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Holarctic phylogeography of the root vole (*Microtus oeconomus*): implications for late Quaternary biogeography of high latitudes

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Abstract

A species-wide phylogeographical study of the root vole (*Microtus oeconomus*) was performed using the whole 1140 base pair mitochondrial (mt) cytochrome *b* gene. We examined 83 specimens from 52 localities resulting in 65 unique haplotypes. Our results demonstrate that the root vole is divided into four main mtDNA phylogenetic lineages that seem to have largely allopatric distributions. Net divergence estimates (2.0–3.5%) between phylogroups, as well as relatively high nucleotide diversity estimates within phylogroups, indicate that the distinct phylogeographical structure was initiated by historical events that predated the latest glaciation. European root voles are divided into a Northern and a Central mtDNA phylogroup. The mtDNA data in concert with fossil records imply that root voles remained north of the classical refugial areas in southern Europe during the last glacial period. The currently fragmented populations in central Europe belong to a single mtDNA phylogroup. The Central Asian and the North European lineages are separated by the Ural Mountains, a phylogeographical split also found in collared lemmings (*Dicrostonyx*) and the common vole (*M. arvalis*). The Beringian lineage occurs from eastern Russia through Alaska to northwestern Canada. This distribution is congruent with the traditional boundaries of the Beringian refugium and with phylogeographical work on other organisms. In conclusion, similarities between the phylogeographical patterns in the root vole and other rodents, such as Arctic and subarctic lemmings, as well as more temperate vole species, indicate that late Quaternary geological and climatic events played a strong role in structuring northern biotic communities.

Keywords: Beringia, colonization history, cytochrome *b*, *Microtus oeconomus*, mtDNA, phylogeography

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Introduction

The Northern Hemisphere has experienced dramatic climatic fluctuations over the past 2.5 million years (Andersen & Borns 1997). The effects of such climatic perturbations on biological diversity can be investigated by studying widely distributed species. In rodents, large-scale phylogeographical studies of Arctic, as well as more subarctic and temperate species, have demonstrated distinct phylogeographical patterns that can be interpreted readily in terms of recent glacial history (Fedorov *et al*. 1999a,b; Jaarola & Searle 2002; Haynes *et al*. 2003). This study represents the first species-wide phylogeographical investigation of an interzonal small mammal that occurs in wet grasslands of both Arctic and temperate biota, the root or tundra vole (*Microtus oeconomus*). The root vole is the only extant Holarctic species of the species-rich genus *Microtus*. Root voles occur from northwest Europe, eastward through north and central Asia to Alaska and northwest Canada and are found on many north Pacific islands (Fig. 1).

Root voles are thought to have originated in Asia and only recently entered the Nearctic via the Bering Land Bridge (Rausch 1963; MacPherson 1965). Low allozymic variation in North American populations is consistent with

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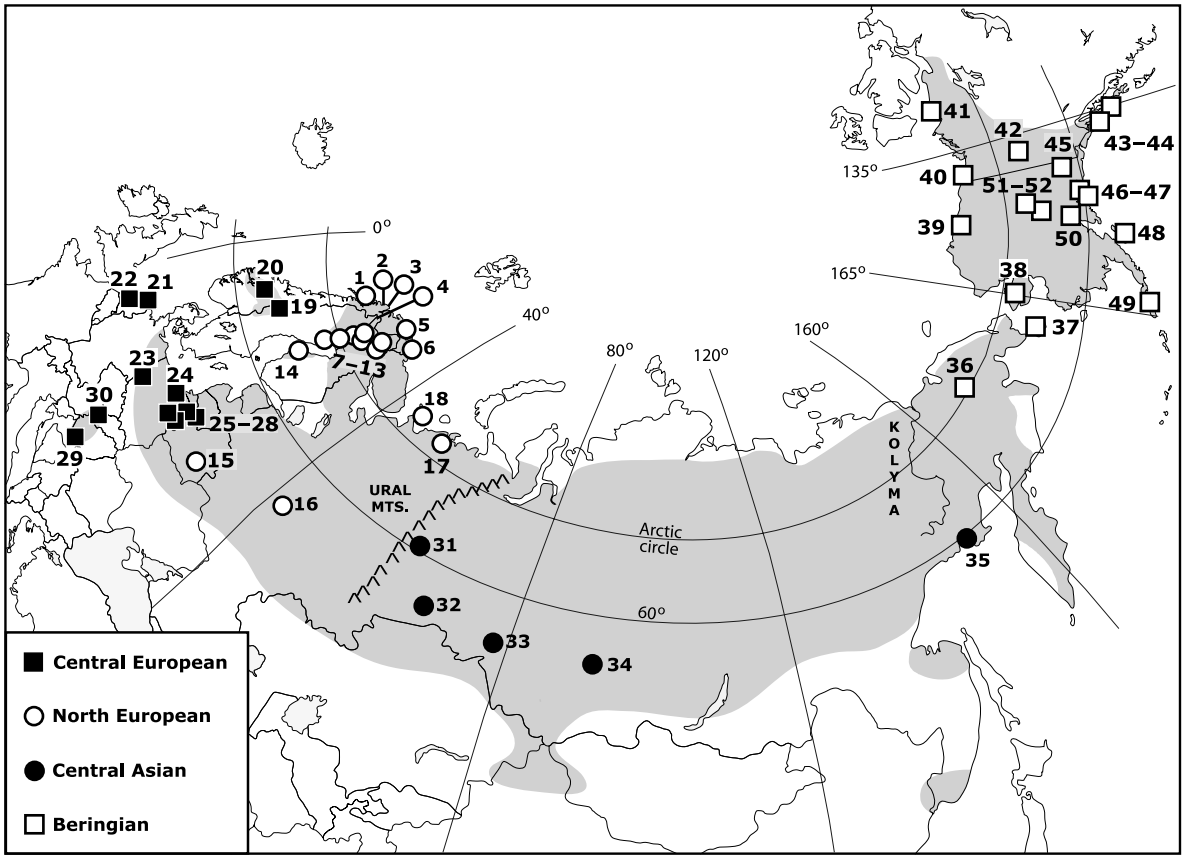

Fig. 1 The root vole (*Microtus oeconomus*) distribution is shown in grey (Gromov & Polyakov 1992; Whitaker 1995; Panteleyev 1998; Mitchell-Jones *et al*. 1999). Numbers correspond to the localities in Table 1. The four mtDNA lineages are represented by different symbols.

a late colonizing scenario for these populations (Lance & Cook 1998), although a fossil in Canada dating to the late Illinoian glacial stage at 200 000–130 000 BP (Jopling *et al*. 1981) indicate that colonization predated the latest glaciation at 115 000–10 000 BP.

Phylogeographical studies of various organisms in Europe have demonstrated that the southern Mediterranean areas and Caucasus served as refugia during the last glaciation (reviewed in Taberlet *et al*. 1998; Hewitt 1999, 2000). However, the universality of this model has been questioned recently (e.g. Bilton *et al*. 1998; Stewart & Lister 2001; Jaarola & Searle 2002; Kropf *et al*. 2002). According to Bilton *et al*. (1998), the mtDNA phylogeographies of several small mammals suggest additional glacial refugia in central Europe. Fossil data show that the root vole occurred over much of central Europe during the last glacial period, including the last glacial maximum 21 000–17 000 BP (Chaline 1987). In fact, the species even expanded its range during cold and humid periods (Chaline 1987; Kordos 1990).

The overall aim of this study is to assess the phylogeographical structure of root voles and begin to explore the extent of congruent phylogeographical patterns for northerly distributed species. If congruent, these patterns might reflect common biogeographical events (cf. Taberlet *et al*. 1998; Hewitt 1999; Riddle *et al*. 2000). Species-specific patterns, in contrast, may reflect individualized responses (cf. Graham *et al*. 1996) or simply reflect the history of the gene studied. We compare the root vole data with previous phylogeographical investigations of collared lemmings (*Dicrostonyx*; Fedorov *et al*. 1999a), true lemmings (genus *Lemmus*; Fedorov *et al*. 1999b), field voles (*M. agrestis*; Jaarola & Searle 2002) and common voles (*M. arvalis*; Haynes *et al*. 2003) to identify phylogeographical structure shared across these high latitude taxa. These small mammals have been partially codistributed with the root vole during the late Quaternary.

Materials and methods

Samples and DNA extraction

This study includes a total of 83 root vole samples from 52 localities across the species' range in Europe, Asia and North America (Table 1, Fig. 1). Samples were provided by individuals or museum archives or were collected during the Swedish–Russian Tundra Expedition 1994 (localities

Table 1 Geographic information, number of specimens from each locality and haplotype designation of the specimens. The data are separated according to the four root vole (*Microtus oeconomus*) mtDNA phylogroups. Figure references correspond to the localities in Fig. 1; *n* denotes the number of specimens

Country	Fig ref.	Locality (region or closest city)	Haplotype	*n*	Museum no.	GenBank Accession no.
North European						
Norway	1	Andøya	Nor-1, Nor-2	2		
	2	Kvaløya	Nor-3, Nor-4	3		
	3	Ringvassøya	Nor-5	1		
Finland	4	Kilpisjärvi	Fin-1	3	AF1944, AF1948	
Norway	5	Porsanger	Nor-6	2		
	6	Hamningberg	Nor-7	1		
Finland	7	Inari	Fin-Swe	1		
	8	Laanila	Fin-2, Fin-3	3		
	9	Muonio	Fin-4	1		
	10	Kolari	Fin-5	1		
Sweden	11	Pajala	Swe-1	1		
	12	Överkalix	Fin-Swe	1		
	13	Luleå	Swe-2, Swe-3	3		
Finland	14	Vaasa	Fin-6	1		
Belarus	15	Bobrujsk	Bel-1	1		
Russia	16	Vladimir	Rus-1	1		
	17	Petjora	Rus-2	1		
	18	Kanin Peninsula	Rus-3, Rus-4	2		
Central European						
Sweden	19	Mittådalen	Swe-4	1		
Norway	20	Dovre	Nor-8, Nor-9	2		
Netherlands	21	Texel	Neth-1	1		
	22	Vlietlanden	Neth-2	2		
Poland	23	Krosno Odrzańskie	Pol-1	1		
	24	Iława	Pol-2	1		
	25	Ciszewo	Pol-3	3		
Lithuania	26	Zuvintas	Lith-1	1		
Poland	27	Czarna Białostocka	Pol-4	1		
	28	Biebrza	Pol-5	1		
Hungary	29	Szigetköz	Hun-Slo	1		
Slovakia	30	Bratislava	Hun-Slo	1		
Central Asian						
Russia	31	Sverdlovsk	Rus-5	1		
	32	Kurgan	Rus-6	1		
	33	Novosibirsk	Rus-7	1		
	34	Krasnoyarsk	Rus-8	1		
	35	Magadan	Rus-9, Rus-10	2	AF6640, AF6700	
Beringian						
Russia	36	Chukotka	Rus-11, Rus-12	2	AF3758, AF3762	
USA	37	S:t Lawrence Island	USA-1, USA-2	2	AF20805, AF20818	
	38	Pilgrim Springs	USA-3	2	AF7462, AF7463	
	39	Colville River Delta	USA-4, USA-5	2	AF22101, AF22104	
Canada	40	Ivvavik National Park	Can-1	2		
	41	Amundsen Gulf South	Can-2, Can-3	3		
	42	Ogilvie Mountains	Can-4	1		
USA	43	Chichagof Island	USA-6	2	AF16082, AF16083	
	44	Baranof Island	USA-5, USA-7	2	AF17082, AF17085	
	45	McCarthy	USA-8, USA-9	2	AF3277, AF3289	
	46	Hinchinbrook Island	USA-10	2	AF460, AF476	
	47	Montague Island	USA-11	2	AF517, AF535	
	48	Kodiak Island	USA-12, USA-13	2	AF798, AF801	
	49	Cold Bay	USA-14, USA-15	2	AF14978, AF14985	
	50	Anchorage	USA-16, USA-17	2	AF11320, AF11600	
	51	Healy, AK	USA-18	1	AF347	
	52	Fairbanks	USA-19	1	AF1110	

17–18), the Swedish Tundra Northwest Expedition 1999 (localities 40–41), and the Beringian Co-evolution Project 1998–2000 (localities 35–52). Total DNA was extracted from dried, frozen or ethanol preserved tissues, such as tail tips, ears, heart, kidney or liver using either the DNeasy™ Tissue Kit (Qiagen) or a modified sodium chloride extraction protocol (Fleming & Cook 2002). Purified mtDNA was isolated from two individuals (localities 11, 13) using the method described in Jaarola & Tegelström (1995).

PCR amplification

To ensure the mitochondrial origin of sequences, we amplified the cytochrome *b* gene from both purified mtDNA and total DNA from two individuals. The entire cytochrome *b* gene [1140 base pairs (bp)] from each specimen was amplified in either one polymerase chain reaction (PCR) or, when the DNA was degraded, in two to three PCR reactions that produced overlapping fragments. The primers used for PCR and sequencing are given in Table 2. PCR reagents included AmpliTaq Gold™ DNA Polymerase and GeneAmp PCR Buffer Gold (Applied Biosystems). Amplifications were performed using either methods described in Jaarola & Searle (2002) or in Galbreath (2002).

DNA sequencing

Direct sequencing was performed using ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The entire cytochrome *b* gene was sequenced in both directions. Sequencing products were purified according to the manufacturers' recommendations and run on an ABI Prism 310 or 3100 capillary automated sequencer, or a 373 or 377 gel-based automated sequencer (Applied Biosystems).

Sequence and phylogenetic analysis

Sequences were aligned and ambiguous sites were resolved using the SeqManII module of the Lasergene99 program or the program Sequence Navigator™ (Applied Biosystems). The computer program DNA Sequence Polymorphism (DnaSP) version 3.53 (Rozas & Rozas 1999) was used to translate nucleotide sequences into amino acid sequences and to calculate sequence statistics. The numbers of transitions and transversions were calculated using MacClade version 4.02 (Maddison & Maddison 2000). MODELTEST version 3.06 (Posada & Crandall 1998) was used to establish the model of DNA substitution that best fitted the data. The Akaike information criterion estimates (AIC) and the hierarchical likelihood ratio tests (hLRT) that are implemented in MODELTEST resulted in the same models of nucleotide substitution. The Tamura–Nei (TrN) model (Tamura & Nei 1993) and the GTR model (Rodríguez *et al*. 1990) were selected (with or without outgroups, respectively). The best models included both the gamma shape parameter (α) and the proportion of invariable sites (*I*). However, we also tested the models using only α and excluding α and *I*.

Table 2 Primers used for PCR and sequencing

Primer	Sequence (5′–3′ direction)	Reference
L14724B	CGAGATCTGAAAAACCATCGTTG	Irwin *et al*. (1991)
L14727-SP	GACAGGAAAAATCATCGTTG	Jaarola & Searle (2002)
L15162M	GCAAGGTACTTCCATGAGGACAAATATC	Modified from Irwin *et al*. (1991)
L15162M2	GCTACGTACTTCCATGAGGACAAATATC	Jaarola & Searle (2002)
L15162MO	CTTCCATGAGGCCAAATATC	Modified from Irwin *et al*. (1991)
Lem3	TTCTTCGCATTCCA(TC)TT	Fedorov *et al*. (1999a)
L15287MO	TCACACGATTCTTCGCCT	*
L15408M	GCAGACAAAATCCCGTTCCA	Jaarola & Searle (2002)
MVZ 05	CGAAGCTTGATATGAAAAACCATCGTTG	Smith & Patton (1993)
Micro06	GGATTATTTGATCCTGTTTCGT	Galbreath (2002)
Arvic07	AAAGCCACCCTCACACGATT	*
H15177MO	AGGAGGTTTGTGATTACTG	*
H15408M	TGGAACGGGATTTTGTCTGC	Jaarola & Searle (2002)
H15408MO	TGGAATGGGATTTTGTCTGT	*
H15576M	GACCGTAAAATGGCGTAGG	Jaarola & Searle (2002)
H15576MO	GATCGTAGGATGGCGTAGG	*
H15915	AACTGCAGTCATCTCCGGTTTACAAGAC	Irwin *et al*. (1991)
VOLE 14	TTTCATTACTGGTTTACAAGAC	Conroy & Cook 1999
H-PRO	AAGTAGTTTAATTAGAATATCAG	*
H-ISO-MO	AAGTAGTTTAATTAGAATGTCAG	*

*Primers designed for this study.

Phylogenetic analyses were conducted using neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) algorithms. Various combinations of *M. middendorffi* (GenBank Accession no. AF163898), *M. kikuchii* (AF163896) and *M. montebelli* (AF163900) (Conroy & Cook 2000a) were tested as outgroups. The cytochrome *b* sequence of another sister-species, *M. fortis* (Conroy & Cook 2000a), contains an unresolved nucleotide and was therefore excluded from the analyses.

NJ trees were constructed in MEGA version 2.1 (Kumar *et al*. 2001). We compared the TRN and GTR models with the less sophisticated Jukes–Cantor (Jukes & Cantor 1969) and Kimura two-parameter (Kimura 1980) models. Bootstrap analyses were performed with 10 000 replicates.

MP analyses were conducted using PAUP* version 4.0b8 (Swofford 1998) with the heuristic search algorithm, 10 random addition replicates, tree bisection–reconnection (TBR) swapping and the steepest descent option. The maximum number of trees was constrained to 10 000–35 000, and at least five searches were performed for each combination of outgroup. 500 bootstrap replicates were generated with the maximum number of trees constrained to 5000. The ML tree search was conducted using PAUP as described for MP but with the 'as is' addition replicate. The substitution models determined by MODELTEST were used.

Rate heterogeneity within and among lineages was evaluated by comparing log likelihood scores of ML trees (without outgroups) constructed with and without a molecular clock constraint (Felsenstein 1988). Transition/transversion ratios for lineages were estimated using ML trees in MODELTEST.

Nucleotide diversity (π) within phylogroups and estimates of DNA net and raw divergence (Da and Dxy) (Nei 1987) were calculated using the TrN model in MEGA version 2.1. Standard errors (SE) were estimated by the bootstrap method using 10 000 replicates. Divergence time (T) between phylogeographical groups was estimated as $T = Da/2\mu$ where 2μ is the divergence rate. Ninety-five percent confidence intervals for the divergence times were calculated as ± 1.96 SE of the net distances.

Results

Nucleotide and amino acid sequence composition and variation

The sequences obtained from total genomic DNA and isolated mtDNA from two individuals were identical in both cases and matched the previously published cytochrome *b* sequence for this species (Conroy & Cook 2000a). Furthermore, there were no anomalies of the type commonly associated with pseudogenes, such as sequence ambiguities, frameshift mutations or internal stop codons (cf. DeWoody *et al*. 1999; Bensasson *et al*. 2001). We are thus confident that the cytochrome *b* sequences represent the mitochondrial gene rather than a nuclear pseudogene.

The entire cytochrome *b* gene (1140 bp) was sequenced in 83 specimens resulting in 65 unique haplotypes. Sequences have been deposited in GenBank (Accession nos AY219981–AY220045). Altogether 146 segregating sites were found, representing 13% of the nucleotides analysed, and 104 of the sites were informative for the parsimony analyses. The data did not fit an infinite-site model (Kimura 1969); we observed six polymorphic sites with three different nucleotides. The majority, 118 (78%), of the variable sites occurred at the third codon position, six at the second position and 27 at the first. Most of the nucleotide substitutions were transitions: 87% when counting directly from the sequence matrix and 93% according to the parsimony trees (using MacClade). Composition and variation in the root vole cytochrome *b* sequences were very similar to that of other species of *Microtus* (Conroy & Cook 2000b; Jaarola & Searle 2002; Haynes *et al*. 2003). The data set comprised 26 variable amino acid residues. A total of five sites coded for three different amino acids. Altogether 19 of the 26 variable amino acid residues, including the five sites that coded for three different amino acids, have previously been described as hypervariable in mammals (Irwin *et al*. 1991).

Phylogenetic analyses

All phylogenetic methods (NJ, MP and ML) discriminated four distinctive cytochrome *b* lineages with high bootstrap values (95–100%) (Fig. 2). These four lineages were retrieved regardless of outgroup(s) or substitution model employed and only minor topological differences within lineages were observed.

The MP searches of the 104 informative sites generated more than 30 000 trees irrespective of the outgroup(s) used, the large number of trees reflecting homoplasy within lineages. The highest consistency index (CI = 0.665, 328 steps) was obtained when using *M. montebelli* for outgroup rooting.

Including the proportion of invariable sites (I) in the ML analyses yielded lower log likelihood scores in MODELTEST, but it also lowered the percentage of recovery for all main branches and one of the lineages changed its position in the tree (cf. Lockhart *et al*. 1996). The longer distances, mainly the outgroup distances, increased significantly when I was included in the analyses. Hence, including I in the analyses made the outgroups too distant to function properly for rooting.

The ML trees constructed under a molecular clock constraint differed significantly from the unconstrained ML trees ($\chi^2 = 114–104$, d.f. = 63, $P < 0.0005$). However, the molecular clock hypothesis was not rejected for individual lineages ($P > 0.05$) except for the Beringian lineage. Exclusion of the USA-10 and USA-12 haplotypes also yielded nonsignificance for this lineage.

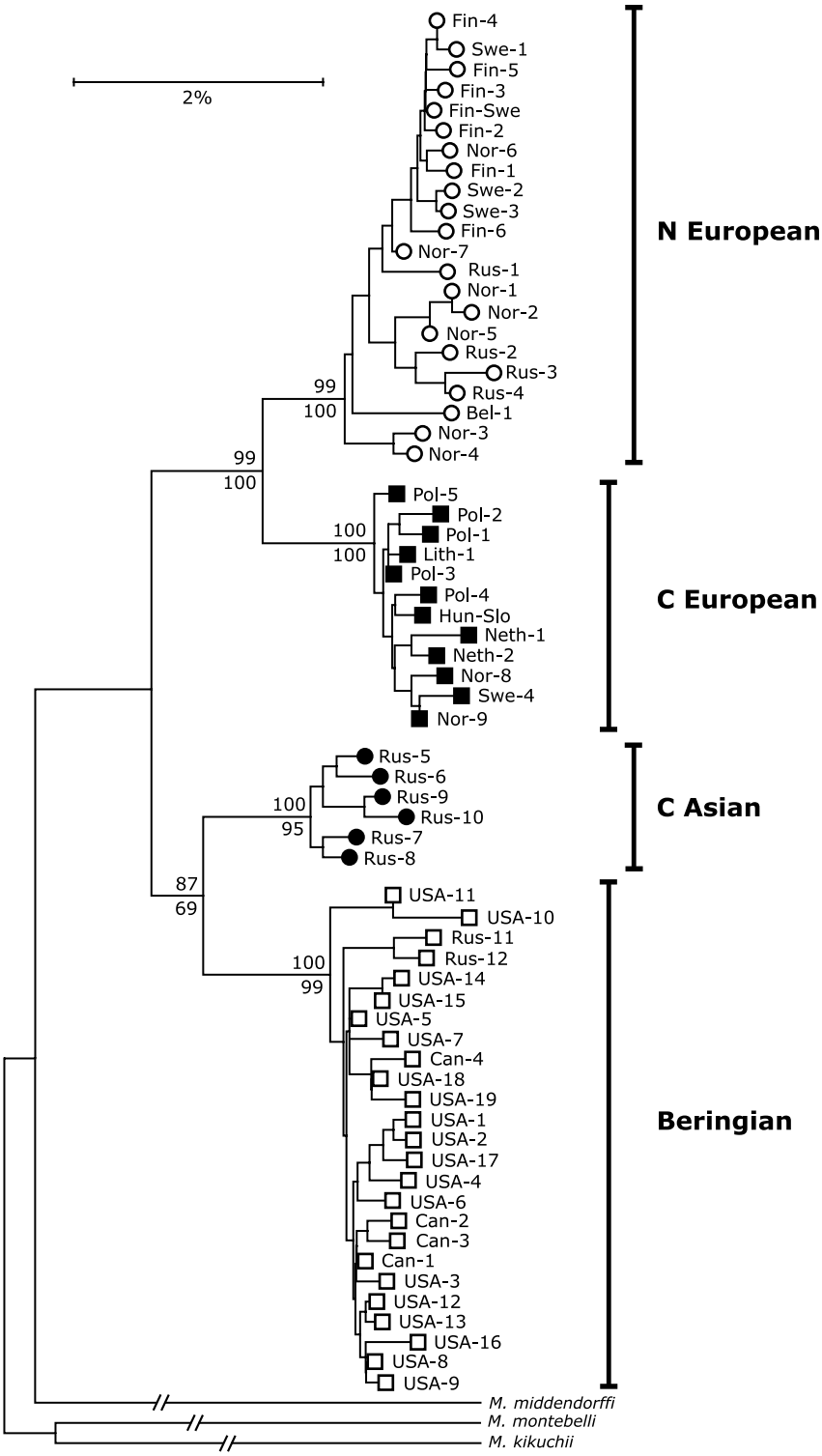

Fig. 2 Neighbour-joining tree illustrating the phylogenetic relationships among the 65 cytochrome *b* gene haplotypes in the root vole (*Microtus oeconomus*). The tree is constructed in MEGA version 2.1 using the TrN substitution model. Bootstrap resampling support (10 000 iterations) is listed above main branches. Values under branches denote the bootstrap support (500 iterations) for the parsimony analysis when using only *M. montebelli* as outgroup. Haplotype designations are the same as in Table 1.

Phylogeography and diversity

The four mtDNA phylogenetic lineages showed distinct geographical distributions (Fig. 1). Specimens from northern Fennoscandia (Sweden, Norway and Finland), western Russia (west of the Ural Mountains) and Belarus belong to a North European group. Specimens from central Sweden, southern Norway, the Netherlands, Poland, Hungary, Slovakia and Lithuania belonged to a Central European group. All specimens from Russia between the Ural

Table 3 Measures of intrapopulation variability for the root vole (*Microtus oeconomus*) mtDNA phylogroups. The nucleotide diversities are calculated using MEGA version 2.1 assuming a TrN model of sequence evolution. Standard errors (SE) are estimated by bootstrap method using 10 000 replications

Phylogroup	*n*	No. of haplotypes	Nucleotide diversity (π) (% ± SE)	No. of segregating sites (S)
C. European	16	12	0.51 (0.12)	27
N. European	29	22	0.82 (0.14)	51
C. Asian	6	6	0.72 (0.17)	19
Beringian	32	25	0.70 (0.10)	58
Total	83	65	2.82 (0.32)	146

Table 4 Raw distance (Dxy) and net distances (Da) between root vole (*Microtus oeconomus*) phylogroups are found above and below the diagonal, respectively. All values are calculated using MEGA version 2.1 assuming a TrN model of sequence evolution. Standard errors were estimated by bootstrap method (10 000 replications) and are given in parentheses. All estimates are expressed as percentages

Phylogroup	Central European	North European	Central Asian	Beringian
Central European	—	2.7 (0.4)	3.9 (0.6)	4.1 (0.6)
North European	2.0 (0.4)	—	3.7 (0.6)	4.3 (0.6)
Central Asian	3.3 (0.6)	2.9 (0.5)	—	2.8 (0.4)
Beringian	3.5 (0.6)	3.5 (0.6)	2.1 (0.4)	—

Mountains and the 151° E longitude belonged to a Central Asian group. Specimens from easternmost Russia, northwest Canada and Alaska formed a Beringian group centred on the Bering Strait.

The MODELTEST analyses indicated that the transition/transversion (ti/tv) ratios varied among lineages. The Central and North European lineages had high ti/tv ratios (38 and 25) whereas the Central Asian and Beringian lineages exhibited relatively low ratios (9 and 11).

Nucleotide diversity (π) within each of the four mtDNA phylogroups was similar, ranging from 0.5 to 0.8% (Table 3). Nucleotide diversity across the entire species was estimated at 2.8%. The number of segregating sites in each lineage varied between 19 and 58 (Table 3). Total (raw) divergences between the mtDNA phylogroups were estimated at 2.7–4.3%, whereas the net distances ranged from 2.0% to 3.5% (Table 4). The North European and Central European phylogroups were the least divergent, whereas the largest distance was observed between the Beringian and North European phylogroups.

Discussion

Our data show that the root vole constitutes an excellent candidate for examining the consequences of more northern glacial survival on current patterns of genetic differentiation and levels of genetic diversity. The root vole comprises four distinct mtDNA lineages that seem to be largely allopatric (Fig. 3). The corresponding four phylogroups are designated the North European, the Central European, the Central Asian and the Beringian.

There is no coincidence between the distribution of root vole subspecies and mtDNA phylogroups (cf. Gromov & Polyakov 1992). However, we argue that the root vole mtDNA genealogy identifies populations with specific and ancient evolutionary histories, because independent loci and phylogeographical patterns across multiple codistributed species show similar patterns (cf. Barton & Wilson 1995; Avise 2000; Nordborg 2001). First, Y chromosome sequence variation (Brunhoff *et al.* unpublished) and sequences from the ALDH1 nuclear intron (Galbreath 2002) in the root vole have demonstrated that at least three of the four mtDNA groups can be retrieved using other genetic markers. Second, the phylogeographical splits between the four mtDNA phylogroups are also found in several other high latitude species of small mammals such as lemmings and voles as well as other organisms (see below).

History of the Central and North European groups

Root voles in Europe form a northern and a central mtDNA phylogroup. Although the phylogeographical pattern could be interpreted as a result of postglacial expansions from southern European refugia, fossil findings throughout the last glaciation suggest a different story. During the glacial maximum, 21 000–17 000 BP, northern Europe was completely covered by ice (Andersen & Borns 1997) and collared lemmings (*Dicrostonyx*) and true lemmings (*Lemmus*) constituted the most common small mammals in central Europe. These species were regularly accompanied by several vole species, including the root vole, as far north as southern Poland (Jánossy 1986; Chaline 1987; Nadachowski 1989; Kordos 1990).

These fossil data show that the root vole expanded south and west in central Europe during cold and humid periods of the last glacial advance. More importantly, the species always remained north of the classical refugia in southern Europe. Thus, it is clear that central European root vole populations constituted sources of northward expansion once the climate ameliorated.

The root vole's postglacial distribution in Europe was wider than today, including the North Sea coast of Germany, east central Sweden and Britain (von Tast 1982; Mitchell-Jones *et al.* 1999; Yalden 1999). Accordingly, the threatened and geographically isolated populations in Hungary, Austria, Slovakia and the Netherlands (Fig. 1) represent glacial and postglacial relicts (von Tast 1982; Chaline 1987). Our phylogeographical data corroborate

this historical model with the fragmented populations in the Netherlands, Slovakia and Hungary all being part of a single mtDNA phylogroup.

The current distribution of the North European group probably reflects late glacial and postglacial expansion from several refugial areas. Fossils from northern Eurasia indicate that mammal species richness was highest in the mountain areas with the Urals, Caucasus and Carpathians forming centres of high densities of small mammal surrounded by areas of lower densities and patchy distributions (Markova *et al*. 1995). Root vole fossils from the last glacial maximum have been registered in Crimea (Markova 1984) and from the south Middle Urals, where the species was common (Markova *et al*. 1995).

The distribution of the Central and North European root vole mtDNA groups corresponds to patterns observed in field voles from north and central Europe (Jaarola & Searle 2002), suggesting that these two species have experienced similar glacial and postglacial history in Europe.

Colonization history of Fennoscandia

The presence of both the North European and the Central European phylogroups in Fennoscandia (Finland, Norway and Sweden) suggests that the root vole recolonized this area from both the northeast and from the south. Similar north–south phylogeographical patterns in Fennoscandia have been described for several other mammals (reviewed in Jaarola *et al*. 1999) and a variety of other taxa such as virus (Hörling *et al*. 1996), birds (Bensch *et al*. 1999), snakes (Carlsson & Tegelström 2002), fish (Koskinen *et al*. 2000; Kontula & Väinölä 2001) and plants (Nyberg Berglund & Westerbergh 2001; Malm 2001), strongly corroborating this recolonization model.

Root voles in western Sweden and southern Norway belong to the Central European mtDNA group and are thus descendants of root voles that colonized Sweden from the south. Today, southern Sweden is separated from continental Europe by the Öresund Strait, but land bridges between Denmark and Sweden existed for a combined period of 2500 years during 11 200–8200 BP (Björck 1995a,b). There are currently no root voles in the Öresund area (see Fig. 1), but remains from Denmark and southernmost Sweden (Liljegren 1975; Aaris-Sørensen 1995, 1998) demonstrate that root voles colonized southern Sweden via Denmark at the very end of the Pleistocene. Our mtDNA data suggest that the Robertsonian fissions that characterize the chromosome polymorphisms found in the geographically isolated populations in western Sweden and southern Norway (Fredga & Bergström 1970; Fredga *et al*. 1980, 1986) have occurred after the colonization of southern Fennoscandia, as the source populations in central Europe do not carry the fissions.

The subspecies *M. o. finnmarchicus* is thought to have survived the end of the last glaciation *in situ* in ice-free areas, nunataks, along the Norwegian coast (Siivonen 1968, 1982 and references therein). However, our mtDNA analyses suggest that the haplotypes from the Norwegian islands Andøya and Ringvassøya (Nor-1,2 and 5; Fig. 2 and Table 1), descend from root voles that immigrated along the north coast of Russia and Norway during the late glacial period. This scenario is possible considering that the area north and east of the White Sea was the first part of northern Fennoscandia to be deglaciated (cf. Svendsen *et al*. 1999; Boulton *et al*. 2001). A similar colonization route has been suggested for the wild reindeer (*Rangifer tarandus*) based on fossil findings (Rankama & Ukkonen 2001). We are currently undertaking a more detailed genetic investigation of root voles from northernmost Fennoscandia to test this hypothesis.

History of the Central Asian group

The Central Asian phylogroup is separated from the North European group by the Ural Mountains. The Urals has been suggested by Hewitt (1996) as a possible suture zone, i.e. an area with a clustering of contact zones (Remington 1968), and several small mammal studies corroborate this hypothesis. A phylogeographical split in the Urals is also found in collared lemmings (Fedorov *et al*. 1999a) and the common vole (Haynes *et al*. 2003). Root voles were common in the Urals during the end of the last glacial maximum (Markova *et al*. 1995), so the location of this split may predate the Holocene (< 10 000 BP).

Although the specimens that represent the Central Asian group originate from localities extending across more than 4600 km, this group of root voles seems to be no more diverse than the other three groups that cover smaller geographical areas (Table 3). Historical restriction of mesic grasslands followed by a rapid late glacial expansion may be responsible for this pattern. Although the major part of Russia was not covered by ice during the last glacial period, large parts were exposed to extremely cold and arid conditions — and especially so during the last glacial maximum (Svendsen *et al*. 1999; Schirrmeister *et al*. 2002).

Colonization of North America

During the last glacial maximum, southern Alaska and northwestern Canada were covered by ice (Andersen & Borns 1997) and there is no fossil evidence to suggest that root voles were present south of the ice sheet (Zakrzewski 1985). If the root vole was already present in North America at this time, we would expect this cold-tolerant species to have persisted in the Beringian refugium in northwest Alaska, as shown for several other high latitude mammals and plant species (Fedorov *et al*. 1999a,b; Tremblay &

Schoen 1999; Barnes *et al*. 2002; Fleming & Cook 2002). In fact, the distribution of the Beringian mtDNA phylogroup in the root vole is striking in its congruence with the traditional boundaries of the Beringian refugium (i.e. eastern Siberia, Alaska and northwestern Canada), suggesting a history of glacial isolation within Beringia. The same phylogeographical pattern in Beringia has been observed in a variety of other species, including the lemming *Lemmus trimucronatus* (Fedorov *et al*. 1999b) and the avian wader *Calidris alpina* (Wenink *et al*. 1996; Wennerberg *et al*. 1999; Wennerberg 2001).

Lack of mtDNA differentiation in the root vole across the Bering Strait corroborates previous findings based on karyotypic (Nadler *et al*. 1976), allozymic (Nadler *et al*. 1978; Lance & Cook 1998) and morphological (Paradiso & Manville 1961) data that suggest that populations in Siberia and Alaska arose recently from a relatively homogeneous ancestral population. Similarities between Siberian and Alaskan root voles have historically been cited as evidence that they are recent colonizers of North America, having crossed the Bering Land Bridge during the last glacial period (termed the Wisconsin in North America) (Rausch 1963; MacPherson 1965). Alternatively, the root voles could have entered Beringia and North America at an earlier time and simply not differentiated across the strait due to interglacial periods intermittently permitting population admixture. To date, the only evidence for a pre-Wisconsin colonization of North America has been from a single fossil assemblage in Canada (Jopling *et al*. 1981; Zakrzewski 1985) that may represent the late Illinoian glacial period, 200 000–130 000 BP. Our time estimate for the separation between the Beringian and Central Asian mtDNA groups suggests that a pre-Wisconsin colonization of North America is plausible (see below).

The molecular clock and age estimates of population splits

The cytochrome *b* gene of the root vole does not seem to be evolving in a clock-like manner, a result in line with recent evidence (Strauss 1999; Gissi *et al*. 2000). Although it is unclear how reliable the likelihood ratio test is (Nei & Kumar 2000), it is evident that there is significant rate heterogeneity in transition/transversion (ti/tv) rates among root vole lineages. The relatively low ratios within the Central Asian and Beringian lineages vs. the high ratios in the two European lineages cannot be explained by a general overestimate of the ti/tv ratio at low sequence divergence or saturation at high sequence divergence (cf. Yang & Yoder 1999) as the nucleotide diversity is similar among all lineages.

We estimated separation times between phylogroups to show that these splits are ancient and probably dating back to several glacial periods; however, the relative time frame may differ. The overall rate of the cytochrome *b* gene in *Microtus* has been estimated to be almost seven times higher (Conroy & Cook 2000a) than the widely used mtDNA standard rate of 2% sequence divergence per million years (Myr; Avise 1998). This *Microtus* clock probably represents an overestimate (see Conroy & Cook 2000a), but data from other rodent taxa (e.g. Smith & Patton 1993; Lessa & Cook 1998; Fedorov & Goropashnaya 1999; Fedorov & Stenseth 2001) suggest that the cytochrome *b* gene of rodents in general may evolve at least three to five times faster than the standard mammal rate (cf. Irwin *et al*. 1991). We therefore used a moderate cytochrome *b* divergence clock of 6–10% per Myr.

To determine minimum population divergence times, we used net divergence estimates (Nei & Li 1979; Table 4) under the assumption that the ancestral effective population sizes were the average of the current population sizes (see Edwards & Beerli 2000). The results suggest that there have been two bouts of splitting of phylogroups. First, the North European and the Central Asian groups, now separated by the Ural Mountains, would have diverged 0.29–0.49 Myr ago (95% CI = 0.19–0.66 Myr ago), i.e. three to five glacial periods ago. Second, the two European groups, and the Central Asian and the Beringian groups, respectively, would have separated 0.20–0.33 Myr ago (CI = 0.13–0.46 Myr ago) and 0.21–0.35 Myr ago (CI = 0.13–0.48 Myr ago). These dates correspond to two to three glacial periods ago.

Conclusions

The root vole exhibits substantial mtDNA phylogeographical structure across its Holarctic range. The four phylogeographical groups identified are ancient, reflecting isolation during several glacial periods. The current phylogeographical structure reflects late glacial and postglacial range expansions and shifts as well as fragmentation of populations. However, there are some indications that elements such as locations of phylogeographical splits may have been geographically static for longer periods than expected. Although the phylogeographical pattern could be interpreted as a result of postglacial expansions from southern refugia in Europe, fossil findings throughout the last glaciation suggest a distinctly different story. Thus, fossil data show that the root vole shifted its range southwards and westwards during the last glacial period but always remained north of the classical European refugia. Root vole remains also indicate that the four phylogroups would have been quite widespread during the coldest and driest periods of the last glaciation, with mountain areas forming centres of density. Similarities between the phylogeographical patterns of lemmings and two other species of voles suggest similarities in these species' responses to large-scale climate change.

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