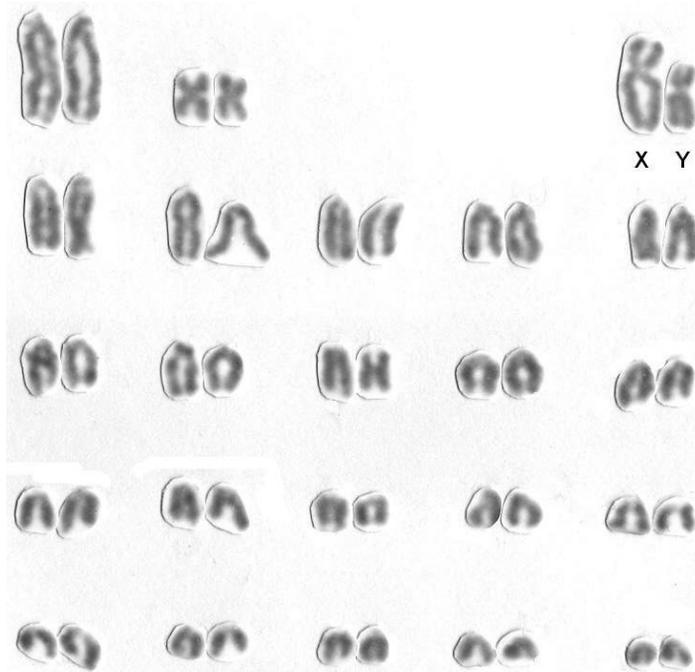
Fig. 1. Karyotype of a male *Microtus bavaricus* from Rofangebirge, northern Tyrol, Austria analysed by conventional Giemsa staining.

sequences. Phylogenetic analysis of this alignment, including three *M. bavaricus* and two *M. liechtensteini*, shows that *M. bavaricus* and *M. liechtensteini* are reciprocally monophyletic (data not shown).

For the 1143 bp cytochrome *b* alignment, a total of 371 (32 %) polymorphic sites were observed and 325 (28 %) of those were parsimony informative. Phylogenies inferred with ML, MP, NJ and Bayesian methods had similar topologies. The phylogenetic analysis shows that *M. bavaricus* is most closely related to *M. liechtensteini* and the result is supported by high bootstrap support and posterior probability values (Fig. 2). Monophyly of the *M. multiplex* complex had bootstrap support of 71 % in the NJ tree, 75 % in the MP trees and a Bayesian posterior probability of 1.0. Total and net divergence between *M. bavaricus* and *M. liechtensteini* was estimated at 2.3 % and 1.7 %, respectively, which is the lowest between-species divergence reported within the subgenus *Terricola* (Table 2). Despite the limited sample size, nucleotide diversity of the *M. bavaricus/liechtensteini* group was within the range of intraspecific nucleotide diversity in other *Terricola* species (Fig. 3).

Discussion

The karyotype of the *M. bavaricus* male studied is almost identical with those reported from various parts of the range of *M. liechtensteini* (e.g., P e t r o v & Ž i v k o v i ć 1971, S t o r c h & W i n k i n g 1977). The autosome sets are apparently quite similar, and the only difference between the karyotypes reported for individual populations of *M. liechtensteini* and *M. bavaricus* can be found in the size and the centromeric position of

Table 2. Total (Dxy; below the diagonal) and net (Da; above the diagonal) divergence (Kimura 2-parameter distances) between species of the *Terricola* subgenus based on partial cytochrome *b* gene sequences (650 bp). Numbers in bold represent total and net divergence between *Microtus bavaricus* and *M. liechtensteini*.

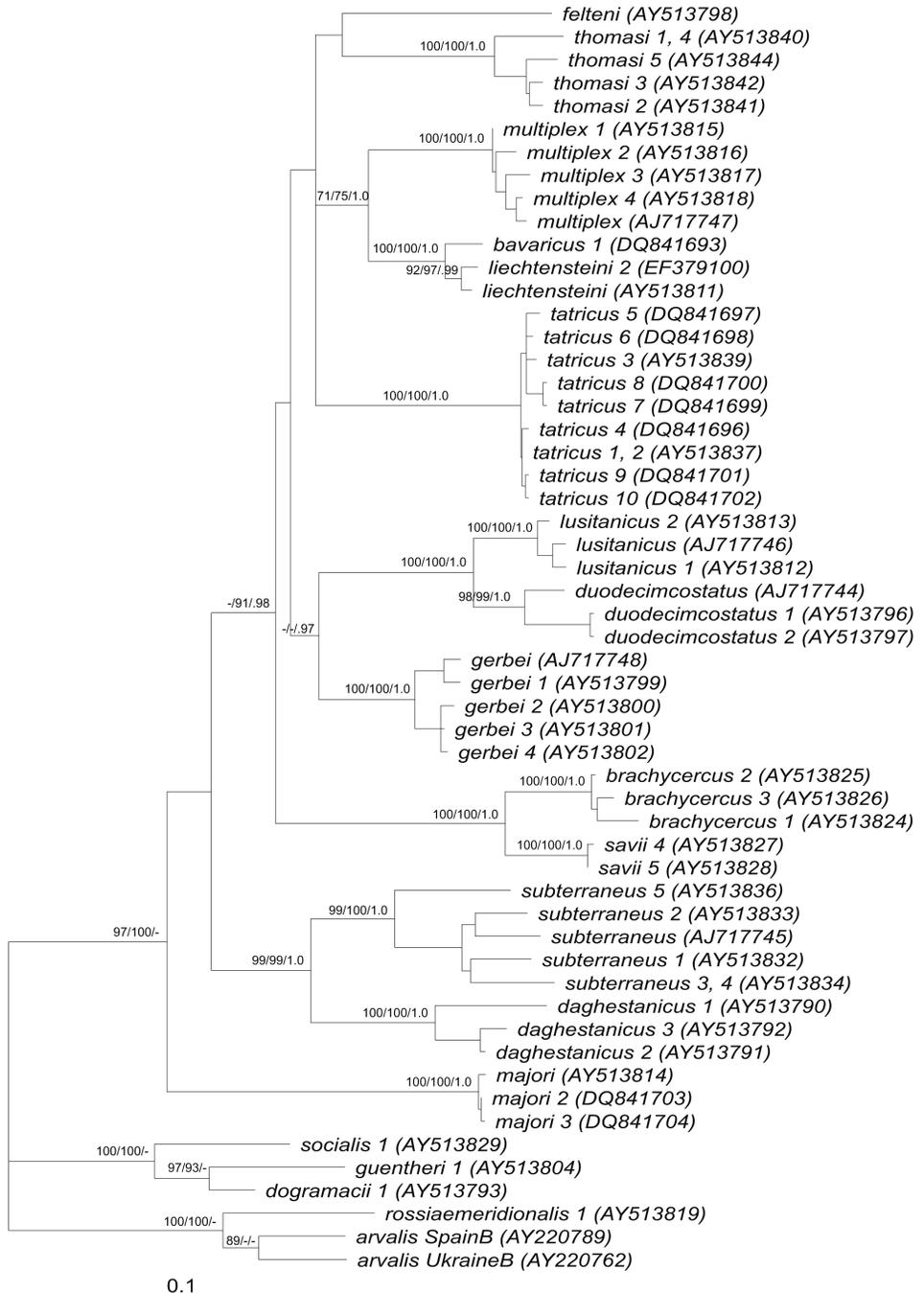| Dxy/Da	*Microtus*													
	bav.	brach.	dagh.	duod.	felt.	gerbei	liecht.	lusit.	majori	multipl.	savii	subt.	tatr.	thom.
M. bavaricus (3)		0.090	0.076	0.074	0.070	0.058	**0.017**	0.078	0.089	0.058	0.098	0.067	0.076	0.074
M. brachycercus (3)	0.099		0.109	0.094	0.108	0.092	0.077	0.094	0.114	0.100	0.044	0.098	0.102	0.094
M. daghestanicus (3)	0.092	0.129		0.097	0.078	0.073	0.080	0.100	0.090	0.078	0.115	0.052	0.088	0.077
M. duodecimcostatus (4)	0.088	0.112	0.123		0.083	0.066	0.066	0.030	0.082	0.072	0.101	0.091	0.083	0.080
M. felteni (1)	0.072	0.114	0.092	0.095		0.067	0.076	0.083	0.101	0.070	0.106	0.078	0.077	0.061
M. gerbei (5)	0.065	0.104	0.092	0.083	0.072		0.053	0.069	0.075	0.067	0.090	0.069	0.068	0.059
M. liechtensteini (2)	**0.023**	0.087	0.098	0.082	0.080	0.063		0.069	0.077	0.056	0.087	0.066	0.074	0.070
M. lusitanicus (3)	0.084	0.104	0.117	0.045	0.086	0.078	0.077		0.077	0.075	0.106	0.094	0.083	0.079
M. majori (3)	0.092	0.121	0.106	0.096	0.103	0.082	0.083	0.090		0.084	0.125	0.085	0.093	0.099
M. multiplex (5)	0.065	0.111	0.097	0.088	0.075	0.077	0.065	0.083	0.090		0.107	0.075	0.068	0.066
M. savii (2)	0.101	0.051	0.129	0.114	0.107	0.096	0.092	0.111	0.127	0.113		0.098	0.109	0.092
M. subterraneus (6)	0.089	0.124	0.085	0.123	0.098	0.094	0.090	0.117	0.106	0.099	0.118		0.086	0.073
M. tatricus (10)	0.081	0.112	0.105	0.098	0.080	0.077	0.081	0.089	0.098	0.076	0.113	0.109		0.075
M. thomasi (5)	0.088	0.113	0.103	0.104	0.073	0.077	0.086	0.095	0.112	0.083	0.104	0.105	0.090	

Fig. 2. Maximum likelihood phylogenetic tree (-ln L = 7584.58) based on the GTR+I+Γ substitution model showing the inferred phylogenetic relationships among 49 *Terricola* cytochrome *b* haplotypes (1143 bp). The tree was rooted with representatives of *Microtus sensu stricto* subgenus. Numbers above branches represent bootstrap support based on neighbour-joining, maximum parsimony analysis and Bayesian posterior probability, respectively. Only values for major branches greater than 70 % and 0.95, respectively, are shown.
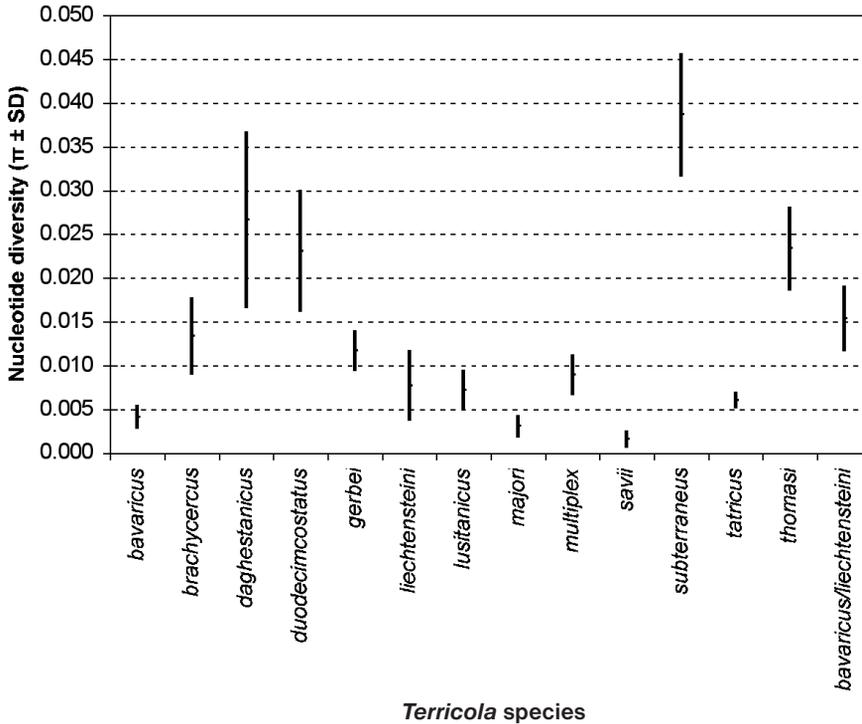
Fig. 3. Comparison of variation of within-species nucleotide diversity (π ± SD) in the *Terricola* subgenus and between-species divergence of the *Microtus bavaricus*/*liechtensteini* group based on partial cytochrome *b* gene sequences (650 bp).

the Y chromosome. The Y chromosome observed in *M. bavaricus* is almost identical to that of a population of *M. liechtensteini* from northern Velebit Mts in Croatia studied by P e t r o v & Ž i v k o v i ć (1974). On the other hand, the Y chromosome of males from *M. liechtensteini* populations from southern Austria (Defereggen-Gebirge, Karnische Alpen) was subtelocentric and distinctly larger than a similar metacentric pair of small autosomes (K r á l et al. 1978). Submetacentric and subtelocentric morphs of the Y chromosome were found also in populations of *M. liechtensteini* from northern Italy (Trento and Belluno provinces; S t o r c h & W i n k i n g 1977). The distribution of individual morphs of the Y chromosome within the range of *M. liechtensteini* has no distinct geographic pattern, and it probably yields no phylogenetic information. Similar variation in the sex chromosomes was reported also among populations of *M. multiplex* (G r a f & M e y l a n 1980, B r u n e t – L e c o m t e & V o l o b o u e v 1994). The karyotype structure in the studied male of *M. bavaricus* thus indicates its close relatedness to populations of *M. liechtensteini*, and no distinct specific features were observed.

The phylogenetic inference derived from complete cytochrome *b* sequences confirms the close relationship of *M. bavaricus* and *M. liechtensteini* reported by H a r i n g et al. (2000) based on mitochondrial control region sequences. Similarly, in accordance with previous phylogenetic analyses (H a r i n g et al. 2000, J a a r o l a et al. 2004), our data demonstrate that *M. multiplex*, *M. liechtensteini* and *M. bavaricus* represent a well supported monophyletic lineage.

The primary divergence within the *M. multiplex* complex occurs between *M. multiplex sensu stricto* and the sister species *M. liechtensteini* and *M. bavaricus*. The total and net divergence between *M. bavaricus* and *M. liechtensteini* is the lowest observed between any pine vole species, indicating a very recent origin of the two taxa.

The branching pattern within the *M. multiplex* complex, namely the sister relationship of *M. multiplex* to closely related but reciprocally monophyletic *M. bavaricus* and *M. liechtensteini*, suggests that the ancestral population of the complex survived the last glaciations at the rims of the ice sheet covering the Alps and/or in the unglaciated mountainous areas. The ancestral population became divided into two glacial refugia, one situated probably in the southwestern and western Alps, while the second refugium occurred in the south, east and north of the Alpine main ridge. This geographic isolation caused speciation of *M. multiplex* and subsequently lead to a split between *M. liechtensteini* and *M. bavaricus*. Additionally, the range of *M. liechtensteini* occurring north of the main alpine ridge became fragmented leading to the apparent isolation of the contemporary populations of *M. liechtensteini* in Niedere Tauern, Salzburg and Totes Gebirge, Styria (S p i t z e n b e r g e r 2002). A similar scenario can also be proposed for the origin of the current *M. bavaricus* populations.

Our cytochrome *b* results and the control region sequence analyses reported by H a r i n g et al. (2000) are congruent with respect to the phylogenetic relationships of the *M. multiplex* complex as well as other species of pine voles. Specifically, the data show that *M. tatricus* and *M. subterraneus* are not closely related to the *M. multiplex* complex and that they belong to other lineages of pine voles (*cf.* K r a t o c h v í l 1970, J a a r o l a et al. 2004). The topology of the phylogenetic tree presented here indicates an apparent discord between molecular and chromosomal data (see Z i m a & K r á l 1984 for review) since monophyletic lineages contain species with distinctly divergent karyotypes. For example, the sister groups of the *M. multiplex* complex with 2N = 46-48 are *M. tatricus* (2N = 32), *M. felteni* (2N = 54), and *M. thomasi* (2N = 40-44), whereas *M. gerbei* (2N = 54) is a sister group of the Iberian species, *M. duodecimcostatus* and *M. lusitanicus* (2N = 62). Altogether, the data strongly suggest that morphological, chromosomal and molecular evolution has proceeded independently during pine vole evolution, and that each evolutionary process has had its own specific rate. This model may be applied for divergence between species as well as between populations within single species. The low mitochondrial DNA variability observed in many pine vole species today suggests that chromosomal evolution in this subgenus could have been facilitated by small historical population sizes.

We conclude that both our chromosomal and mitochondrial DNA findings demonstrate a close affinity between *M. bavaricus* and *M. liechtensteini*. The pattern of evolutionary divergence between populations of this group must have been rather complex in the past, and some populations probably survived the last glaciation period in refugia situated in the northern Alps.

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