



## Evolutionary history of the bank vole *Myodes glareolus*: a morphometric perspective

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The bank vole experienced a complex history during the Quaternary. Repeated isolation in glacial refugia led to the differentiation of several lineages in less than 300 000 years. We investigated if such a recent differentiation led to a significant divergence of phenotypic characters between European lineages, which might provide insight into processes of intraspecific differentiation. The size and shape of the first and third upper molars, and first lower molar, of bank voles genetically attributed to different lineages were quantified using an outline analysis of their occlusal surface. The three teeth present similar trends of decreasing size towards high latitudes. This trend, the inverse of Bergmann's rule, is interpreted as the result of a balance between metabolic efficiency and food availability, favouring small body size in cold regions. Molar shape appeared to differ between lineages despite genetic evidence of suture zones. A mosaic pattern of evolution between the different teeth was evidenced. The analysis of such phenotypic features appears as a valuable complement to genetic analyses, providing a complementary insight into evolutionary processes, such as selective pressures, that have driven the differentiation of the lineages. It may further allow the integration of the paleontological dimension of the bank vole phylogeographic history. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2010, **100**, 681–694.

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### INTRODUCTION

Most of the species from the Northern Palaearctic and Nearctic regions have a complex genetic structure shaped by repeated isolations of populations during the Quaternary (e.g. Hadly *et al.*, 1998; Barnes *et al.*, 2002). The accumulation of genetic divergence through time between populations led to the differentiation of lineages within species (Hewitt, 2000) if the isolation was prolonged, and/or if the contraction

and expansion of populations were repeated in the same regions through successive climatic stages (Carstens & Knowles, 2007). Evidence of lineage differentiation during the Quaternary period was found in a wide range of species from large mammals like the brown bear (Sommer & Benecke, 2005) to small mammals like the field vole (Jaarola & Searle, 2004), mostly using molecular analyses based on the variations in mitochondrial DNA (mtDNA) (e.g. Avise, 2000). Expectations regarding the phenotypic differentiation among these phylogeographic lineages are balanced. Significant phenotypic differences between genetic lineages were found in a wide range of taxa

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from mammals to insects (Good *et al.*, 2003; Garnier *et al.*, 2005). In contrast, many species present a morphological homogeneity contrasting with a pronounced intraspecific genetic divergence (e.g. Austin *et al.*, 2002; Jockusch & Wake, 2002). Such discrepant patterns of genetic and phenotypic differentiation might result from differences in selection regimes. Genetic markers are often considered as neutral regarding the differentiation of phylogeographic lineages. In contrast, adaptation to similar environments can lead to phenotypic convergence or promote stabilizing selection despite genetic divergence (Rychlik, Ramalhinho & Polly, 2006), and inversely, competition for resources or habitat shift may lead to a significant phenotypic differentiation despite reduced genetic differences (Caumul & Polly, 2005; Renaud, Chevret & Michaux, 2007).

The present paper investigates if phenotypic characters combined with genetic analyses might allow us to decipher the evolutionary history of the bank vole *Myodes glareolus* (Schreber, 1780), formerly known as *Clethrionomys glareolus* (Wilson & Reeder, 2005). This arvicoline rodent is associated with forest habitats and suffered repeated isolation and expansion phases during the Quaternary. This shaped a complex intraspecific genetic structure (Kotlík *et al.*, 2006; Deffontaine *et al.*, 2005, 2009). The first question to be addressed is whether or not phenotypic characters have diverged according to this genetic structure. If such morphological differentiation is evidenced, then questions about the processes of phenotypic differentiation may be addressed: did the morphological divergence between lineages occur as a result of the accumulation of genetic differences, or did selective processes contribute to the observed pattern of morphological differentiation?

To answer these questions the size and shape of the first upper molar (UM1), the third upper molar (UM3), and the first lower molar (LM1) were selected as relevant characters to be considered. These teeth were chosen because UM1 and LM1 are the most widely used teeth in paleontological and biogeographic studies (Kitahara, 1995; Chaline *et al.*, 1999; Luo *et al.*, 2004; Martin, Crockett & Marcolini, 2006; Tougaard *et al.*, 2008). We added UM3 because a few studies have used it for systematic purposes (Kaneko, 1992) and evolutionary studies (Barnosky, 1993), and because this tooth displays a large intraspecific variation (Bauchau & Chaline, 1987; Guérécheau *et al.*, 2010).

The patterns of morphological differentiation in the bank vole molars were investigated based on specimens from Europe and Russia, covering most of the documented lineages. The shape of the teeth was quantified by Fourier analyses of their occlusal surface outline. The emerging pattern of morphologi-

cal differentiation was compared with the phylogeographic structure based on previous mtDNA analyses (Deffontaine *et al.*, 2005).

## MATERIAL AND METHODS

### MATERIAL

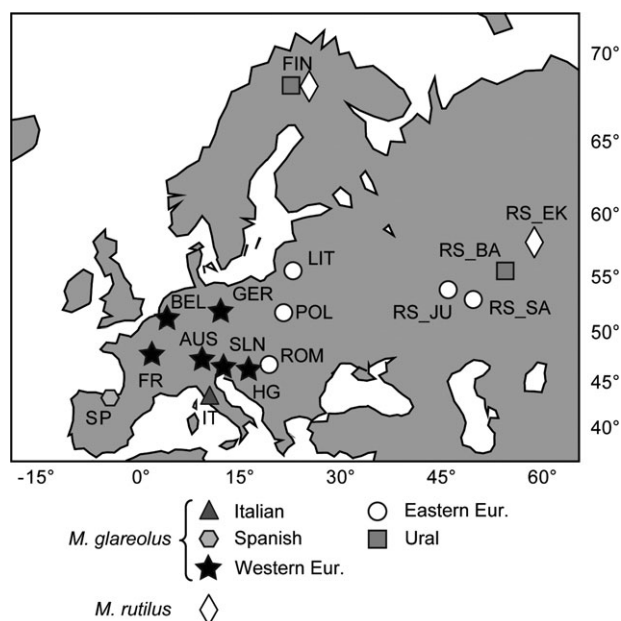
#### *Molecular analyses*

Among the published sequences in GenBank, we selected specimens matching our own sampling to provide a phylogenetic reconstruction to be compared with the morphological data set. A total of 75 mitochondrial cytochrome *b* (*cyt b*) haplotype sequences previously published were downloaded from the GenBank database, and were used for the genetic analyses (accession numbers: AJ867979, AF367074, AF367079, AF367080, AF367081, AF367082, AF367083, AF367084, AF429781, AF429782, AF429783, AF429784, AF429785, AF429786, AF429787, AF429788, AF429789, AF429790, AJ639661, AJ639708, AY062900, AY062901, AY062902, AY062903, AY062904, AY062905, AY062906, AY062907, AY185786, AY185800, VYD021, VYD023, VYD024, VYD029, VYD033, VYD034, VYD041, VYD042, VYD043, VYD051, VYD052, VYD053, VYD055, VYD057, VYD058, VYD076, VYD077, VYD092, VYD123, VYD124, VYD125, VYD128, VYD145, VYD146).

These haplotypes correspond to 154 *M. glareolus* specimens (one sequence corresponding to one or more specimens) from different European and Russian regions (Fig. 1): Spain (Navarre, Granollers), France (Py Mantet, Montpellier, Bourdeilles, St-Aignan), Belgium (Dalhem, Liège), Italy (Pietraporzio, Trentino, Chiuse della Verna, Lucretili Mountains), Germany (Konstanz, Gera, Parchim), Sweden (Batskarsnas), Austria (Titole, Ost Tirol, Karnten, Salzburg), Hungary (Zala, Nagycsany), Romania (Timisoara, Moneasa, Targu Mures, Zarnesti, Bacau, Maramures), Lithuania (Alytus, Zemaitijos National Park), Poland (Pulawy, Bialowieza), Russia (Novgorod, Samara, Omsk, Novosibirsk), and Finland (Pallasjärvi). These specimens are distributed into five *M. glareolus* lineages (Ural, Western and Eastern European, Spanish, and Italian). Two northern red-backed voles (*Myodes rutilus* Pallas, 1779) and two grey red-backed voles (*Myodes rufocanus* Sundevall, 1846), available in the GenBank database (respectively, AB072223 and AB072224, and AF429815 and AF429816), were used as out-groups in the phylogenetic reconstruction.

#### *Morphometric analyses*

A total of 145 bank voles (*M. glareolus*) were sampled in 15 localities in Europe and Russia (Fig. 1; Table 1).



**Figure 1.** Geographic distribution of the bank vole (*Myodes glareolus*) samples and genetic lineages. This study includes five bank vole mitochondrial lineages: the Spanish, Italian, Western European, Eastern European, and Ural groups. The symbols correspond to species and lineages within *M. glareolus*.

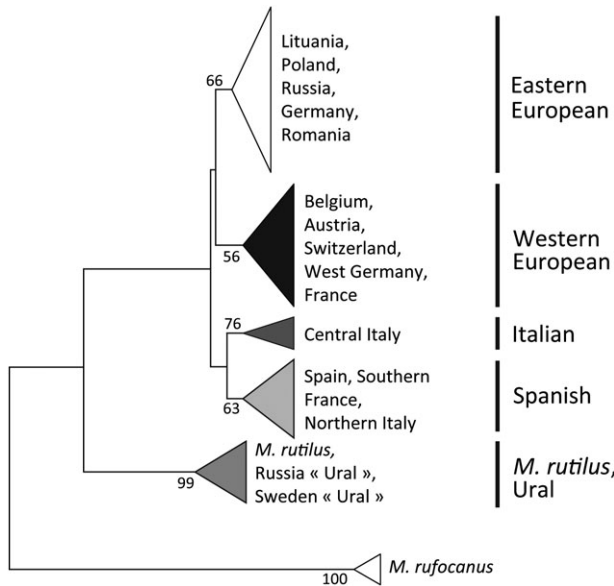
A total of 79 specimens were genetically attributed to a lineage, documenting five of the known mtDNA phylogroups (Fig. 2): the Spanish, Italian, Western European, Eastern European, and Ural lineages. Localities where mtDNA analyses evidenced the co-occurrence of several lineages were discarded from our sampling to avoid mixing lineages that might blur any morphometric differentiation between them. In localities without evidences of mixing, all available specimens were considered, including those that were not genetically identified. For each animal three morphological characters were considered, when intact: UM1, UM3, and LM1. For comparison purposes, 31 specimens of the related red vole (*M. rutilus* Pallas, 1779) were included in the study.

#### PHYLOGENETIC RECONSTRUCTIONS

We used MODELTEST 3.0 (Posada & Crandall, 1998) to determine the most suitable model of DNA substitution for the *cyt b* data set studied. Phylogenetic reconstructions were performed using the maximum likelihood criterion (ML; Felsenstein, 1981) implemented in PHYML (Guindon & Gascuel, 2003). Phylogenetic trees were rooted with *cyt b* sequences from two northern red-backed voles (*M. rutilus*) and two grey red-backed voles (*M. rufocanus*). The robustness of the tree was assessed by bootstrap support (BP) (1000 random pseudoreplicates).

**Table 1.** Sampling localities with their label and country of origin. The lineage of most specimens was genetically identified, and the number of first upper (UM1), third upper (UM3), and first lower (LM1) molars measured is indicated

Species	Country	Locality	Label	Lineage	Number of measured features		
					UM1	UM3	LM1
<i>Myodes glareolus</i>	Austria	Pfunds, Ventetal, Zemmatal	AUS	W	11	16	13
	Belgium	Blégny, Dahlem, Liège, Virelles	BEL	W	23	24	22
	Finland	Pallasjärvi	FIN	UR	11	12	12
	France	Loiret	FR	W	2	2	2
	Germany	Langenberg	GER	W	3	3	3
	Hungary	Bak	HG	W	4	4	3
	Italy	Tuscany	IT	IT	10	10	10
	Lithuania	Alytus	LIT	E	4	4	4
	Poland	Lublin	POL	E	3	9	5
	Romania	Sovata	ROM	E	9	9	9
	Russia	Bashkiria	RS_BA	UR	25	25	23
	Russia	Zhiguli	RS_JU	E	4	4	4
	Russia	Ozerki	RS_SA	E	7	8	7
	Slovenia	Delnice, Livek	SLN	W	3	3	2
	Spain	Asturias	SP	SP	12	12	12
<i>Myodes rutilus</i>	Finland	Pallasjärvi	RUT_FIN	–	24	27	24
	Russia	Shigaev	RUT_EK	–	4	4	4
			Total		159	176	173



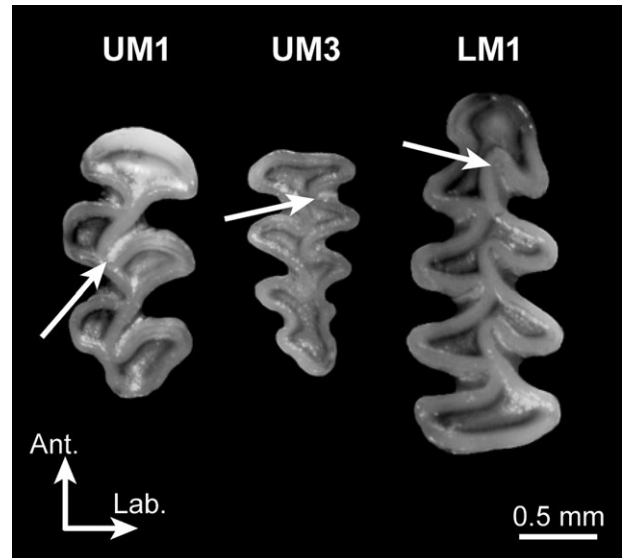
**Figure 2.** Simplified maximum-likelihood tree summarizing the genetic relationships among the *Myodes glareolus* studied, and based on 154 specimens coming from different European and Russian regions.

Mean sequence divergences among the four main *M. glareolus* (Western, Eastern, Spanish, and Italian) genetic lineages were calculated in MEGA v.4 (Tamura *et al.*, 2007), using the corrected K2P distance matrix (Kimura, 1981), as proposed by MODELTEST 3.0. The Ural group was not considered in this analysis as it displays *M. rutilus* mtDNA.

#### SHAPE ANALYSIS

Landmark-based morphometrics (Bookstein, 1991) and outline analysis (Renaud *et al.*, 1996) are among the most widely used methods to quantify morphological divergence. For molars with complex shapes, such as those of bank voles (Fig. 3), the elliptic Fourier transform (EFT) appears to be the most appropriate method to describe them (Navarro, Zatarain & Montuire, 2004). The occlusal surface of the molars was considered with the labial side to the right, for upper as well as lower molars. The starting point was defined at the minimum of curvature between the first and second anterior labial triangles (Fig. 3). When a molar was damaged or missing, a mirror image of the opposite tooth was measured.

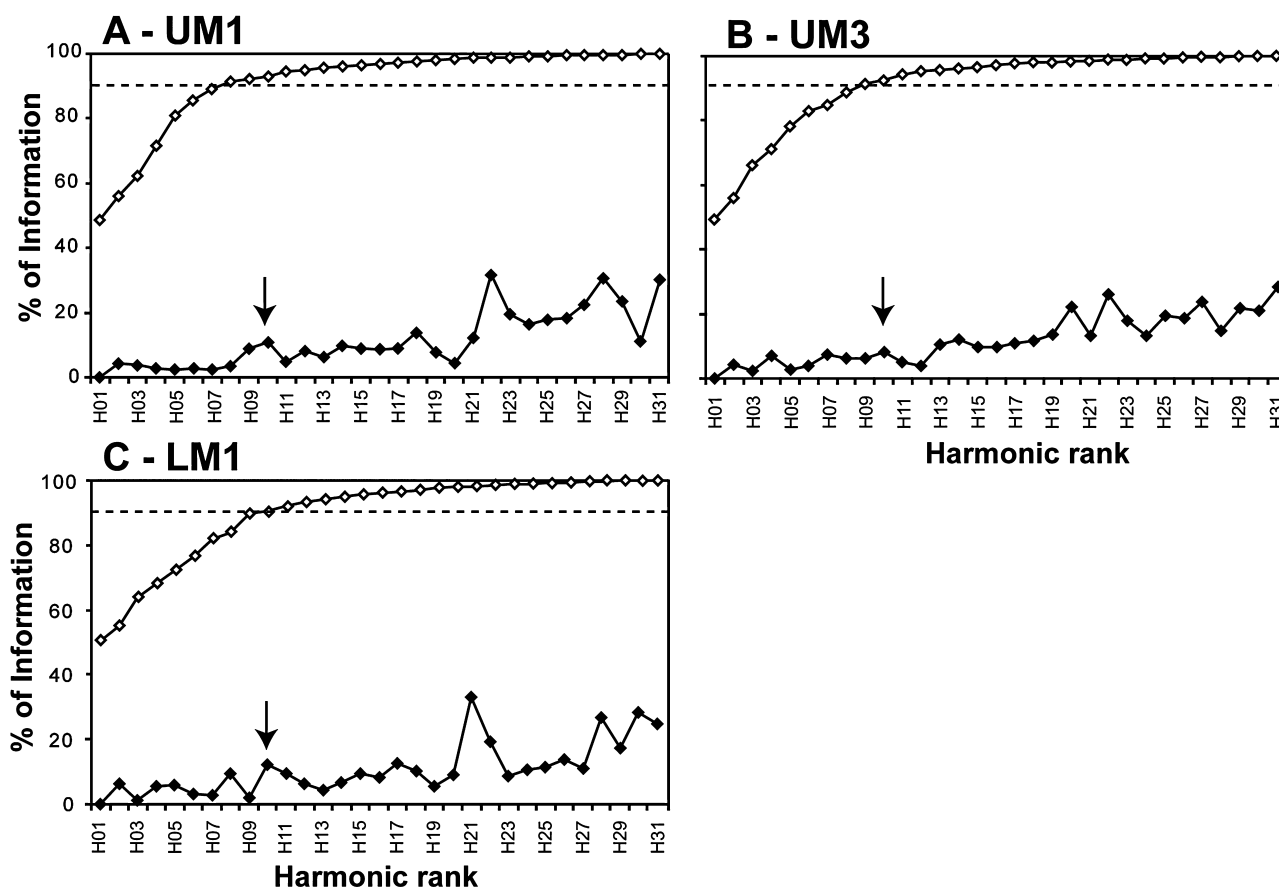
For each molar, 64 points at equally spaced intervals along the outline were sampled and analysed by an EFT using EFAwin software (Ferson, Rohlf & Koehn, 1985). This method is based on the separate Fourier decomposition of the incremental changes of the  $x$  and  $y$  coordinates as a function of the cumulative length along the outline (Kuhl & Giardina, 1982).



**Figure 3.** Occlusal surface of the first upper molar (UM1), third upper molar (UM3), and first lower molar (LM1) of the bank vole (*Myodes glareolus*), represented with the labial side to the right. The starting point of the outline of each tooth is indicated by an arrow.

Using this method the outline is approximated by a sum of trigonometric functions of decreasing wavelength: the harmonics. Each harmonic is weighted by four Fourier coefficients defining an ellipse:  $A_n$ ,  $B_n$ ,  $C_n$  and  $D_n$ . The first harmonic ellipse corresponds to the best-fitting ellipse to the outline, and its area can be considered as a reliable size estimator. It was used to standardize the Fourier coefficients for size differences. The major axis of the first harmonic ellipse was taken as new  $x$ -axis to adjust the orientation of the outline (Rohlf, 1990). As coefficients  $A_1$ ,  $B_1$  and  $C_1$  correspond to residuals after standardization (Crampton, 1995), they were not included in the subsequent statistical analysis. The coefficient  $D_1$  still retains information about the elongation of the outline (Michaux, Chevret & Renaud, 2007). Hence, it was included in the statistical analyses.

Using a Fourier analysis, the higher the rank of the threshold harmonic, the more detailed is the description of the outline. The number of harmonics selected for the analysis should take into consideration the level of measurement error occurring during the data acquisition and the information content of each harmonic. The shapes of the UM1 (Fig. 4A), UM3 (Fig. 4B), and LM1 (Fig. 4C) of one specimen were measured ten times, and the measurement error was calculated for each harmonic. The content of information of each harmonic (Fig. 4) provides an estimation of the amount of shape information described by that harmonic (Crampton, 1995), calculated as follows: the



**Figure 4.** Measurement error (black diamonds) and information content (white diamonds) as a function of the harmonic rank for (A) the first upper molar (UM1), (B) the third upper molar (UM3), and (C) the first lower molar (LM1). The measurement error corresponds to the coefficient of variation of the harmonic amplitude for one specimen measured ten times. The percentage of information corresponds to the contribution of each harmonic (amplitude %) to the total of information (i.e. the sum of all harmonic amplitudes = 100%). For the three molars, considering the first ten harmonics provides more than 90% of the information content (dotted lines), and a measurement error of less than 15%, which was thus chosen as the common threshold.

amplitude of each harmonic  $n$  [ $= \sqrt{(A_n^2 + B_n^2 + C_n^2 + D_n^2)}$ ] is cumulated over the total range of harmonics, and the information brought by each harmonic is then estimated as the percentage of this sum represented by the amplitude of rank  $n$ . For the three teeth used here, more than 90% of the information content is reached by considering the first ten harmonics, in agreement with previous studies on arvicoline molars (Marcolini, 2006), showing that considering this set of harmonics offers a good compromise between measurement error (less than 15%), the number of variables, and information content. Therefore, a data set of 37 variables (40 Fourier coefficients minus  $A_1$ ,  $B_1$ , and  $C_1$ ) was retained for subsequent analyses.

A visualization of shape changes can be provided by the reconstruction of outlines using the inverse Fourier method (Rohlf & Archie, 1984).

#### STATISTICAL ANALYSIS

The size of the three molars, estimated by the square root of the 2D outline area, was investigated using univariate statistics. Unfortunately the body size was not available for comparison. Inter- and intraspecific differences in size as well as the occurrence of sexual dimorphism were tested by analyses of variance (ANOVA) completed by Student's  $t$ -tests for pairwise differences.

Multivariate analyses were used to investigate the shape of the molars, estimated by the set of 37 Fourier coefficients. Multivariate analyses of variance (MANOVA) were performed on these variables to minimize the influence of intragroup variation, and to evidence the patterns of among-group differentiation on a few synthetic canonical shape axes. Pairwise comparisons between lineages were also performed

using a Hottelling  $T^2$  test (test considered: Wilks' Lambda). Finally, multivariate regressions were performed between size and shape to test for an allometric effect.

Statistics were performed using Systat v.11 and NTsys-pc 2.1 (Rohlf, 2000).

## RESULTS

### PHYLOGENETIC ANALYSES

The ML tree is presented Figure 2. The ML analyses were performed using the K2P model estimated using MODELTEST 3.0, with the proportion of invariable sites set to 0.52 and with a gamma distribution shape parameter of 0.69. As previously observed (Deffontaine *et al.*, 2005), the studied *M. glareolus* sequences are divided into five main lineages. The Eastern and Western European groups (bootstrap/BP values: 66 and 56%, respectively) associate animals from: (1) Russia, Germany, Romania, Lithuania, and Poland; and (2) Belgium, Austria, Switzerland, Germany, and France, respectively. Two other groups correspond to the Mediterranean peninsular lineages (Spanish and Italian, BP values: 63 and 76%, respectively). Finally, some bank vole sequences from Russia and Sweden are closer to red-backed vole sequences than to other *M. glareolus* sequences forming the previously described bank vole 'Ural group' (Deffontaine *et al.*, 2005). The Western and Eastern European lineages seem to be more closely related as compared with the two Mediterranean peninsular populations, which are associated together. However, these relationships do not have strong support (BP < 50) and should be taken with caution, as the four lineages are genetically so close that it is difficult to precisely define their relationships. The levels of genetic divergence among the four European lineages summarized in Table 2 evidence such difficulties, as these results suggest the Western European group is more closely related to the Mediterranean peninsular populations (1.01% K2P distance) than to the Eastern lineage (1.34%). A similar result was already observed in Deffontaine *et al.* (2005) on the basis of a more complete data set.

**Table 2.** Mean sequence divergence among genetic lineages calculated in MEGA v.4 (Tamura *et al.*, 2007), using the corrected K2P distance matrix. The Ural group was not considered in this analysis as it displays red vole mitochondrial DNA

	#WESTERN	#ITALIAN	#SPANISH
#ITALIAN	0.0101		
#SPANISH	0.0105	0.0073	
#EASTERN	0.0134	0.0155	0.0165

In contrast, the low levels of genetic divergence (0.73%) between the Italian and Spanish groups tend to confirm a close relationship between them.

### SEXUAL DIMORPHISM IN MOLAR SIZE AND SHAPE

Molar size and shape differences between males and females were tested in 25 specimens trapped in the same Russian locality of Bashkiria (Table 1). No sexual dimorphism was evident on UM1 (ANOVA:  $P = 0.462$ ), UM3 ( $P = 0.280$ ), or LM1 size ( $P = 0.060$ ). No evidence of sexual dimorphism in molar shape was found (MANOVA:  $P_{UM1} = 0.654$ ,  $P_{UM3} = 0.748$ , and  $P_{LM1} = 0.606$ ). Therefore, males and females were pooled together in the subsequent analyses.

### INTERSPECIFIC MORPHOLOGICAL DIFFERENCES

Significant size differences between *M. glareolus* and *M. rutilus* were found for the three molars ( $P < 0.001$ ), with the molars of *M. rutilus* being smaller than those of *M. glareolus* (Fig. 5). The two species further differ in shape ( $P < 0.001$ ). The level of interspecific difference was compared with variation among *M. glareolus* specimens by performing an analysis including the *M. rutilus* sample and the five *M. glareolus* lineages as groups. In all cases, the two species differ along the first axis (Fig. 6). The molars of *M. rutilus* were characterized by a larger anterior part of UM1, a more developed posterior lingual triangle on UM3, and a shorter anterior part and less marked triangles on LM1.

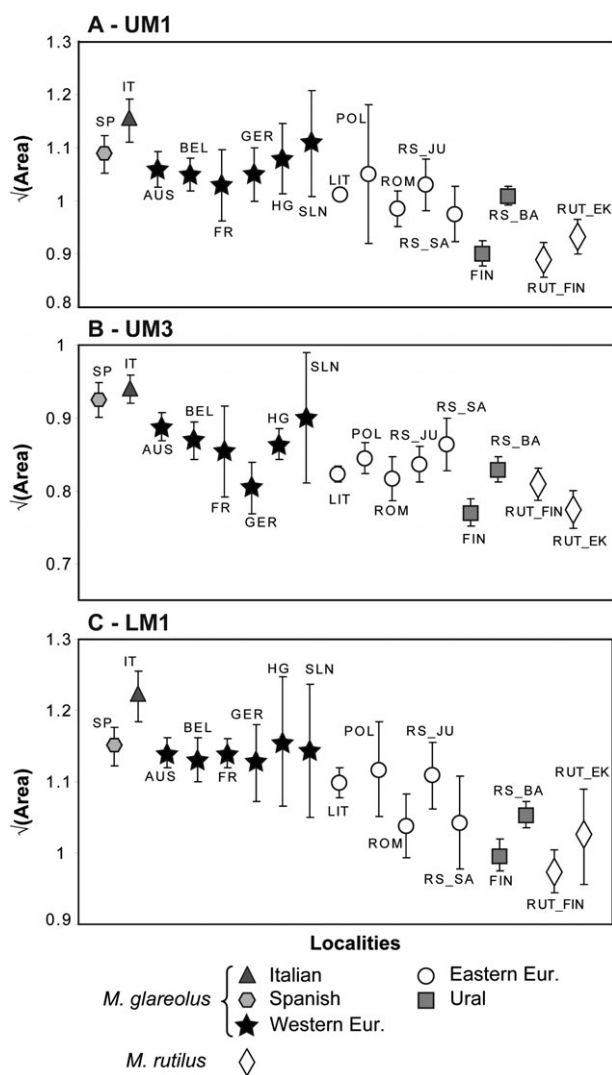
The intraspecific differences within *M. glareolus* emerge along the second canonical axis, which represents from half to a third of the variation expressed by the first axes (Fig. 6). The molar shape of the Ural lineage is clearly associated with other *M. glareolus* lineages, and is particularly close to the Eastern European lineage.

To investigate the intraspecific differences that emerged from this analysis in more detail, further analyses were performed on bank vole samples alone.

### INTRASPECIFIC DIFFERENTIATION OF *M. GLAREOLUS*

#### *Size differences among lineages*

Differences in molar size among lineages were investigated (Table 3). Size is significantly different between lineages in most of the cases ( $P < 0.01$ ). The Eastern European and Ural lineages are the least differentiated, only showing a weak size difference in UM3 and LM1 ( $P = 0.012$  and  $P = 0.020$ , respectively). By contrast, the Western European and Spanish groups show a high differentiation in UM3 size ( $P = 0.003$ ), but not in UM1 ( $P = 0.164$ ) and LM1 ( $P = 0.474$ ). These differences among lineages corre-



**Figure 5.** Geographical differences in the size of the occlusal surface of (A) the first upper molar (UM1), (B) the third upper molar (UM3), and (C) the first lower molar (LM1). The square-root of the two-dimensional outline area is used as the size estimator. Each dot corresponds to the mean of a geographical group  $\pm$  the confidence interval. The symbols correspond to species and to lineages within *Myodes glareolus*.

spond to an overall decrease in molar size from Mediterranean peninsulas (Spanish or Italian groups) to northernmost localities, where a convergence between the Ural lineage and *M. rutilus* was observed (ANOVA, UM1,  $P = 0.750$ ; UM3,  $P = 0.760$ ; LM1,  $P = 0.236$ ; Fig. 5). This is confirmed by a significant and negative relationship between molar size and latitude ( $P < 0.001$ ).

#### Patterns of shape differentiation

Since interlineage differences are tenuous compared with intragroup variability, a canonical analysis was

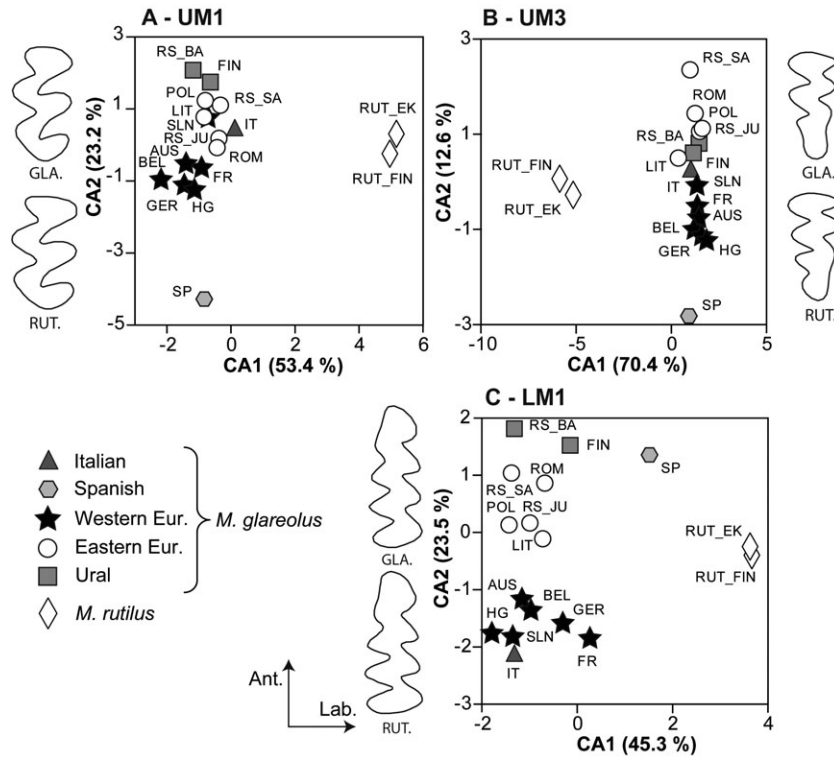
**Table 3.** Two-by-two tests (Student's *t*-tests) of size differences between lineages for the first upper (UM1), third upper (UM3), and first lower (LM1) molars

	UM1	UM3	LM1
E_W	0.001***	0.004**	< 0.001***
E_IT	< 0.001***	< 0.001***	< 0.001***
E_SP	< 0.001***	< 0.001***	0.001***
E_UR	0.146	0.012*	0.020*
W_IT	< 0.001***	< 0.001***	< 0.001***
W_SP	0.164	0.003**	0.474
W_UR	< 0.001***	< 0.001***	< 0.001***
IT_SP	0.030*	0.373	0.005**
IT_UR	< 0.001***	< 0.001***	< 0.001***
SP_UR	< 0.001***	< 0.001***	< 0.001***

Probabilities are given with significance thresholds (\*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ). E, Eastern European group; W, Western European group; IT, Italian group; SP, Spanish group; UR, Ural group.

performed on the Fourier coefficients of each molar to focus on differences among lineages that were significant in the three molars ( $P < 0.001$ ). The patterns of differentiation were visualized on the first three canonical axes (Fig. 7). Even if the grouping variable corresponds to the lineages, the average value per locality has been represented. Clear patterns of shape differentiation emerge on the three teeth, supported by two-by-two comparisons between lineages (Table 4).

The analysis of UM1 evidenced a segregation of the Spanish and Italian lineages from the other groups (Fig. 7A) along the first and second shape axes, respectively ( $CA1 = 45.6\%$  and  $CA2 = 22.4\%$  of the among-group variance). The Italian lineage is close to the Eastern European group along  $CA1$ , but is clearly differentiated along  $CA2$ . The Spanish lineage differentiates in the  $CA1$ – $CA2$  plane, with the shape tending to be more similar to the Western European group than to the others, but still with a significant divergence ( $P = 0.008$ ; Table 3). The Eastern European and Western European groups weakly differentiate along the first and third shape axes ( $CA3 = 19.2\%$ ) with a low level of divergence ( $P = 0.012$ ). The Ural group, being close to the Eastern European lineage in morphospace, still differentiates along  $CA1$  from the other lineages, and is significantly different from the other groups. Finally, despite its extreme average position along  $CA2$ , the Italian lineage is only weakly differentiated from the other groups ( $P < 0.05$  or non significant), possibly because of its limited sample size. The differences observed on the reconstructed outlines are tenuous. The main regions of variation are the re-entrant



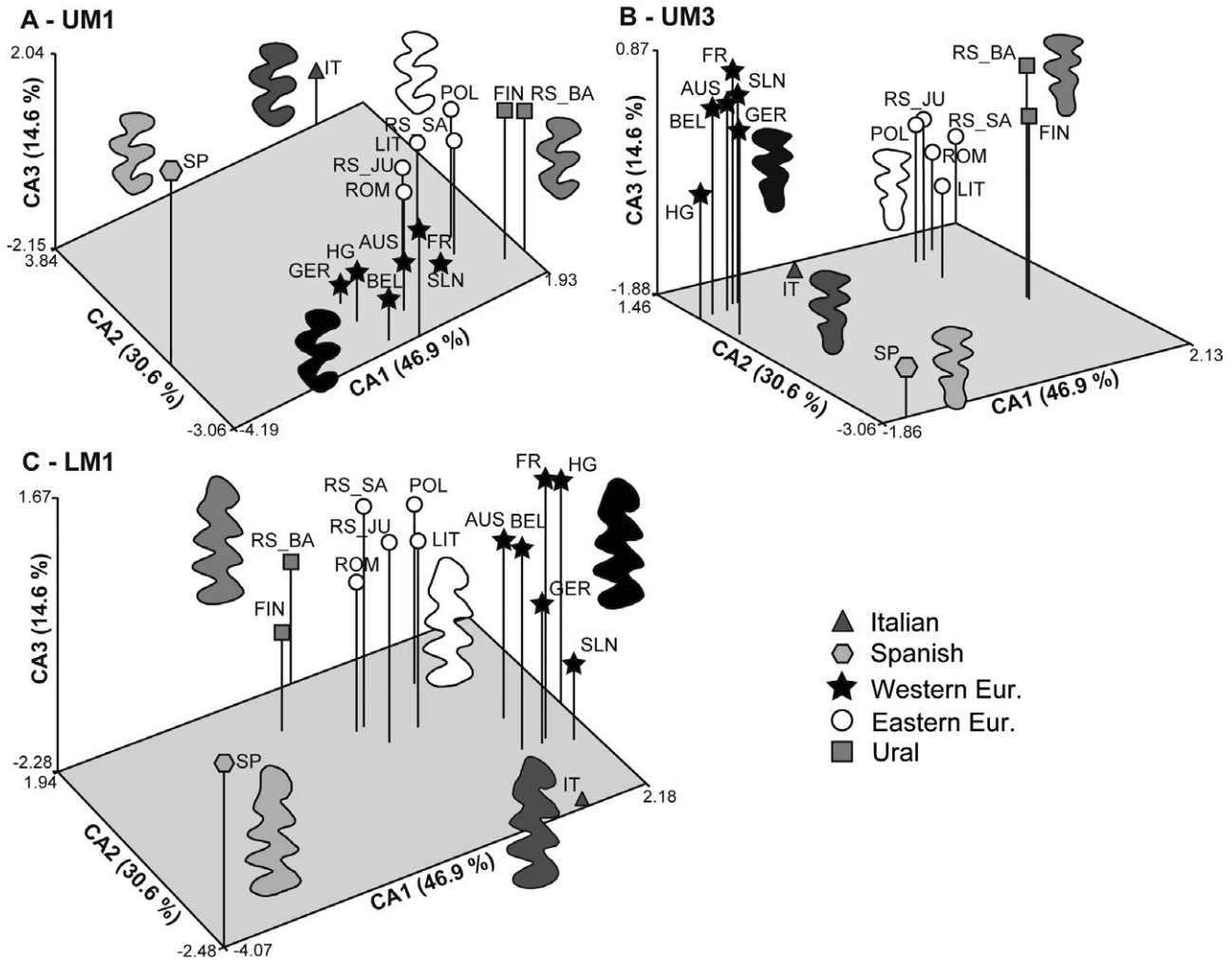
**Figure 6.** Shape differences between *Myodes rutilus* and *Myodes glareolus*. The variations are displayed on the first two axes of a canonical analysis on the Fourier coefficients of the molar outline. The grouping variable used for the analysis includes six groups: the five lineages of *Myodes glareolus* and the sample of *Myodes rutilus*. To have a better representation of the intraspecific variability, each dot corresponds to the mean by localities. Reconstructed outlines visualize the difference between the two species.

**Table 4.** Two-by-two tests of shape differences between lineages for the first upper (UM1), third upper (UM3), and first lower (LM1) molars

	UM1		UM3		LM1	
	FCs	Res.	FCs	Res.	FCs	Res.
E_W	0.012*	0.027*	< 0.001***	< 0.001***	0.024*	0.503
E_IT	0.447	0.215	0.205	0.121	0.091	0.231
E_SP	0.005**	0.0002***	0.066	0.028*	0.151	0.060
E_UR	0.006**	0.076	0.033*	0.111	0.098	0.247
W_IT	0.027*	0.040*	0.146	0.007**	0.036*	0.027*
W_SP	0.008**	0.004**	0.007**	< 0.001***	< 0.001***	< 0.001***
W_UR	< 0.001***	< 0.001***	< 0.001***	< 0.001***	< 0.001***	0.018*
IT_SP	0.471	0.361	0.527	0.337	0.434	0.602
IT_UR	0.030*	0.072	0.017*	0.216	0.030*	0.430
SP_UR	< 0.001***	0.003**	0.003**	0.034*	0.029*	0.009**

Shape was estimated by the set of Fourier coefficients (FCs), and differences were tested using a Hotelling  $T^2$  test. To test the occurrence of an allometric effect on the shape of the molars, a multivariate regression was performed. The two-by-two tests were performed on the residuals (Res) and compared with the previous results. Probabilities are given with significance thresholds (\*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ). E, Eastern European group; W, Western European group; IT, Italian group; SP, Spanish group; UR, Ural group.





**Figure 7.** Shape variations of (A) the first upper molar (UM1), (B) the third upper molar (UM3), and (C) the first lower molar (LM1) within *Myodes glareolus*, displayed on the first three axes of a canonical analysis of the Fourier coefficients, the grouping variable being the five lineages of *Myodes glareolus*. Each symbol corresponds to the mean of a geographical group. Reconstructed outlines visualize the mean shape of each lineage.

angles between each triangle, the size of the triangles and the posterior part of the molar, which is more or less rounded (Fig. 7A).

Compared with the results obtained from UM1, similar patterns are observed in UM3 (Fig. 7B), but the probabilities are in general less significant (Table 4). The Mediterranean lineages also show the most pronounced differentiation, but this time the Spanish group segregates along the second shape axis (CA2 = 30.6%), with a clear differentiation from the Western European ( $P = 0.007$ ) and Ural lineages ( $P = 0.003$ ), corresponding to a broader posterior part of the Spanish UM3. The first axis (CA1 = 46.9%) mainly represents the differentiation between the Western and the Eastern European lineages (Table 4;  $P < 0.001$ ), which present a lengthened posterior part.

The Italian lineage has an intermediate position between these two groups along CA1, but differentiates along the third axis (CA3 = 14.6%). The Ural lineage is close to the Eastern European lineage, but slightly differentiates along the CA2–CA3 plane ( $P = 0.033$ ).

As for UM1 or UM3, LM1 shapes of the peninsular lineages are the most differentiated (Fig. 7C). They diverge along the first shape axis (CA1 = 46.2%) for the Spanish lineage and along the second and third shape axes (CA2 = 32.8% and CA3 = 14.2%) for the Italian lineage. The Spanish LM1 is characterized by a slight flattening of its anterolingual part, and the Italian LM1 is characterized by a more rounded posterior part. The Italian group is the closest to the Western European lineage, but is still significantly

different (Table 4;  $P = 0.036$ ). The Eastern and Western European lineages are weakly differentiated ( $P = 0.024$ ) on the CA1–CA2 plane. The Ural lineage is close to the Eastern European lineage but slightly shifted along CA1. Overall, the analysis of LM1 evidences weaker among-lineage differentiation than in UM1 and UM3. However, LM1 is the only tooth supporting the Western-Spanish differentiation ( $P < 0.001$ ).

*Possible effect of an allometric component on the size and shape differentiation patterns*

Our results indicate that the Spanish, Italian, and Ural lineages were clearly divergent from the other lineages in shape, but were also the most divergent in size. The possible influence of an allometric component on the shape differentiation of these lineages was tested using a multivariate regression between size and shape variables. It was significant for the three molars ( $P < 0.001$ ). However, canonical analyses performed on residuals showed very similar results to those obtained on raw Fourier coefficients (Table 4). Thus, the patterns of shape differentiation were not attributable to allometric effects.

## DISCUSSION

### A GEOGRAPHICAL TREND IN SIZE

The best-known trend in biogeography is Bergmann's rule (Bergmann, 1847) stating that warm-blooded animals tend to increase in size in cold environments, with larger animals obtaining a smaller surface-to-volume ratio and thereby improving heat conservation in a cold climate. Yet, some groups tend to depart from this general trend, especially carnivores (Meiri, Dayan & Simberloff, 2004) and small mammals (Meiri & Dayan, 2003). Assuming that molar size can be considered a proxy for body size at the interpopulation scale of variation, as suggested in wood mice (Renaud & Michaux, 2007), our data evidenced that the bank vole may be a further exception to Bergmann's rule. This trend emerged between lineages, with Mediterranean groups tending to be larger than northern ones. Several factors may contribute to this apparently surprising trend. First, predators such as stoats (Erlinge, 1987) and pine martens (Zalewski, 2005) also tend to become smaller towards high latitudes, possibly as an adaptation to winter conditions including snow cover. Being one of the main prey of these predators, the concomitant decrease in size of bank voles may be an adaptive response to predation pressure, with bank voles finding shelters in burrows narrower than their predators (Sundell & Norrdahl, 2002). The size decrease is particularly

marked in the Ural lineage, strikingly converging towards the size of the northern red-backed vole *M. rutilus*. High-latitude environments are characterized by low primary production and low food availability, especially in winter (Yom-Tov & Geffen, 2006), so a smaller body size may represent an adaptation to reduce the total energy requirement (Ellison *et al.*, 1993). Furthermore, as mtDNA is involved in metabolic activity, the introgression of *M. rutilus* mtDNA in the Ural lineage might be of selective advantage, by allowing bank voles to display a metabolism similar to that of their cold-adapted relative. An association between the occurrence of *M. rutilus* mitochondrial DNA, smaller body size, and reduced basal metabolic rate has been recently evidenced in Finnish bank voles (Boratynski, Koskela & Mappes, 2009), supporting the interpretation of a complex adaptation to cold conditions in the Ural lineages through evolutionary trade-offs between metabolic rate and body size.

### INTER- AND INTRASPECIFIC DIFFERENTIATION

Molars of arvicoline rodents have long been recognized for their taxonomic value at the interspecific level (e.g. Kaneko, 1992; Kitahara, 1995; Chaline *et al.*, 1999). In the arvicoline genus *Myodes*, 12 species are currently recognized (Wilson & Reeder, 2005) in which differentiation occurred during the second pulse of the arvicoline radiation, around 2.6 Mya (Chaline *et al.*, 1999; Conroy & Cook, 1999). The obvious morphological differentiation observed in the present study between *M. glareolus* and *M. rutilus* confirms that this time span has been long enough for important differences in molar shape to accumulate between species (e.g. Renvoisé *et al.*, 2009). The existence of intraspecific differences among lineages of *M. glareolus* was less expected because of their recent divergence time, estimated to 250–300 Kya (Deffontaine *et al.*, 2005). Furthermore, genetic analyses pointed to a low divergence of the lineages (maximum 1.6% genetic differentiation; Table 2). Cases of morphological differentiation in an even shorter time span are known, but they usually correspond to peculiar conditions such as small, isolated populations in insular conditions (Renaud & Millien, 2001) or fragmented populations as a result of anthropization (Mikulová & Frynta, 2001). The context of divergence between bank vole lineages is very different, as they came into contact regularly during interglacial periods, allowing for significant gene flow between large populations. In a similar context, evidences of molar shape differentiation among wood mice (*Apodemus sylvaticus*) lineages were found, with the level of differentiation being low, however, despite a date of divergence of around 1 Mya

(Renaud & Michaux, 2007). Compared with the wood mouse, the bank vole lineages present an even more recent date of divergence, and it is thus remarkable that we evidenced a significant difference between lineages in the shape of all molars considered. Yet in agreement with the low genetic divergence, the morphological differentiation is tenuous and of the same order of magnitude than local sources of variation within populations (Guérécheau *et al.*, 2010). The question of the processes leading to this differentiation will be addressed in the following sections.

#### MOSAIC EVOLUTION BETWEEN MORPHOLOGICAL CHARACTERS

Many studies have considered patterns of differentiation in arvicoline molars, but usually each study focused on a single tooth (e.g. Marcolini, 2006; Tougaard *et al.*, 2008). An originality of this study was to consider several teeth, and compare their patterns of differentiation. As the different molars share a similar genetic background involved in the dental row development (Kavanagh, Evans & Jernvall, 2007), they cannot be considered as independent characters, and a concerted evolution among molars is expected. Accordingly, many similarities were observed between the patterns of shape differentiation among teeth. The Spanish and Italian lineages were always the most divergent groups. The Eastern and Western European groups were generally close to each other at the centre of the morphological space, and finally the Ural lineage was closely related to the Eastern European lineage. These results complement our interpretation of the phylogenetic reconstructions that present relatively low bootstraps.

However, even if common features emerged from the patterns of shape differentiation of the three teeth, some discrepancies also occurred. Depending on the molar, the Italian lineage appeared to be close either to the Eastern European group (UM1), the Western European group (LM1), or intermediate between the two groups (UM3). The differentiation between the Western and Eastern European lineages was most pronounced on the UM3. These discrepancies between the patterns of differentiation of the three teeth suggest that a mosaic evolution occurred, despite the overall concerted evolution. This may be because of slightly different evolutionary rates for the different characters, as previously observed in rodent teeth (Barnosky, 1993). It suggests a more pronounced divergence of UM1, mainly during the Ural lineage differentiation, whereas UM3 diverged faster than the other teeth between Western and Eastern European groups, and LM1 diverged faster than the other teeth between Western and Spanish lineages.

#### MOLAR SHAPE PROVIDING HINTS ABOUT BANK VOLE PHYLOGEOGRAPHIC HISTORY

*The Mediterranean lineages as sources of endemism*  
For a long time the Mediterranean peninsulas were considered as the main refugia and sources of northward recolonization for temperate forest species during the Quaternary period (Lugon-Moulin & Hausser, 2002; Sommer & Benecke, 2005). An alternative view is to consider these regions as zones of endemism (Bilton *et al.*, 1998). Considering the Mediterranean peninsulas as sources of recolonization would lead to the expectation of the Western and Eastern European molar shapes being close to the Italian and Spanish ones. On the contrary, our results present a marked divergence of the peninsular lineages from the Western and Eastern European groups, thus bringing support to the second hypothesis of Mediterranean endemism. In agreement, genetic analyses showed that the two Mediterranean lineages were independent compared with the other populations from Western and Eastern Europe. Furthermore, the lack of shape similarities between the Italian and Spanish lineages, despite a low degree of genetic divergence (0.73% K2P distance; Table 2), evidenced the absence of parallel evolution, even if they share similar Mediterranean environments. It suggests that molar shape in the two groups differed rather by accumulation of neutral differences in isolated populations than by parallel adaptation to their environments.

#### *Western and Eastern European lineages*

During the Quaternary period, repeated cooling and fluctuations of ice sheets caused shifts in species distribution (Bilton *et al.*, 1998; Hewitt, 2000). Whereas bank vole populations were isolated in refugia during glacial periods, leading to a differentiation between lineages, the deglaciation phases induced an expansion with two possible scenarios. The lineages might have been isolated by geographical barriers, decreasing their dispersal ability and leading to endemism, as illustrated by the Mediterranean lineages. Alternatively, lineages might have come into contact, with gene flow in suture zones swamping out the differentiation accumulated during glaciation phases. The Western and Eastern lineages in bank voles appear to be relevant to this last case. Given the genetic evidence, their suture zone ranges over a thousand kilometres in Central Europe, with the occurrence of Eastern mtDNA in Germany and the occurrence of Western mtDNA as far as Romania (Deffontaine *et al.*, 2005). The morphometric results, however, point to a weak but significant differentiation between the two lineages, in agreement with the level of genetic divergence larger than between the

endemic Mediterranean lineages (1.34% K2P distance between Eastern and Western lineages vs. 0.73% between Spanish and Italian lineages). This suggests that despite extensive mixing nowadays, the repeated isolations during glaciation periods were important enough to shape the pattern of differentiation still observed today.

#### *Ural lineage*

The distinctiveness of the Ural lineage is that it has *M. rutilus* mtDNA despite the fact that its overall morphology, and all nuclear markers investigated so far, are typical of the bank vole (Tegelström, 1987; Potapov *et al.*, 2007; Henttonen & Kaikusalo, pers. com.). In agreement, the molar shape of the Ural group is clearly related to other bank voles. Moreover, the Eastern European lineage is morphologically close to the Ural group, supporting the Eastern European group as the closest relative of the Ural lineage, as suggested on the basis of molecular analyses (Defontaine *et al.*, 2005). The introgression of *M. rutilus* mtDNA within a bank vole lineage is likely to have resulted from hybridization between the two species, a process that can still occur *in natura* and in laboratory experiments (Osipova & Soltin, 2006; Potapov *et al.*, 2007). Despite the possibility of hybridization between the two species, hybrids were not evidenced in our data: all specimens clearly shared typical bank vole morphology, whereas modern hybrids would have been expected to be morphologically intermediate between parental forms (e.g. Albertson & Kocher, 2005). The absence of such intermediate hybrid shapes suggests that either hybridization is very rare in the wild, or that hybrids are strongly counter-selected compared with parental species (Arnold & Hodges, 1995). Hence, the occurrence of *M. rutilus* mtDNA within the Ural bank vole might be the result of an ancient hybridization process, as observed in the mountain hare (Melo-Ferreira *et al.*, 2005). Our data on size suggest another, non-exclusive scenario. The convergence in size between Ural *M. glareolus* and *M. rutilus* was interpreted to be the result of a selective advantage of an *M. rutilus*-like size together with *M. rutilus* mtDNA in a northern environment. This does not exclude the occurrence of a disruptive selection favouring either the *M. rutilus* or the *M. glareolus* phenotype for features coded by nuclear DNA. A selection of the red vole mtDNA might therefore occur without much introgression of the nuclear genome (Potapov *et al.*, 2007).

As a conclusion, morphometric analyses can complement phylogenetic analyses. Two scenarios of post-glacial expansion were evidenced depending on the lineage: endemism of the Mediterranean lineages, characterized by a high morphological interlineages divergence, and a differentiation between the Western

and Eastern European lineages despite a low genetic divergence and a large area of sympatry.

Moreover, if the Ural lineage was defined on the basis of mtDNA, molecular analyses based on mtDNA were not able to describe its history because of an introgression from *M. rutilus* to *M. glareolus*. In this context, morphological data efficiently complemented genetic data, and confirmed the introgression of *M. rutilus* mtDNA into an otherwise *M. glareolus* genome. They also suggested the Eastern group as its closest relative and hence provide insight into the temporal dynamics that led to the emergence of the complex Ural lineage.

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