

Phylogeographical footprints of the Strait of Gibraltar and Quaternary climatic fluctuations in the western Mediterranean: a case study with the greater white-toothed shrew, *Crocidura russula* (Mammalia: Soricidae)

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Abstract

We used mitochondrial *cyt b* sequences to investigate the phylogenetic relationships of *Crocidura russula* (*sensu lato*) populations across the Strait of Gibraltar, western Europe, Maghreb, and the Mediterranean and Atlantic islands. This revealed very low genetic divergence between European and Moroccan populations. The application of a molecular clock previously calibrated for shrews suggested that the separation of European from Moroccan lineages occurred less than 60 000 BP, which is at least 5 million years (Myr) after the reopening of the Strait of Gibraltar. This means that an overwater dispersal event was responsible for the observed phylogeographical structure. In contrast, genetic analyses revealed that Moroccan populations were highly distinct from Tunisian ones. According to the molecular clock, these populations separated about 2.2 million years ago (Ma), a time marked by sharp alternations of dry and humid climates in the Maghreb. The populations of the Mediterranean islands Ibiza, Pantelleria, and Sardinia were founded from Tunisian populations by overwater dispersal. In conclusion, overwater dispersal across the Strait of Gibraltar, probably assisted by humans, is possible for small terrestrial vertebrates. Moreover, as in Europe, Quaternary climatic fluctuations had a major effect on the phylogeographical structure of the Maghreb biota.

Keywords: Europe, human-assisted dispersal, mtDNA, North Africa, phylogeography, Quaternary history, speciation

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Introduction

The relationships between the species currently found in Europe and Maghreb (the northern parts of Africa, i.e. Morocco, Algeria, and Tunisia) are thought to have been influenced mainly by the Strait of Gibraltar which acted as a geographical barrier for gene flow (Beerli et al. 1996; De Jong 1998; Prüser & Mossakowski 1998; Castella et al. 2000;

Palmer & Cambefort 2000; Gantenbein & Largiadèr 2003). During the Messinian period in the late Miocene, 5–6 Ma, the Mediterranean basin largely dried out because of the closing of the Strait of Gibraltar (Hsü et al. 1977; Krijgsman 2002). The resulting land corridors allowed biotic interchanges between southern Europe and the Maghreb. The strait reopened at the start of the Pliocene, about 5 Ma, causing the refilling of the Mediterranean and the closing of the terrestrial connection between Europe and the Maghreb. This event probably accounts for the vicariance observed in many Mediterranean lineages (Cheylan 1990; Zardoya & Doadrio 1998; Blondel & Aronson 1999; Dobson

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& Wright 2000; Sanmartín 2003) and for the genetic differentiation between some Iberian and Maghreb biota (Castella *et al.* 2000; De Jong 1998; Harris & Sá-Sousa 2002; Gantenbein & Largiadèr 2003). Later on, particularly since 2.4 Ma, the Quaternary climatic oscillations profoundly affected the phylogeographical structure of Mediterranean biota (Hewitt 1996; Taberlet *et al.* 1998; Hewitt 1999). The ranges of most European species underwent several consecutive contractions and expansions, with regular extinctions of the northern populations during ice ages, followed by subsequent northward expansions from southern Mediterranean peninsulas during interglacial periods. The isolation of populations in separate Mediterranean regions during the ice ages led to allopatric differentiation and strong phylogeographical structures (Hewitt 2001, 2004; Michaux *et al.* 2001). Comparatively little is known about the Quaternary evolutionary history of the fauna and flora of North Africa (Hewitt 2000). North Africa was regularly affected by alternating humid and hyperarid phases during the mid-Pliocene to Pleistocene (Street & Gasse 1981; Quezel & Barbero 1993) and a few recent studies suggest that these changing climatic conditions led to conspicuous phylogeographical footprints for some terrestrial animals (Brown *et al.* 2002; Guiller *et al.* 2001).

The greater white-toothed shrew (*Crocidura russula*) is a good example of a mammalian species that has undergone drastic changes in its taxonomic definition. The high level of morphological variability within *Crocidura* species resulted in numerous redescrptions of the same species under different names since its first description as *Sorex russulus* Hermann, 1780. The morphological similarity of populations of different species resulted in many erroneous species-level assignments (Ellerman & Morrison-Scott 1966; Corbet 1978). The extensive use of karyological techniques elucidated the distribution of *C. russula*, showing that it is limited to the Maghreb and continental western Europe, from Portugal through Spain and France, Switzerland, Germany and Austria (Reumer & Meylan 1986; Genoud & Hutterer 1990; Vogel *et al.* 1990). Morphological investigations covering the entire range of *C. russula* revealed substantial differences between the populations of eastern and western Maghreb (Sarà & Vogel 1996; Contoli & Aloise 2001). A molecular study (Vogel *et al.* 2003) using the cytochrome *b* gene confirmed a deep split within *C. russula*, with a western clade formed by samples from Spain and Morocco, and an eastern clade represented by a sample from Tunisia. Finally, a study based on mitochondrial simple sequence repeats and 12S *rRNA* gene confirmed the existence of two different clades (Lo Brutto *et al.* 2004).

The evolutionary history of *C. russula* is relatively well documented by palaeontological records (Rzebik-Kowalska 1988). However, as in taxonomy studies, the difficulty of precise species identification using mandibles and skulls can lead to misinterpretation of fossil records. Based on the

compilation of the literature about fossil reports, Dobson (1998) and Dobson & Wright (2000) concluded that *C. russula* was native to Iberia and reached the Maghreb only recently. In contrast, based on chronological analyses of some fossil assemblages, Poitevin *et al.* (1986) stated that *C. russula* arrived in the south of France only after the last glacial age. Along with Catzefflis (1984) and Vogel & Maddalena (1987), Poitevin *et al.* proposed that the species originated in the Maghreb.

The aim of our study was to determine the influence of the major climatic events that have occurred in the western Mediterranean since the reopening of the Strait of Gibraltar at the beginning of the Pliocene, on the evolutionary history of the greater white-toothed shrew. We used a phylogeographical approach to trace the history of the species in the region and to estimate the chronology of diversification events within the species. The phylogenetic relationships among populations were determined by analysis of cytochrome *b* in numerous specimens collected from the entire range of *C. russula*. The evolutionary history of the species was profoundly influenced by Quaternary fluctuations in the Maghreb and by its ability to disperse across different stretches of water to reach continental Europe and the Atlantic and Mediterranean islands. We show that small terrestrial vertebrates dispersed across the Strait of Gibraltar and that, like in Europe, Quaternary climatic fluctuations profoundly influenced the phylogeographical structure of some Maghrebian biota.

Materials and methods

Forty-five *Crocidura russula* samples were used in this study (Fig. 1). Specimens, origins, and identification codes are given in Table 1. We included *Crocidura russula osorio* from Gran Canaria in the data set, as this taxon was recently shown to be conspecific with *C. russula* (Molina *et al.* 2003; Vogel *et al.* 2003). Shrews were brought to the laboratory alive or preserved in liquid nitrogen in the field, then stored at -70°C , and finally preserved in 80% ethanol until DNA extraction. In some cases, we extracted DNA directly from bones collected in owl pellets, as was the case from several localities in France and Belgium.

Total genomic DNA was extracted by digestion with proteinase K for 4–8 h at 37°C . The resulting DNA was purified by extracting twice with phenol–chloroform and once with chloroform. The sample was then desalted and concentrated by ethanol precipitation. DNA was extracted from owl pellets as described by Taberlet & Fumagalli (1996). Double-stranded DNA was amplified with the primers L14841: TCAAACATCTCATCATGATGAAA, H15149: CCTCAGAATGATATTTGTCCTCA and H15915: TCATCTCCGGTTTACAAGAC. Polymerase chain reaction (PCR) conditions consisted of 30–35 cycles of 30–60 s denaturation at 93°C , 30–60 s annealing at 50°C , and 60–120 s extension

Table 1 Species sequenced, geographical origin of the samples, voucher codes (IZEA, Department of Ecology and Evolution, University of Lausanne; JFC, Centre de Biologie et Gestion des Populations, Montferrier), length of the cytochrome *b* gene sequenced and haplotype designations for short and long sequences

	Sample location	Identification codes	Cyt <i>b</i> sequences	
			Length (bp)	Haplotype designation
<i>Crocidura russula russula</i>	Breskens, the Netherlands	IZEA1084	998	Ru1 Ru1.2
<i>Crocidura russula russula</i>	Bonn, Germany	IZEA752	998	Ru1 Ru1.3
<i>Crocidura russula russula</i>	Liège, Belgium	JFC-R10	273	Ru1
<i>Crocidura russula russula</i>	La Chapelle d'Huin, France	JFC-R13	273	Ru1
<i>Crocidura russula russula</i>	Bréhat, France	JFC-R9	273	Ru1
<i>Crocidura russula russula</i>	Roscoff, France	IZEA5541/842	273	Ru1
<i>Crocidura russula russula</i>	Molène, France	JFC-R8	273	Ru1
<i>Crocidura russula russula</i>	Sein Island, France	9/JFC-R7	998	Ru1 Ru1.4
<i>Crocidura russula russula</i>	Sein Island, France	19/JFC-R6	998	Ru1 Ru1.4
<i>Crocidura russula russula</i>	Glénan (St Nicolas), France	JFC-R5	273	Ru1
<i>Crocidura russula russula</i>	Groix, France	JFC-R4	273	Ru1
<i>Crocidura russula russula</i>	Touchay, Cher, France	JFC-R11	273	Ru1
<i>Crocidura russula russula</i>	Morges, Vaux, Switzerland	IZEA812	273	Ru1
<i>Crocidura russula russula</i>	Laubertie, France	JFC-R2	273	Ru1
<i>Crocidura russula russula</i>	Commarque, France	JFC-R3	273	Ru1
<i>Crocidura russula russula</i>	Montpellier, France	JFC-R12	273	Ru1
<i>Crocidura russula russula</i>	Nice, France	JFC-R13	273	Ru1
<i>Crocidura russula russula</i>	Banyuls, France	JFC-R1	273	Ru1
<i>Crocidura russula russula</i>	Candelario, Salamanca, Spain	IZEA5936	998	Ru1 Ru1.1
<i>Crocidura russula russula</i>	Albaracin, Teruel, Spain	IZEA650	273	Ru1
<i>Crocidura russula russula</i>	Albaracin, Teruel, Spain	IZEA652	273	Ru1
<i>Crocidura russula russula</i>	Peniche, Madure, Portugal	IZEA5897	273	Ru1
<i>Crocidura russula russula</i>	Murillo, Logroño, Spain	IZEA5788	998	Ru1 Ru1.2
<i>Crocidura russula yebalensis</i>	Skhirat, Rabat, Morocco	IZEA2641	998	Ru2 Ru2.1
<i>Crocidura russula yebalensis</i>	Moulay Bou Selham, Morocco	IZEA2642	998	Ru2 Ru2.2
<i>Crocidura russula yebalensis</i>	Oukaimeden, Asni, Morocco	IZEA1239	273	Ru2
<i>Crocidura russula yebalensis</i>	Imlil, Asni, Morocco	IZEA2631	273	Ru2
<i>Crocidura russula yebalensis</i>	Imlil, Asni, Morocco	IZEA2633	273	Ru2
<i>Crocidura russula yebalensis</i>	Marrakech, Morocco	IZEA5058	273	Ru2
<i>Crocidura russula yebalensis</i>	Marrakech, Morocco	IZEA5060	273	Ru2
<i>Crocidura russula yebalensis</i>	Casablanca, Morocco	IZEA5073	273	Ru2
<i>Crocidura russula yebalensis</i>	Oukaimeden, Asni, Morocco	IZEA1572	998	Ru3 Ru3.1
<i>Crocidura russula yebalensis</i>	Aguelmane Aziga, Khenifra, Morocco	IZEA1237	998	Ru4 Ru4.1
<i>Crocidura russula yebalensis</i>	Aguelmane Aziga, Khenifra, Morocco	IZEA1582	273	Ru4
<i>Crocidura russula yebalensis</i>	Moulay Bou Selham, Morocco	IZEA2644	273	Ru5
<i>Crocidura russula yebalensis</i>	Skhirat, Rabat, Morocco	IZEA2639	273	Ru6
<i>Crocidura russula agilis</i>	Aïn Draham, Tunisia	IZEA4011	998	Ru7 Ru7.1
<i>Crocidura russula agilis</i>	Tunisia	IZEA4126	273	Ru8
<i>Crocidura russula ichnusae</i>	Gallura, Sardinia, Italy	IZEA649	273	Ru9
<i>Crocidura russula ichnusae</i>	Gallura, Sardinia, Italy	IZEA5680	998	Ru9 Ru9.1
<i>Crocidura russula ibicensis</i>	Ibiza, Balearic Archipelago, Spain	Libois-I36	273	Ru10
<i>Crocidura russula ibicensis</i>	Ibiza, Balearic Archipelago, Spain	Libois-I42	273	Ru10
<i>Crocidura russula cosyrensis</i>	Montagna Grande, Pantelleria, Italy	IZEA3895	998	Ru11 Ru11.1
<i>Crocidura russula cosyrensis</i>	Siba, Pantelleria, Italy	IZEA4184	998	Ru11 Ru11.2
<i>Crocidura osorio</i>	Gran Canaria, Canary Islands, Spain	IZEA3707	998	Os1 Os1.1
<i>Crocidura suaveolens</i>	Candelario, Salamanca, Spain	IZEA1203	998	Su1 Su1.1
<i>Crocidura leucodon</i>	Rechy, Valais, Switzerland	IZEA883	998	Le1 Le1.1

sites (gamma shape parameter = 1.419) and three different substitution types [rate (A-G) = 12.12; rate (C-T) = 17.28; and other rates = 1.00]. ML analyses were performed assuming this model and using the full heuristic search option

with a stepwise addition of sequences. As the sequence of taxon entry in phylogenetic reconstruction can bias the position in the tree (Maddison 1991), we systematically used 10 repeated randomized input orders for ML analyses.

Neighbour-joining (NJ) phylogenetic trees were constructed using the previously selected ML genetic distance using heuristic search with tree-bisection-reconnection (TBR) branch-swapping. Parsimony analyses were performed using the branch-and-bound search option; all characters having the equal weight.

Maximum likelihood, distance and parsimony results were compared for congruence of tree topologies. To test the robustness of nodes, we conducted a thorough bootstrap procedure using ML reconstructions with the TrN + I + G model, TBR branch-swapping algorithm, and 10 random input orders with stepwise-addition of sequences (500 pseudoreplicates for each). Support for nodes was also evaluated with the bootstrapping method using 500 pseudoreplicate data sets for parsimony and distance analyses.

The molecular clock hypothesis was tested using long sequences and according to Posada & Crandall (1998), by calculating the log-likelihood score with molecular clock enforced and comparing it with the log likelihood previously obtained without enforcing the molecular clock. In our case, the degree of freedom for the likelihood ratio test was 13 (number of OTUs = 2). Relative-rate tests were also done between the different lineages observed within *C. russula*. Tests were conducted with each lineage against the remaining lineages on the proportions of synonymous (*Ks*) and nonsynonymous (*Ka*) substitutions using *RTREE* version 1.0 (Robinson *et al.* 1998). The NJ tree rooted with *C. suaveolens* and *C. leucodon* sequences was chosen as the reference.

Divergence time was estimated from the molecular data according to the calibration developed for Soricidae by Fumagalli *et al.* (1999). This calibration is based on an estimate of 20 Myr for the split between Crocidurinae and Soricinae shrews and was developed considering cytochrome *b* sequence divergences based on third-position transver-

sions (tv). The estimate of divergence rate at third-position tv in shrews is 1.36% per Myr.

Results

The sequencing of the PCR fragments revealed 12 different haplotypes among the 998-bp sequences, and 11 different haplotypes among the 273-bp sequences. Within the long sequences, 247 sites were variable, of which 121 (49%) were parsimony-informative (including outgroups).

Pairwise sequence divergences (Kimura 2-parameter distances was chosen for comparison with other studies) varied from 0.001 to 0.091 across the different lineages of *Crocidura russula* (Table 2). Most of these values are within the range of variation observed between mammalian taxa at the intraspecies level, although the maximal values (up to 0.091) are particularly high (Avice *et al.* 1998; Johns & Avice 1998; Bradley & Baker 2001).

Phylogenetic relationships between haplotypes are given in Figs 2 and 3. As all methods, either using short or long sequences, gave similar topologies, only the tree resulting from maximum likelihood is shown, completed for long sequences with the bootstrap values from the NJ and MP analyses. The ML method gave one tree of score $-\ln L = 2771.20$. The distance method gave one tree of minimum evolution score equal to 0.596. The parsimony analysis provided 32 most parsimonious trees (CI = 0.81 excluding uninformative characters) with a tree length of 318 steps. The majority-rule consensus of the 32 MP trees had similar topology as the ML tree shown in Fig. 2.

The three phylogenetic methods strongly support the integrity of the clade grouping all sequences from the specimens referred to as *C. russula*, apart from those of the two outgroups, *Crocidura suaveolens* and *Crocidura leucodon*. Within the *C. russula* lineages, the only differences between

Table 2 Nucleotide divergence values for the 998-bp sequences calculated with the Kimura 2-parameter method

	Ru1.1	Ru1.2	Ru1.4	Ru1.3	Ru1.5	Os1.1	Ru2.1	Ru2.2	Ru3.1	Ru4.1	Ru7.1	Ru11.1	Ru11.2
Ru1.1	—												
Ru1.2	0.00301	—											
Ru1.4	0.00503	0.00201	—										
Ru1.3	0.00503	0.00201	0.00402	—									
Ru1.5	0.00807	0.00503	0.00301	0.00705	—								
Os1.1	0.00705	0.00402	0.00604	0.00604	0.00705	—							
Ru2.1	0.00705	0.00402	0.00604	0.00604	0.00503	0.00604	—						
Ru2.2	0.00604	0.00301	0.00503	0.00503	0.00402	0.00503	0.00100	—					
Ru3.1	0.01419	0.01215	0.01215	0.01419	0.01113	0.01418	0.01012	0.00910	—				
Ru4.1	0.00705	0.00402	0.00604	0.00604	0.00503	0.00604	0.00302	0.00201	0.01115	—			
Ru7.1	0.08951	0.08723	0.08723	0.08951	0.08496	0.08827	0.08503	0.08620	0.08973	0.08620	—		
Ru11.1	0.08951	0.08723	0.08723	0.08951	0.08496	0.08827	0.08503	0.08620	0.08973	0.08620	0.00909	—	
Ru11.2	0.08958	0.08730	0.08730	0.08958	0.08503	0.08834	0.08510	0.08627	0.08981	0.08627	0.00706	0.00201	—
Ru9.1	0.09062	0.08834	0.08834	0.09062	0.08606	0.08938	0.08613	0.08730	0.09084	0.08730	0.01010	0.00503	0.00503

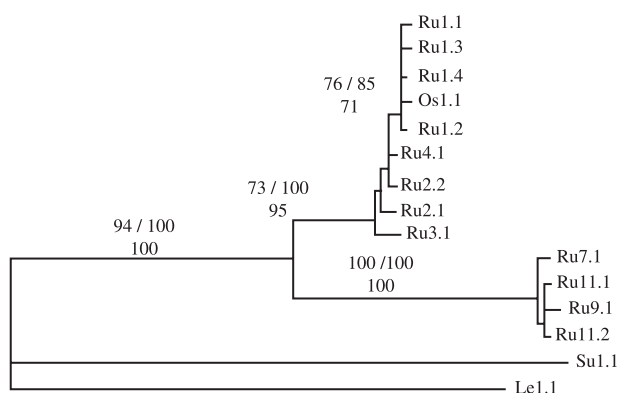


Fig. 2 Phylogeny of the 998-bp cytochrome *b* sequences analysed with the maximum-likelihood method using the TrN + I + G model of substitution and TBR branch swapping. The values at the branches are bootstrap values for maximum likelihood/distance analyses (percentage of 500 replications for each of 10 random orders for stepwise addition of sequences). Bootstrap values for the parsimony analysis using the branch-and-bound option are also indicated (percentage of 500 replications). Codes are as in Table 1.

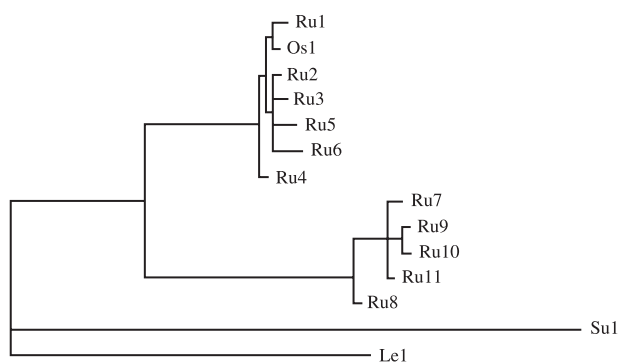


Fig. 3 Phylogeny of the 273-bp cytochrome *b* sequences analysed with maximum-likelihood method using the TrN + I + G model.

the different phylogenetic methods were the relative positions of the sequences into three well-defined clades. The first clade groups all European specimens, referred to as subspecies *Crocidura russula russula*, and specimens of *Crocidura russula osorio* from Gran Canaria. This grouping

is supported by high bootstrap values (76% for ML, 71% for MP, and 85% for NJ). The second clade groups sequences from the first clade with sequences from Moroccan specimens with high bootstrap values (73% for ML, 100% for MP, and 95% for NJ). Finally, the third and independent clade, groups sequences from Tunisia with those from the Mediterranean islands, is also supported by very high bootstrap values (100% for ML, 100% for MP, and 100% for NJ).

The statistical parsimony network estimated that the parsimony connection limit was 13 mutations in our data set (i.e. meaning that the maximum number of differences among haplotypes that are not the result of multiple substitutions at a single site is 13 with a 95% statistical confidence). As a result of their strong differentiation, eastern (Tunisia and Mediterranean islands) and western (Morocco & Europe) lineages are not connected by the network reconstruction (Fig. 4). The number of steps (i.e. mutations) separating the two clades was estimated to be 80. Within the western clade, haplotypes from Europe and Gran Canaria were derived from one ancestral haplotype not sampled in our data set. Within the eastern clade, haplotypes from the Mediterranean islands were derived from a common ancestral haplotype sampled in Pantelleria (as shown also by the phylogenetic reconstruction in Fig. 3).

Haplotype and nucleotide diversities within the 273-bp sequences varied greatly according to geographical origin, with North African ones showing high diversities and European ones showing no diversity at all. Among the North African haplotypes, those from Tunisia and Mediterranean islands were slightly more diverse than those from Morocco (Table 3).

The likelihood ratio test led us to accept the molecular clock hypothesis for the long cytochrome *b* sequence data ($\chi^2 = 12.93$, d.f. = 13, $P = 0.55$). The relative rate tests revealed no significant heterogeneity between the different lineages for synonymous or nonsynonymous substitutions ($P > 0.20$ for each test).

According to Fumagalli *et al.* (1999) the divergence rate at third-position tv in shrews is 1.36% per Myr. This calibration helped us to estimate the divergence times between the main lineages within *C. russula*, although it

Table 3 Number of samples and haplotypes, number of polymorphic sites, haplotype diversities and nucleotide diversities for the 273-bp sequences from different geographical regions

	Europe	North Africa	Morocco	Tunisia & islands
No. of examined samples	23	21	13	8
No. of different haplotypes	1	10	5	5
Polymorphic sites	0	13	7	6
Haplotype diversity	0.000 ± 0.000	0.848 ± 0.069	0.681 ± 0.132	0.893 ± 0.086
Nucleotide diversity	0.0000 ± 0.0000	0.0518 ± 0.0056	0.0046 ± 0.0015	0.0084 ± 0.0016

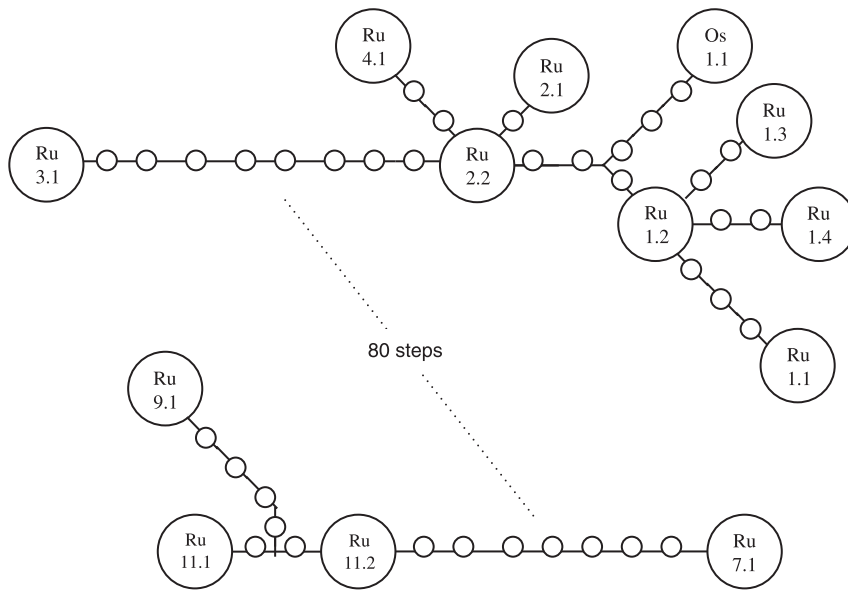


Fig. 4 Relationships between the 998-bp cytochrome *b* sequences reconstructed with the maximum-parsimony method and tcs. Small, white circles represent hypothetical haplotypes not found in the sample.

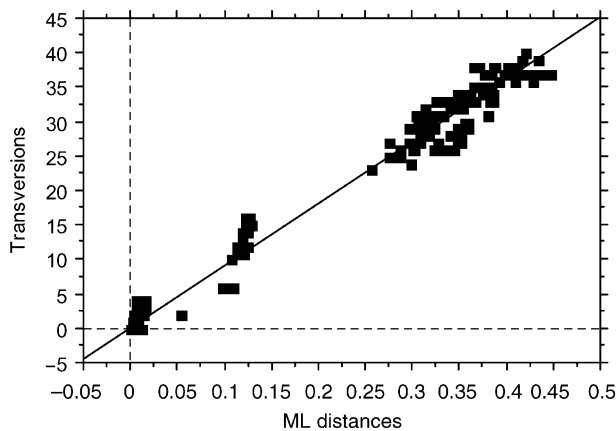


Fig. 5 Correlation between the number of third-position transversions and maximum-likelihood distances inferred with the TrN + I + G model of substitution for published cytochrome *b* sequences of the main lineages of the *Crocidura* species in Europe.

could not be used directly because of the low numbers of third-position tv observed. We thus estimated the correlation between the third-position tv and the maximum-likelihood distances inferred from an ML tree constrained to clock-like evolution after adding published sequences of the main lineages of *Crocidura* species in Europe (Vogel *et al.* 2003). A fairly good correlation was observed ($r = 0.973$, $P < 0.001$, Fig. 5) giving a rate of divergence of 0.051 increase in ML distance per Myr, with a 95% confidence interval of 0.044–0.070 per Myr. The ML distances between main lineages were then estimated using the formula in Edwards (1997) which corrects for ancestral mtDNA polymorphism within lineages. The rate of ML distance increas-

ing with time (0.051 per Myr) was then applied to the different dichotomies within *C. russula*, suggesting that the European and Moroccan populations separated from the Tunisian and Mediterranean island populations 2.21 (1.61–2.57) Ma; and that the European populations separated from the Moroccan populations 0.06 (0.05–0.08) Ma. These divergence dates are only approximate because of the low precision in dating fossil records and stochastic events occurring during lineage evolution within species (Edwards & Beerli 2000; Nichols 2001). However, they give useful information about the timing of the major splits that occurred within *C. russula* over its present range.

Discussion

Basic remarks about the phylogeography of the *Crocidura russula* group

Our investigation was based on a large sample covering the entire distribution of the *Crocidura russula* group. Our results confirm the monophyly of the shrews sharing a karyotype of $2N = 42$ chromosomes, as already described by Vogel *et al.* (2003). Shrews from Gran Canaria, Ibiza, Sardinia, and Pantelleria were clearly included in the *C. russula* clade. Haplotypes from North Africa are far more diverse than those collected in Europe. This supports the hypothesis that the *C. russula* group basically evolved in North Africa (Vogel & Maddalena 1987). We observed a deep split between western haplotypes (Morocco) and eastern ones (Tunisia) in the Maghreb. Lineages from Europe and Gran Canaria were closely related to those from Morocco, whereas haplotypes from Mediterranean islands were closely related to Tunisian lineages.

The colonization of Europe across the Strait of Gibraltar

The divergence between European and Moroccan populations was unexpectedly low, suggesting overwater dispersal across the 10- to 14-km wide Strait of Gibraltar, rather than a vicariance event dating back to the reopening of the strait. This very low diversity (Table 3) and the star-like topology of the network of European haplotypes (Fig. 4) suggest a strong bottleneck event, consistent with the recent invasion of the area by a fewer number of individuals. The relationship between European and Moroccan haplotypes suggests that the source population evolved in Morocco. However, the lack of samples from the south of the Iberian Peninsula does not make it possible to rule out the possibility of divergence of populations from the south of Spain followed by a recent expansion to the northern part of Europe, presumably following the last glacial age. Further studies are required to resolve this question.

The Strait of Gibraltar is deep and steep-sided. Its width did not decrease much when sea levels were low, and the strait did not dry out during the Quaternary. However, bathymetric maps of the western part of the strait indicate the occurrence of substantial shoals that became large islands during ice ages when sea levels were low. The length of the strait itself was increased to the west where some islands reduced the passage towards the Atlantic Ocean, forming a somewhat small interior and safe sea (Fig. 4 in Nehren 1992; Fig. 1 in Collina-Girard 2001). Islands probably formed visible land masses covered by vegetation, completely changing the appearance of the strait from either shore, and providing a stepping stone for humans to cross from Africa to Europe (Flemming *et al.* 2003). When sea levels were low, the maximum distance between two land masses from Morocco to Spain was only about five kilometres.

Crocidura russula is clearly unable to swim the distance between Africa and Europe, and even between either of the two continents and the Mediterranean islands. However, rafting on a natural support may potentially have occurred even though biogeographical data concerning the western Mediterranean (Dobson 1998) and other parts of the world (Heaney 1986) suggest that such events are extremely rare. The alternative means of crossing the Strait of Gibraltar are even less plausible. The connection of Moroccan and Iberian populations through a terrestrial route along the southern Mediterranean shore, via corridors along the Mediterranean coasts of the Sahara and the Levant, up to eastern Mediterranean, then back to the western Mediterranean along the northern shores, is hard to believe based on the existing palaeontological literature for the period and the area (Kowalski 1991; Geraads 1995; Tchernov 1996) and given that the species is currently absent from eastern parts of Europe and North Africa. Likewise, a crossing through the Sicily channel, which separates Tunisia from

Sicily (Italy), is not really compatible with the current ranges of the two *C. russula* lineages, as Tunisian lineages are well differentiated from Iberian and Moroccan ones.

Given the number and variety of small mammal species introduced to the Mediterranean islands (Vigne 1992), and the recent introduction of the North African wood mouse (*Apodemus sylvaticus*) from western Europe (Libois *et al.* 2001), it is possible that human activities led to the translocation of *C. russula* from Morocco to Spain. Our molecular dating analysis suggested that this species was transferred from Morocco to Europe across the Strait of Gibraltar about 60 000 (50 000–80 000) BP. This estimate coincides with possible human dispersal across the Strait of Gibraltar in the middle and upper Pleistocene. This question has been debated periodically for decades and still remains a hot issue for current archaeologists (Straus 2001). The records of human contacts between North Africa and Iberia are patchy and ambiguous, but there are data indicating that humans have migrated between these two areas since the middle Pleistocene (Alimen 1975). According to Straus (2001), the Strait of Gibraltar was a very effective natural barrier to human movements throughout the Pleistocene (between c. 30 000 and 40 000 BP). The first solid evidence of humans crossing the Strait of Gibraltar (similarity between lithic assemblages, marine fishing, and probable navigation) appears in the terminal Palaeolithic, about 25 000 BP (Bouzouggar *et al.* 2002; Collina-Girard 2003).

The discrepancy between our molecular timing analysis and the oldest documented trans-Strait of Gibraltar human movements may result from an overestimation of population divergence time from our molecular data, or just from lack of archaeological data. It is worth noting that haplotype divergence analyses systematically overestimate the amount of time that has passed before populations separated because haplotypes can diverge within the ancestral population well before the populations separate (Taberlet *et al.* 1998). A number of factors linked to demography, genetic drift, and genetic structure within the source population may have strong effects, making it hard to estimate the amplitude of the discrepancy between haplotype and population splits (Nichols 2001).

The phylogeographical structure within the Maghreb

The genetic differentiation between the western and eastern clades is particularly high (mean = 8.5%) in comparison with the general range of variation observed between mammalian taxa at the intraspecific level (Avise *et al.* 1998; Johns & Avise 1998; Bradley & Baker 2001). This genetic differentiation is far greater than that observed within each of these clades (mean = 0.4% for western clade, and mean = 0.6% for eastern clade). This explains also the high Nei's genetic distances observed in allozyme studies between continental European populations (western clade)

and insular populations (eastern clade) of Sardinia ($D = 0.084$, Catzefflis 1984), Ibiza ($D = 0.102$, Catalan *et al.* 1988), and Pantelleria ($D = 0.15$, Vogel *et al.* 2004). Based on our molecular data, the split occurred around 2.2 Ma, a time of great palaeoclimatic variability in the area, which was characterized by rapid alternations of dry and humid periods (Kowalski 1991). These climatic fluctuations may have played a central role in local adaptation and gene flow disruption between ancient *C. russula* populations in Algeria.

Because of the lack of sampling in Algeria, we do not know exactly where the two observed lineages meet in the field. The investigation by Sarà & Vogel (1996), based on multivariate analysis of mandibles and skulls collected in several sites from Tunisia to Morocco, showed a stepped cline with a transition zone situated in eastern Algeria. The fact that one outlier specimen from a Tunisian locality (Lac Melleguè) grouped with the Moroccan cluster (Sarà & Zanca 1992) may be an indication of a patchy pattern of distribution of morphotypes within the area. Further investigations along the Tunisian–Algerian border are needed to determine the characteristics of the contact zone of both clades and morphotypes in the field.

The colonization of the Atlantic and Mediterranean islands

According to our data, the Atlantic islands were recently colonized by source populations from the western clade (Europe–Morocco). *C. russula* probably reached some of the continental islands along the west coast of France during the regression of the Holocene sea, given its presence on all islands separated from the continent by a shallow stretch of sea (Cosson *et al.* 1996). Some more remote islands, subjected to regular boat traffic with the continent, were colonized later on with the accidental help of humans. This was probably the case for Gran Canaria following the Spanish colonization (Vogel *et al.* 2003) and for the tiny Sein Island in Brittany (Cosson *et al.* 1996).

Greater white-toothed shrews found on the Mediterranean islands (Ibiza, Sardinia, and Pantelleria) were all derived from the eastern clade (Tunisia). The most recently discovered taxon was *Crocidura cossyrensis* Contoli, which was found on Pantelleria (an island situated between Sicily and Tunisia) in 1989. There is some debate about whether this is indeed a real species (Contoli *et al.* 1989; Contoli 1990, 1992; Vogel *et al.* 1992; Hutterer 1993; Vogel *et al.* 2004). Our study shows that *C. cossyrensis* Contoli is closely related with *C. russula* populations from Tunisia.

No fossils of *C. russula* from before the Holocene, and more precisely from before the development of regular and intensive boat traffic between the islands and the continents, have been found on the Mediterranean islands. Whether introduction was passive or deliberate is still a matter of debate, but there is good evidence and a general

consensus that *C. russula*, like other mammal species, was introduced by humans (Alcover 1980; Cheylan 1984; Vigne & Alcover 1985). Small mammals like shrews probably reached islands during the trade of agricultural goods, as has previously been suggested for the introduction of *Crocidura suaveolens* in Crete during the Minoan period (Vogel *et al.* 1986). According to Alcover & Vesmanis (1985), *C. russula* arrived in Sardinia around 8000 BP. Its arrival in Ibiza is not documented (Alcover 1980).

Insights into the Quaternary legacy within the Maghreb

Hewitt (2000) used the term 'Quaternary legacy' to encompass evolutionary events related to climatic fluctuations during the Quaternary. In contrast to other continents and even sub-Saharan Africa, very little is known about the footprints left by the Quaternary climatic fluctuations in North Africa. The Maghreb region provides an interesting example in this regard. The region is limited to the west by the Atlantic Ocean and to the north by the Mediterranean Sea. The geographically close Iberian Peninsula is separated by the Strait of Gibraltar to the north. East and south, the Maghreb is bordered by an arid zone that extends for several thousand kilometres across the Sahara Desert. Palaeontological records indicate that numerous major ecological changes have occurred within the region in the last million years (Jamet 1991). Recent climatic models and studies of Holocene plant fossils suggest that the western Sahara desert was considerably smaller during the time between 2 and 3 Ma. A long-lasting wet phase that ended around 1.6 Ma was immediately followed by a hyperarid period around 1.5 Ma. The size and aridity of the Sahara Desert then continued to fluctuate considerably, with a frequency of about 41 000 years over between 0.9 and 1.5 Ma, then slowly (100 000 years) from 0.1 to 0.9 Ma, and more rapidly again thereafter. The western Sahara was probably particularly small in the early and mid-Holocene (6000–9000 BP) with the expansion of grassland into the Sahara (Jolly *et al.* 1998; Claussen *et al.* 2003). Throughout the Quaternary, North Africa and the Sahara were characterized by an *Artemisia* steppe intermingled with very big lakes (Suc *et al.* 1995; Faure 1987). This environment expanded and retracted according to climatic oscillations, until the last glacial Würmian age, when two refuge areas in northwestern and northeastern Africa existed (Brown & Gibson 1983; Jolly *et al.* 1998).

It is possible that Maghreb biota regularly expanded to the south during mesic periods when most of the western part of the Sahara was vegetated by xerophytic woods/shrub and warm grass (Claussen & Gayler 1997). During arid periods, the isolation of populations in separate Maghreb refuges (Brown & Gibson 1983) may have led to allopatric differentiation and strong phylogeographical structures. This phenomenon is currently poorly understood,

but is thought to explain morphological variations in the lizard *Acanthodactylus erythrurus* (Bons & Geniez 1995) and the land snail *Helix aspersa* (Madec *et al.* 1996), as well as genetic differentiation in the agamid lizards *Agama impalearis* (Brown *et al.* 2002) and *H. aspersa* (Guiller *et al.* 2001). Our study of the *C. russula* group provides further evidence of morphological and genetic variations related to the Quaternary period within the Maghreb.

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