

Beyond the Mediterranean peninsulas: evidence of central European glacial refugia for a temperate forest mammal species, the bank vole (*Clethrionomys glareolus*)

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Abstract

This study details the phylogeographic pattern of the bank vole, *Clethrionomys glareolus*, a European rodent species strongly associated with forest habitat. We used sequences of 1011 base pairs of the mitochondrial DNA cytochrome *b* gene from 207 bank voles collected in 62 localities spread throughout its distribution area. Our results reveal the presence of three Mediterranean (Spanish, Italian and Balkan) and three continental (western, eastern and 'Ural') phylogroups. The endemic Mediterranean phylogroups did not contribute to the postglacial recolonization of much of the Palaearctic range of species. Instead, the major part of this region was apparently recolonized by bank voles that survived in glacial refugia in central Europe. Moreover, our phylogeographic analyses also reveal differentiated populations of bank voles in the Ural mountains and elsewhere, which carry the mitochondrial DNA of another related vole species, the ruddy vole (*Clethrionomys rutilus*). In conclusion, this study demonstrates a complex phylogeographic history for a forest species in Europe which is sufficiently adaptable that, facing climate change, survives in relict southern and northern habitats. The high level of genetic diversity characterizing vole populations from parts of central Europe also highlights the importance of such regions as a source of intraspecific genetic biodiversity.

Keywords: central Europe, *Clethrionomys glareolus*, glacial refugia, mitochondrial DNA, phylogeography, Quaternary glaciations

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Introduction

The Quaternary has experienced important climatic changes that deeply modified the distribution range of species within the Palaearctic region (Webb & Bartlein 1992). During the glacial events of the Quaternary in Europe, central regions tended to be steppe-tundra, whereas deciduous forests were generally confined to the Mediterranean peninsulas (Zagwijn 1992; Blondel 1995). For temperate forest mammal species such as the bank voles studied here, it is expected that

species shifted their range according to their habitat, so that they would have survived glacial maxima in the Mediterranean peninsulas. Interglacial and postglacial recolonizations of central and northern Europe could therefore have arisen from these Mediterranean glacial refugia (Taberlet *et al.* 1998; Hewitt 1999, 2001; Michaux *et al.* 2003). However, southern glacial refugia have not always been the source of postglacial recolonization of central and northern Europe. Several phylogeographic and palaeontological studies have suggested that some mammal species survived in eastern or northern glacial refugia: the brown bear *Ursus arctos* in the Carpathians (Taberlet & Bouvet 1994; Sommer & Benecke 2004), voles in

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central Europe and the Ural mountains (*Microtus agrestis* and *Microtus oeconomus*; Jaarola & Searle 2002; Brunhoff *et al.* 2003) and the Norwegian lemming *Lemmus lemmus* in periglacial area (Fedorov & Stenseth 2001). However, postglacial recolonization from northern and eastern refugia seems to be associated with animal species adapted to open habitats which were probably less affected than others by the disappearance of forests during the Quaternary Ice Age. Stewart & Lister (2001) have suggested that forest species (bank vole *Clethrionomys glareolus*, wood mouse *Apodemus sylvaticus*, red squirrel *Sciurus vulgaris*) occupied glacial refugia in Belgium and the Norwegian coast on the basis of palaeontological data, but their interpretation of radiocarbon dates is controversial (J.-M. Cordy, personal communication). Until now, such glacial refugia are suspected to have played a role in the phylogeographic structure only of Arctic species such as the Norwegian lemming and some species of Alpine–Arctic flora (Tollefsrud *et al.* 1998; Fedorov & Stenseth 2001).

The aim of this study is to use phylogeographic and palaeontological approaches to examine if a rodent species strongly associated with forest habitat, the bank vole, survived the glacial periods in ‘northern’ or eastern refugia. The bank vole is common, easy to catch and widespread all over the western Palaearctic region except in the Mediterranean biome (Corbet 1978; Fons *et al.* 1980; Bashenina 1981; Spitzenberger 1999). Moreover, previous studies (Tegelström 1987; Tegelström *et al.* 1988; Van Rompaey 1989; Bilton *et al.* 1998) have suggested a complex phylogeographic pattern for this species in Europe. In particular, the Europe-wide study of Bilton *et al.* (1998) proposed that the bank vole may have occupied the northern refugia, although this was based on a small data set (20 specimens, 500 bp mitochondrial DNA sequence). Here, we present a more substantial study in terms of number of specimens and length of sequence to re-examine the question of northern refugia in this species.

Materials and methods

Samples and laboratory procedures

The genetic polymorphism of the mitochondrial cytochrome *b* (*cyt b*) gene was analysed for 153 bank voles trapped in 51 localities. These specimens were obtained from collaborators, museums and the collection of the Zoogeography Research Unit of the University of Liège. Fifty-four additional sequences, mainly from Russia, were downloaded from the GenBank database (Conroy & Cook 1999; Martin *et al.* 2000; Iwasa *et al.* 2002; Wickliffe *et al.* 2002; Dekonenko *et al.* 2003) (Table 1 and Fig. 1).

Total genomic DNA was extracted from ethanol-preserved muscle, liver or spleen as described by Sambrook *et al.* (1989). The *cyt b* gene was amplified using specific

primers designed for the bank vole: LCLE 1 (5′-ATCAT-CAACCACKCATTATT-3′) and MCLE 3 (5′-ATTAT-GCCTGCTATTGGTATG-3′). Amplifications were carried out following the protocol of Michaux *et al.* (2003). Sequencing was performed using the external LCLE 1 primer and an internal LCLE 2 primer (5′-GGACAACCCTAGTA-GAATG-3′) that produced unambiguous results. A single strand was sequenced using a BigDye Terminator kit (Applied Biosystems) on an ABI Automated Sequencer and using an ET-Dye Terminator kit on a MegaBACE 1000 (Amersham Biosciences). The sequences were aligned using the ED editor of the MUST package (Philippe 1993).

Phylogenetic analyses

Phylogenetic reconstructions were performed by a distance method using the neighbour-joining algorithm (NJ) (Saitou & Nei 1987) and using maximum-parsimony criterion (MP) (Fitch 1971) algorithm implemented in PAUP version 4.0b10 (Swofford 2000), as well as the maximum-likelihood criterion (ML) (Felsenstein 1981) implemented in the PHYML program (Guindon & Gascuel 2003). We used MODELTEST version 3.0 (Posada & Crandall 1998) to determine the most suitable model of DNA substitution for the *cyt b* data set studied. MP analysis was conducted with the heuristic search algorithm, tree-bisection–reconnection (TBR) swapping and a maximum number of trees constrained to 1000. Phylogenetic trees were rooted with *cyt b* sequences from ruddy voles (*Clethrionomys rutilus*) and grey-sided voles (*Clethrionomys rufocanus*). The robustness of the trees was assessed by bootstrap resampling (BS) (10 000 random replications for NJ analysis, 1000 for MP and ML analyses; Felsenstein 1985). A Bayesian approach to phylogeny reconstruction (Yang & Rannala 1997; Huelsenbeck *et al.* 2001) was also used, implemented in MRBAYES version 2.01 (Huelsenbeck *et al.* 2001). Metropolis-coupled Markov chain Monte Carlo sampling was performed using four chains run for 1 000 000 iterations and using default model parameters as starting values. Bayesian posterior probabilities (BPP) were picked from the 50% majority rule consensus of trees sampled every 20 generations, after removing trees obtained before the chains reached an apparent plateau (‘burn in’ determined by empirical checking of likelihood values).

Phylogeographic analyses

A network was constructed using the median-joining method (NETWORK version 4.1.0.6 program available at www.fluxus-engineering.com; Bandelt *et al.* 1999).

Demographic histories of different bank vole phylogroups were inferred first by a pairwise mismatch distribution analysis between individuals (Rogers & Harpending 1992) computed under a population growth–decline model

Table 1 Map references, geographical locations, sample abbreviations and GenBank Accession numbers of *Clethrionomys* haplotypes used in this study. Accession numbers with an asterisk were downloaded from the GenBank database (see text for references)

Map. ref.	Geographic origin		Total number of animals	Abbreviations	GenBank Accession numbers (for haplotypes only)
Bank voles (<i>Clethrionomys glareolus</i>)					
1	Britain	Kielder	3	Brit1	AJ639662–AJ867972
2		Derbyshire	2	Brit2	AJ639666
3		Gwent	2	Brit3	AJ639665–AJ867971
4		Cambridgeshire	1	Brit4	AJ867970
5		Essex	4	Brit5	AJ867968–969
6	Spain	Asturias	4	Sp1	AJ867979
7		Navarre	2	Sp2	AJ639671–695
8		Granollers	3	Sp3	AJ639672
9	France	Py Mantet	8	Fr1	AJ639678–691–AJ867980
10		Montpellier	2	Fr2	
11		Bordeilles	2	Fr3	AJ639682–683
12		Saint-Aignan	2	Fr4	
13	Belgium	Virelles	2	Be1	AJ867965–973
14		Liège	4	Be2	AJ639661
15	Switzerland	Evolene	3	Swi	AJ639674–675
16	Italy	Pietraporzio	1	Ita1	AJ639681
17		Trentino alto Adige	1	Ita2	AJ639692
18		Chiusi della Verna	6	Ita3	AJ639663–664
19		Lucretili mountains	1	Ita4	AJ639667
20	Germany	Konstanz	1	Germ1	AF159401*
21		Gera	3	Germ2	AJ867978
22		Parchim	6	Germ3	AJ867974–975–976–977
23	Austria	Tirol	12	Aus1	AJ639685–687–688–689–690–696
24		Osttirol	2	Aus2	AJ639676–694
25		Karnten	1	Aus3	AJ639693
26		Salzburg	2	Aus4	
27	Slovenia	Livek	3	Slo	AJ867953
28	Hungary	Zala	2	Hung1	AJ867954–955
29		Nagycsany	3	Hung2	AJ639707
30	Montenegro	Biogradsko Jezero	3	Mont	AJ639706
31	Macedonia	Pelister mountains	3	Mac	AJ639660–668–670
32	Bulgaria	Rhodopi mountains	2	Bulg1	AJ639702
33		Rila mountains	2	Bulg2	AJ639699–700
34		Vitosha	1	Bulg3	AJ639703
35		Balkan mountains	2	Bulg4	AJ639701
36		Elena	1	Bulg5	AJ639705
37		Solnik	2	Bulg6	AJ639704
38	Romania	Baile Herculane	3	Ro1	AJ867952–958
39		Timisoara	1	Ro2	AJ867963
40		Moneasa	7	Ro3	AJ867957–962–981
41		Targu Mures	5	Ro4	AJ867950–956–960
42		Zarnesti	4	Ro5	
43		Bacau	2	Ro6	
44		Maramures	4	Ro7	AJ867951–961
45	Slovakia	Kosice	8	Sk	AJ867948–949–959–964
46	Ukraine	Chernobyl	2	Ukr	AF318584–585*
47	Poland	Pulawy	1	Po1	AJ639669
48		Bialowieza	4	Po2	AJ639698–708
49	Lituania	Alytus	4	Lit1	AJ639684
50		Zemaitijos national park	3	Lit2	AJ639686–AJ867966–967
51	Finland	Pieksämäki	1	Fin1	AY185798*
52		Sotkamo	3	Fin2	AF119272*, AF429812–813*
53		Puhos	1	Fin3	AY185796*
54		Kittila	2	Fin4	
55	Sweden	Batskarsnas	1	Swe	AY185800*
56	Russia	Nizhniy Novgorod	14	Ru1	AF429798–799–800–801–802–803–804–805–806–807–808–809*, AF367083–084*
57		Samara	11	Ru2	AY062900–901–902–903–904–905–906–907*, AF367080–081–082*
58		Bashkortostan	4	Ru3	AF429794–795–796–797*

Table 1 Continued

Map. ref.	Geographic origin	Total number of animals	Abbreviations	GenBank Accession numbers (for haplotypes only)
59	Ural mountains	1	Ru4	AJ639673
60	Omsk	12	Ru5	AF429781-782-783-784-785-786-787-788-789-790*, AF367074-079*
61	Turkey	4	Ru6	AF429810-811*, AF367075-076*
62	Turkey	1	Turk	AJ639697
Ruddy voles <i>Clethrionomys rutilus</i> (Outgroup 1)				
	Russia	1	Rut	AB072224*
	Russia	1	Rut	AB072223*
Grey-sided vole <i>Clethrionomys rufocanus</i> (Outgroup 2)				
	Finland	1	Ruf	AF429815*
	Russia	1	Ruf	AF429816*

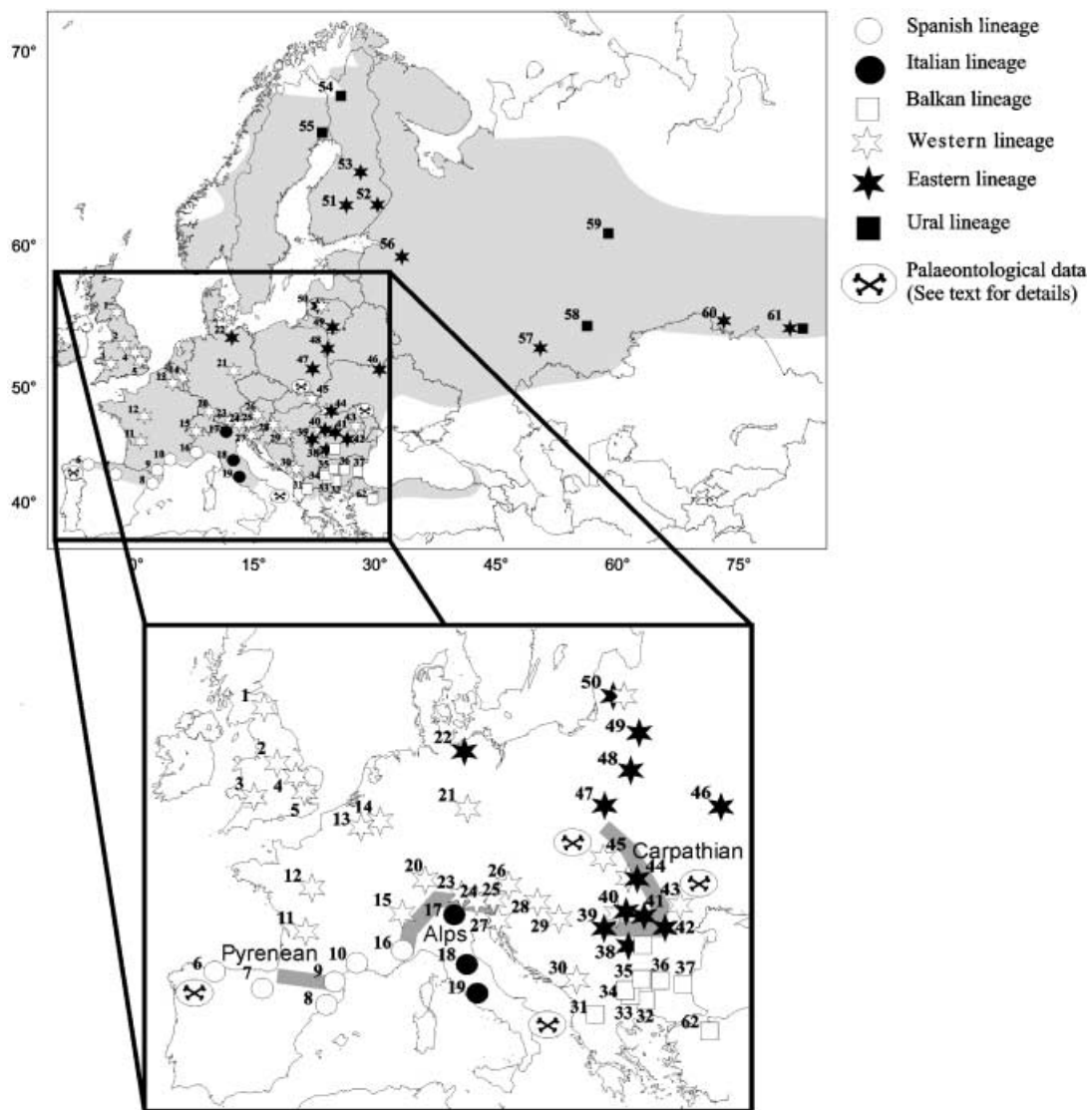


Fig. 1 Geographic distribution of the bank vole (*Clethrionomys glareolus*) samples and the genetic phylogroups revealed by cytochrome *b* gene analyses and palaeontological data (Kowalski 2001; Nadachowski *et al.* 2003) in this study. The shaded zone corresponds to the distribution range of the bank vole (Corbet 1978; Fons *et al.* 1980; Bashenina 1981; Spitzenberger 1999).

in DNASP version 4.0 (θ initial = 2, θ final = 200, $\tau = 5$; Rozas *et al.* 2003). Multimodal distributions were consistent with demographic stability, while sudden expansion would generate a unimodal pattern (Slatkin & Hudson 1991). Second, we used a coalescent approach to validate the patterns of growing or stable populations revealed by the mismatch distributions (Emerson *et al.* 2001). ML was used to test if the data fit a model of exponentially growing or stable populations, estimating the parameter $\theta = N_e\mu$ (where N_e is the effective population size and μ the mutation rate) and g (the growth rate) (Kuhner *et al.* 1995; Kuhner 1998). These analyses were performed using the programs, COALESCE and FLUCTUATE from the LAMARC package (<http://evolution.genetics.washington.edu/lamarc.html>). The two demographic models were compared using a likelihood-ratio test which follows a χ^2 distribution with one degree of freedom, the null hypothesis being that the populations were stable. The programs were run several times with different numbers of short and long Markov chains to check the consistency of the estimated parameters.

The genetic structure of populations was examined using an analysis of molecular variance (AMOVA) performed in the ARLEQUIN version 2.0 program (Schneider *et al.* 2000). AMOVA was conducted at three hierarchical levels of populations subdivision: among geographically delimited groups (Iberian; Italian; Balkan: Montenegro, Macedonia, Bulgaria, Turkey; western Europe: Britain, France, Belgium, Switzerland, Germany, Austria, Slovenia, Hungary, Slovakia, Romania; eastern Europe and Russia: Lithuania, Ukraine, Poland, Fennoscandia, Russia; Ural; see Table 1 and Fig. 1), among populations within groups and within populations. The significance of these parameters were estimated by 10 000 permutations of the distance matrix. Haplotype (h) and nucleotide (π in percentage) diversities (Nei 1987) and their standard deviations (\pm SD; Tajima 1993) were estimated using the DNASP version 4.0 program. The difference between the two observed values of nucleotide diversity was compared statistically using a randomization test. The individuals were pooled and then randomly assigned to the two populations. The difference between these random values was stored and this procedure was repeated 1000 times. The observed differences were compared with this random distribution: the P value (p) is given by the proportion of random values greater than the observed one (using absolute values because the test is two-tailed).

An approximate timing of divergence between the bank vole phylogroups was calculated on the basis of the percentage of genetic divergence. First, to assess that the bank vole *cyt b* sequences evolve in a clock-like way, relative-rate tests were performed with RRTREE version 1.0 (Robinson *et al.* 1998), which improves the test of Wu & Li (1985) by taking taxonomic sampling and phylogenetic relationships into account. The relative rate tests were

performed on the proportions of synonymous (K_s) and nonsynonymous (K_a) substitutions of the *Clethrionomys* phylogroups and samples. Second, a matrix of distances between *Clethrionomys* sequences generated in PAUP version 4.0b10 was used to calculate mean genetic divergences (P_{mean} in percentage K_3P) within and between phylogroups. K_3P distances were corrected for ancestral mtDNA polymorphism using the formula (Edwards 1997):

$$P_{\text{net}} = P_{\text{AB}} - 0.5 (P_{\text{A}} + P_{\text{B}})$$

where P_{net} is the net genetic distance between phylogroups, P_{AB} the mean genetic distance in pairwise comparisons of individuals from A vs. B and P_{A} and P_{B} are mean genetic distances among individuals within phylogroups. Finally, the separation time between *C. rufocanus* and the other *Clethrionomys* estimated as 4.25 million years Ma (Matson & Baker 2001) was used as a calibration point.

To compare the genetic findings with subfossil records, spatial and temporal information on the distribution of the bank vole during the Pleistocene was obtained from the database of the project, 'Holocene History of the European Vertebrate Fauna' (Benecke 1999).

Results

Pattern of sequence variation

A total of 104 haplotypes (including 80 previously unpublished) was identified among the 207 bank vole specimens. Of the 1011 base pairs sequenced, 268 sites were variable and 197 parsimony informative. The average transitions/transversion ratio (5.5) and base composition (T 28.1%, C 29.2%, A 29.2%, G 13.4%) are in agreement with other data on the cytochrome *b* gene of small mammals (Conroy & Cook 2000; Michaux *et al.* 2003; Cook *et al.* 2004).

Phylogenetic and phylogeographic analyses

NJ and ML analyses were performed using the K_3P (K81) model (Kimura 1981) suggested for the data by the Akaike information criterion (AIC) estimates and the hierarchical likelihood ratio tests (hLRT) in MODELTEST version 3.0 (Posada & Crandall 1998), with a gamma distribution shape parameter of $\alpha = 0.925$ (Yang 1996) and a proportion of invariable sites of $I = 0.507$. Because the K_3P model was not available in PHYML, the TN93 model (close to the K_3P model in the three substitution types; Tamura & Nei 1993) was used for ML analysis. For comparison, the NJ and ML analyses were also conducted with few simpler substitution models (JC; Jukes & Cantor 1969; K_2P ; Kimura 1980) as MODELTEST does not always guarantee the best model and as more parameter-rich models do not always produce better trees (Brunhoff *et al.* 2003). The NJ reconstruction of

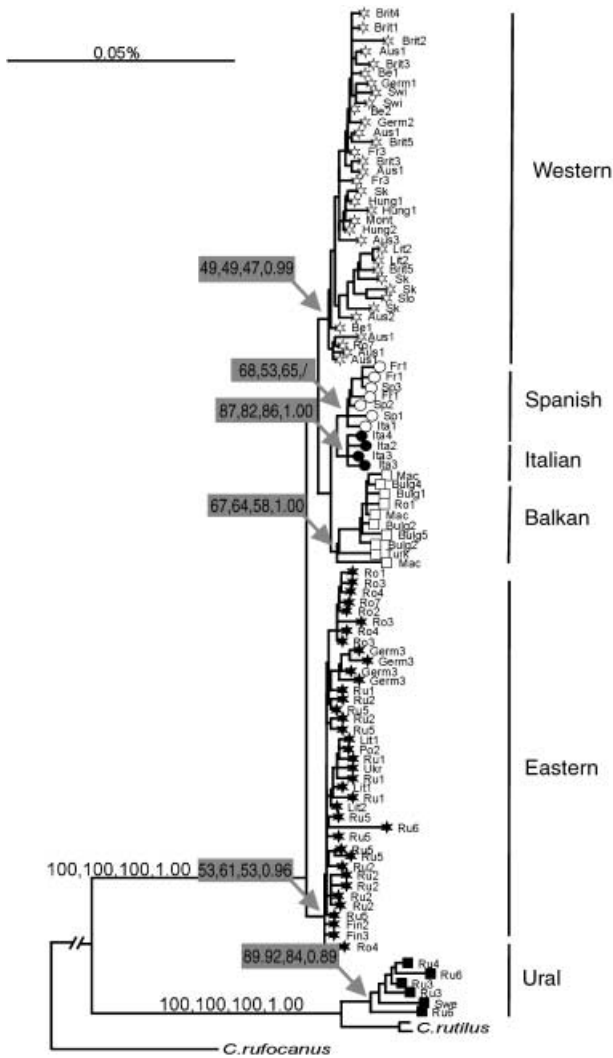


Fig. 2 Neighbour-joining tree of the 108 *Clethrionomys* mtDNA haplotypes. Bootstrap values (%) obtained by the NJ, MP and ML analyses as well as Bayesian probabilities are shown in grey shading for the major phylogroups. See Table 1 for the haplotype designations.

phylogenetic relationships between haplotypes is shown in Fig. 2, the MP (one most parsimonious tree, number of parsimony steps = 441, consistency index = 0.663, retention index = 0.869), ML and Bayesian trees showing identical

topologies. All other substitution models tested in the distance and ML analyses (JC, K_2P) provided the same tree topologies and branch lengths. The bank voles split into two major groups: a Ural group including animals from northern Sweden, the Ural Mountains and the Russian region of Novosibirsk (BS: $\approx 88\%$, BPP: 0.89) and a large group including the other bank voles. Moreover, the Ural group was related to one of the outgroup species (the ruddy vole, BS: 100%, BPP: 1.00). The other bank vole haplotypes are separated into three peninsular and two continental phylogroups: the Spanish group made up of animals from Spain, southern France and one individual from western Italy (BS: $\approx 62\%$, BPP: < 0.3), the Italian group with animals exclusively from Italy (BS: $\approx 85\%$, BPP: 1.00), the Balkan group including animals from the Balkans, Turkey and one individual from southern Romania (BS: $\approx 63\%$, BPP: 1.00), the western group covering Britain, Belgium, central France, a part of Germany and Lithuania, Austria, Switzerland, Slovakia, Hungary, parts of Romania, and Montenegro (BS: $\approx 48\%$, BPP: 0.99), and finally, the eastern group, which ranges from Poland to Novosibirsk (BS: $\approx 56\%$, BPP: 0.96) (Figs 1 and 2). The median-joining tree also generated these five European bank vole phylogroups, although with less differentiation (Fig. 3). The eastern group shows star-like topology, suggesting exponential growth of populations from a small numbers of individuals. The Ural as well as ruddy vole haplotypes formed a group distinct from the main European phylogroups (distance = 47 mutations).

The mismatch distribution (Fig. 4) and the coalescence tests (Table 2) also suggested varied demographic histories for the bank vole phylogroups. The eastern and western groups both presented a bell-shaped distribution suggesting a sudden expansion of these populations. The coalescence analyses confirmed the mismatch distributions as they rejected the null hypothesis of a stable population for these two continental groups (positive g values, $P < 0.001$ and $P = 0.004$ for the western and eastern phylogroups, respectively). On the other hand, the populations from the Balkan and Ural phylogroups showed a more heterogeneous mismatch distribution suggesting long-term stability. No signature of population stability was clearly obtained

Bank vole phylogroups	Sample size	Log likelihood			
		Stable population model	Exponential change model	χ^2	P value
Spanish	20	-0.115	1.802	3.83	0.050
Italian	8	-0.001	0.053	0.11	0.741
Balkan	15	0.022	0.057	0.07	0.793
Western	72	0.053	16.700	29.29	< 0.001
Eastern	81	0.112	4.370	8.52	0.004
Ural	10	0.048	0.122	0.15	0.700

Table 2 Results of the coalescence analysis on the six phylogroups of bank vole (*Clethrionomys glareolus*)

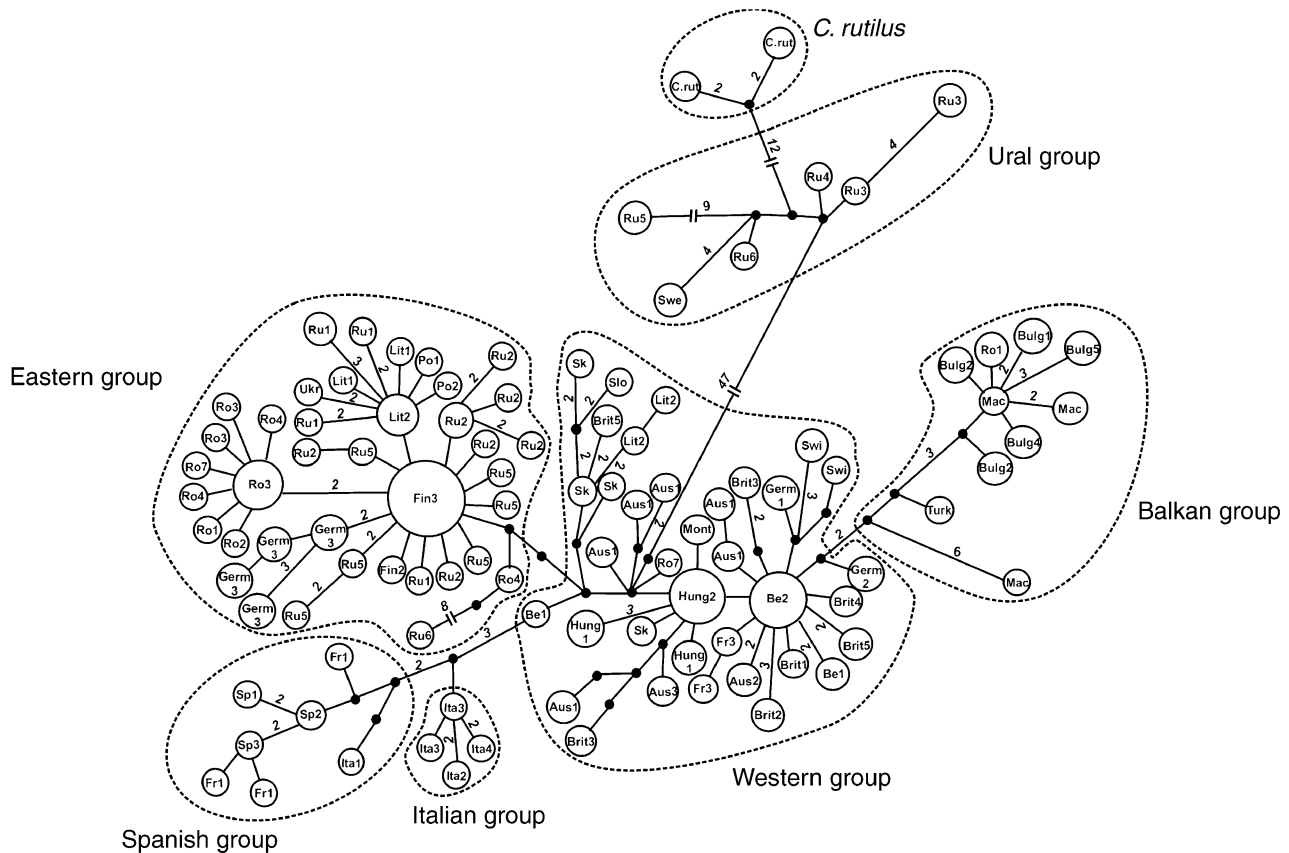


Fig. 3 Median-joining tree of the bank vole (*Clethrionomys glareolus*) and ruddy vole (*Clethrionomys rutilus*) mtDNA haplotypes. Numbers of mutations (greater than one) between haplotypes are indicated on branches. See Table 1 for the haplotype designations.

Table 3 Analyses of molecular variance based on mtDNA data from geographical groups of bank vole (*Clethrionomys glareolus*)

Source of variation	Variance components	Percentage of variation	P value	φ statistics
Among groups	1.659	56.15	< 0.001	φ _{CT} = 0.561
Among populations/groups	0.271	9.19	< 0.001	φ _{SC} = 0.209
Among populations	1.024	34.67	< 0.001	φ _{ST} = 0.653

for the Spanish and Italian phylogroups. However, the coalescence test suggests that the Spanish group has undergone expansion ($P = 0.05$).

Population structure and genetic diversities

Population structure analyses reinforced the genetic pattern found with the different phylogenetic reconstructions. The AMOVA showed that the majority of the total mtDNA variation (56.15%) was distributed among geographical groups whereas a low percentage (9.19%) was observed among populations within the groups (Table 3). Moreover, the φ statistic suggests a low level of gene flow between populations (φ_{ST} = 0.653, $P < 0.001$). In contrast, the genetic

divergence among phylogroups was low (max 1.15% K_3P genetic distance, excepted for the Ural group which shows ≈ 10% K_3P distance from other phylogroups) (Table 4).

To assess whether population genetic diversity was high within the potential glacial refugia, the western and eastern phylogroups were divided into geographically delimited subgroups represented by a minimum of eight individuals (see Table 5). Nucleotide and haplotype diversities were calculated for each of these subgroups as well as for the Mediterranean phylogroups. Spanish and Italian phylogroups display low nucleotide and haplotype diversity values ($P < 0.05$; Table 5). On the other hand, the central European subgroup of the western phylogroup displays significantly higher level of nucleotide diversity ($\pi =$

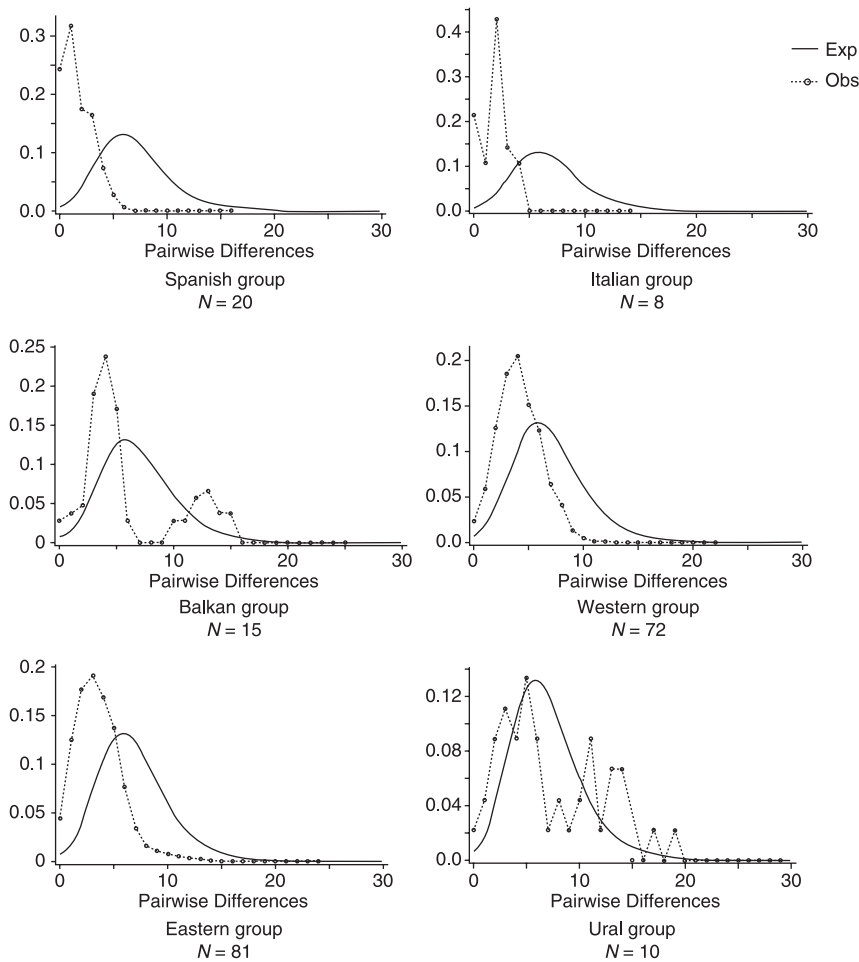


Fig. 4 Mismatch distribution analysis for the five major phylogroups of the bank vole (*Clethrionomys glareolus*) and for the Ural phylogroup linked to the ruddy vole (*Clethrionomys rutilus*).

Table 4 Genetic divergences within and between the main phylogroups of bank voles (*Clethrionomys glareolus*) and between bank vole, ruddy vole (*Clethrionomys rutilus*) and grey-sided vole (*Clethrionomys rufocanus*) (% K_3P). Mean genetic distances (P_{mean}) are given below the diagonal, corrected genetic distances (P_{net}) above the diagonal and intralineage genetic distances in bold

	Spanish	Italian	Balkan	Western	Eastern	Ural	<i>C. rutilus</i>	<i>C. rufocanus</i>
Spanish	0.21	0.62	0.93	1.15	1.12	10.36	10.49	14.27
Italian	0.81	0.18	0.96	1.05	0.94	10.62	10.69	14.78
Balkan	1.34	1.36	0.62	0.95	1.06	11.39	11.45	15.52
Western	1.66	1.45	1.66	0.81	0.66	10.62	10.71	15.49
Eastern	1.47	1.28	1.62	1.31	0.50	10.56	10.75	14.42
Ural	10.83	11.07	12.06	11.39	11.17	0.73	1.95	15.93
<i>C. rutilus</i>	10.79	10.98	11.96	11.31	11.20	2.51	0.40	16.69
<i>C. rufocanus</i>	15.72	16.22	17.18	17.24	16.02	17.64	18.23	2.70

0.74 ± 0.05%) than the other continental subgroups ($P < 0.05$).

Divergence time

The relative rate tests indicated that rates of evolution (both K_s and K_a) for cytochrome *b* were not significantly heterogeneous between the different *Clethrionomys* phylogroups

and samples. Correcting for ancestral mtDNA polymorphism, the mean K_3P distance between *C. rufocanus* and all the other *Clethrionomys* is 15.30 ± 0.87% K_3P (95% CI: 14.66–15.94% K_3P) (Table 4). Assuming a separation time between the two species of 4.25 Ma, the molecular clock rate was estimated at 3.6% K_3P distance per My. This estimate is similar to those observed in rodents of the genus *Apodemus* (*Apodemus sylvaticus* and *Apodemus flavicollis*;

Table 5 Genetic variability observed within the main genetic phylogroups of bank vole (*Clethrionomys glareolus*)

Phylogroups and their subgroups	Sample size	Number of haplotypes	Nucleotide diversity π (\pm SD percentage)	Haplotype diversity (h, \pm SD)
Spanish	20	8	0.21 \pm 0.05	0.758 \pm 0.08
Italian	8	4	0.18 \pm 0.04	0.786 \pm 0.11
Balkan	15	12	0.6 \pm 0.14	0.971 \pm 0.03
Western phylogroup	72	45	0.50 \pm 0.04	0.977 \pm 0.01
Western subgroup 1 (central Europe)	38	25	0.74 \pm 0.05	0.977 \pm 0.01
Western subgroup 2 (France, Belgium, Britain, Germany, Lithuania)	27	19	0.42 \pm 0.07	0.946 \pm 0.03
Eastern	81	44	0.45 \pm 0.04	0.956 \pm 0.02
Eastern subgroup 1 (Germany, Poland, Lithuania, Ukraine)	18	10	0.5 \pm 0.07	0.915 \pm 0.04
Eastern subgroup 2 (Romania)	20	13	0.26 \pm 0.05	0.911 \pm 0.05
Eastern subgroup 3 (Novgorod, Samara)	25	16	0.37 \pm 0.04	0.947 \pm 0.03
Eastern subgroup 4 (Omsk, Novosibirsk)	14	10	0.35 \pm 0.11	0.945 \pm 0.05
Ural	10	9	0.71 \pm 0.02	0.978 \pm 0.05

Michaux *et al.* 2003, 2004). Therefore, the separation time between the Ural phylogroup and the others is estimated to be 2.98 Ma (95% CI: 2.88–3.07 Ma) and that between the European phylogroups of bank vole to 0.27 Ma (95% CI: 0.25–0.30 Ma).

Discussion

A phylogeographic history associated with Pleistocene glaciations

One of the greatest interests of phylogeography is associating a genetic pattern with major environmental changes. In this way, contraction and expansion of species' ranges during Pleistocene glacial events has been shown to explain the intraspecific genetic pattern of many European species (Hewitt 1996, 1999). Our molecular clock analyses estimate that the major European phylogroups of bank voles differentiated during the late Pleistocene (0.30–0.25 Mya) and thus preceded the last glacial cycle (Williams *et al.* 1998). The fragmentation of forests during glaciations would have favoured differentiation in bank voles. As palaeontological data showed that bank voles were present in Europe since at least the middle Pleistocene (1.2 Ma) (Bauchau & Chaline 1987), the low level of genetic divergence among the main European phylogroups could be explained by extinction of ancestral lineages during one of the main middle or late Quaternary glaciations. Alternatively, we cannot exclude the possibility that ancestral bank vole populations have experienced an mtDNA selective sweep leading to the invasion of a positively selected mtDNA group (Maynard-Smith & Haigh 1974; Avise 2000). It might appear that selective effects have played a role in the history of bank vole lineages from the evidence of ruddy vole mtDNA capture by the Ural phylogroup. However,

such capture could also result from differences in the size of the two vole populations (Rhymer & Simberloff 1996). Distinguishing such historical extinction is beyond the scope of the present study. Whatever the process that caused the loss of ancient mtDNA polymorphism, this extinction must have been followed by recent intraspecific genetic differentiation that generated the shallow topology of our phylogenetic reconstructions (Fig. 2).

Endemism in the classical southern glacial refugia

The Mediterranean peninsulas (Iberia, Italy and the Balkans) are classically recognized as major glacial refugia in Europe for temperate species during the Ice Age (Taberlet *et al.* 1998; Hewitt 1999). Our phylogeographic analyses reveal mtDNA phylogroups of bank vole in these three regions (Figs 1 and 2). As fossil remains of bank voles have been reported from southern Europe (Spain and Italy) during the late glacial maximum (Fig. 1; Kowalski 2001), it seems likely that the Mediterranean peninsulas played a role as glacial refugia for this species.

One generally expects that populations of temperate species surviving in Mediterranean refugia during the Quaternary display high genetic variability (Hewitt 1999; Michaux *et al.* 2003). This is not observed for the Spanish and Italian phylogroups in comparison with that from the Balkans (Table 5). The low nucleotide and haplotype diversities characterizing the first two phylogroups could be associated with population fragmentation followed by severe population bottlenecks during the glaciations. Indeed, the topology of the phylogenetic reconstructions suggests that Spanish and Italian phylogroups appeared after a fragmentation of a larger Mediterranean population during the late Pleistocene. After that, gene flow between Spanish and Italian populations would have been reduced

by the presence of geographical barriers (the Pyrenees and the Alps), leading to an allopatric differentiation during the last glacial periods. The crash in Spanish and Italian bank vole populations could be also related to arid and cold climatic events during the late Quaternary in these regions (Petit *et al.* 2003), as well as to recent anthropologic disturbances (evolution from forest to agricultural landscapes, increasing desertification, etc.) (Groves & Di Castri 1991; Gitay *et al.* 2002). In contrast, stable and more diversified bank vole populations persisted in the Balkans, thanks to the presence of a varied topography and humid conditions that created long-term suitable habitats for many temperate species (Tzedakis *et al.* 2002). The bank vole sampled from western Turkey was genetically very similar to those from the Balkans suggesting recent contact between these populations during the last glaciation when the Marmara Sea was dry land (Aksu *et al.* 1999).

Beyond the Mediterranean peninsulas: central European glacial refugia

The high endemism of the Mediterranean bank vole phylogroups strongly suggests that they did not contribute to the postglacial recolonization of central and northern Europe, in agreement with the conclusions of Bilton *et al.* (1998). The major part of this region was rather recolonized by bank vole phylogroups surviving the last glaciations in other glacial refugia. Indeed, the two other phylogroups (Western and Eastern) were subjected to a recent population expansion (Fig. 3, Table 2) and presently cover most of the bank vole distribution range (Fig. 1). As predicted by the expansion/contraction model (Nichols & Hewitt 1994; Ibrahim *et al.* 1996), the analysis of nucleotide diversity (Table 5) suggests that the central European regions (Austria, Hungary, Slovakia and Romania) acted as glacial refugia for the western bank vole group. Indeed, these populations are characterized by a genetic diversity that is significantly higher ($P < 0.05$) than the other western and eastern subgroups. However, as a large ice cap covered Austria during the Last Glacial Maximum (LGM), glacial refugia were most likely located in the river systems present near the Alps or in the Carpathian mountains and the Hungarian plain. Indeed, (i) bone remains of bank vole dated to the coldest epoch of the LGM (21 000–18 000 years BP) were found in the northern Polish Carpathians and in northern Moldova (Fig. 1) (Nadachowski *et al.* 2003); (ii) during the LGM, the southern slope of the Carpathians but also Hungary were covered with patches of mixed coniferous and deciduous forests instead of a uniform steppe-tundra landscape (Berger 1992; Willis *et al.* 2000; K. Neumann, personal communication). The western phylogroup also spreads into the western Balkan region (Montenegro), but this population seems to display low genetic diversity (one haplotype is shared by three

individuals). More samples from this region as well as the Carpathians and the Hungarian plain are essential to confirm the hypothesis of a central European glacial refugium for the western phylogroup.

Genetic diversity values in the eastern phylogroup do not provide evidence for a particular region as a glacial refugium (Table 5). Nevertheless, a recent genetic study on the Puumala viruses of Russian bank voles (Dekonenko *et al.* 2003) hypothesized a European glacial refugium in areas thought to have been forested during the LGM, in the regions of eastern Romania, western Azov Sea and the Crimea (Zagwijn 1992; Blondel 1995; Jaarola & Searle 2002). However, it seems unlikely that the eastern phylogroup of bank vole survived glacial cycles in central Europe unless it experienced a drastic reduction in the effective population size, as suggested by its low level of nucleotide diversity value ($\pi = 0.26 \pm 0.05\%$) within this region. Therefore, glacial refugia for this phylogroup should be sought in more eastern regions.

Up to now, central European glacial refugia were proposed for mammals adapted to open habitat such as the root vole (Brunhoff *et al.* 2003) and the field vole (Jaarola & Searle 2002) (see Introduction). The present genetic study, as well as fossil remains, indicates that a temperate forest species such as the bank vole was also able to survive in central European regions during glacial periods. This could be explained by its resistance to cold environments, a fact supported by past and present ecological observations: (i) the succession of small mammal fossils in Belgium shows that the bank vole was the most abundant forest rodent during the last cold stadials of the late glacial Dryas I, II, III (Cordy 1991); (ii) the current range distribution of the bank vole includes coniferous forests and cold environments (e.g. Fennoscandia; Reichhoff 1984), where populations have developed survival strategies such as tracking rocky microhabitats during winter (Karlsson 1987). Compared to other forest rodents (*Apodemus sylvaticus*, *Apodemus flavicollis*; Michaux *et al.* 2003, 2004), the bank voles would not have followed the dwindling forest cover during the glacial advances. Therefore, bank vole populations would have survived the Quaternary glaciations in cryptic 'northern' forests. This led to the present complex phylogeographic pattern including multiple glacial refugia situated in central Europe, in the Mediterranean peninsulas and maybe in more eastern regions. Moreover, the presence of the bank vole in more northern glacial refugia would explain the fact that this species was one of the earliest mammals to colonize northern regions at the end of the last glaciation (Tegelström 1987; Jaarola *et al.* 1999).

The Ural phylogroup

Our study clearly demonstrates the existence of a third continental bank vole mtDNA lineage that ranges from

northern Sweden to central Siberia, centred on the Ural mountains. This phylogroup is clearly related to haplotypes of ruddy vole and is characterized by very different demographic history, genetic diversity and genetic divergence as compared to the other bank vole phylogroups (Figs 2, 3 and 4; Tables 3, 4 and 5). It also appears that the separation time between the Ural phylogroup and the other European phylogroups dates back to the mid-Pliocene (2.98 Mya). Tegelström (1987) explained the existence of this particular phylogroup as the introgression of ruddy vole mtDNA into bank vole, about 30 000–60 000 years BP, during their post-glacial recolonization of Scandinavia. This phylogeographic group of Ural bank voles should be explored further by a wider geographic sampling with complementary analyses using nuclear markers and a comparison with the phylogeographic patterns of the ruddy vole. Moreover, a comparative phylogeographic study between the two vole species and the Puumala hantavirus, currently underway, will probably give useful information concerning the evolutionary history of these different vole phylogroups.

Conclusion

Phylogeographic advances tend towards an increasing complexity and a multiplication of models explaining the impact of climatic changes on the genetic diversity of species. This study suggests the existence of multiple continental refugia for a forest rodent and the adaptability of a species that, facing climate changes, survives by tracking *in situ* relict habitats. This does not exclude the importance of Mediterranean regions as hotspots of genetic biodiversity and, in the case of the bank vole, as a source of endemic lineages. However, attention should be paid in the future to more northern and eastern regions with mountain ranges or valley systems as potential refugia for European forest species. As for the Mediterranean regions, the conservation of their high levels of genetic diversity should become a priority for biodiversity management programs.

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