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Evolutionary history of the greater white-toothed shrew (*Crocidura russula*) inferred from analysis of mtDNA, Y, and X chromosome markers

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Abstract

We investigate the evolutionary history of the greater white-toothed shrew across its distribution in northern Africa and mainland Europe using sex-specific (mtDNA and Y chromosome) and biparental (X chromosome) markers. All three loci confirm a large divergence between eastern (Tunisia and Sardinia) and western (Morocco and mainland Europe) lineages, and application of a molecular clock to mtDNA divergence estimates indicates a more ancient separation (2.25 Myr ago) than described by some previous studies, supporting claims for taxonomic revision. Moroccan ancestry for the mainland European population is inconclusive from phylogenetic trees, but is supported by greater nucleotide diversity and a more ancient population expansion in Morocco than in Europe. Signatures of rapid population expansion in mtDNA, combined with low X and Y chromosome diversity, suggest a single colonization of mainland Europe by a small number of Moroccan shrews >38 Kyr ago. This study illustrates that multilocus genetic analyses can facilitate the interpretation of species' evolutionary history but that phylogeographic inference using X and Y chromosomes is restricted by low levels of observed polymorphism.
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Keywords: Sex-specific loci; Y-chromosome; X-chromosome; mtDNA; Gibraltar Strait; Colonization; Expansion; Phylogeography; Bayesian analysis

1. Introduction

Surprisingly few mammal species are shared between Morocco and Iberia (Dobson, 1998), despite similar habitats and close geographic proximity. Though only a few kilometres wide, the Strait of Gibraltar has apparently imposed a very important barrier to dispersal, preventing the passage of terrestrial fauna since its reopening at the start of the Pliocene about 5 million years (Myr) ago.

One species that is shared between these two regions, the greater white-toothed shrew *Crocidura russula* (Hermann, 1780), deserves attention since its distribution suggests that it has overcome the Mediterranean sea as a barrier to dispersal several times. The species is found in northern Africa from Tunisia to Morocco, and in Europe from the Iberian Peninsula to the western regions of Switzerland and Germany (Hutterer, 1986), as well as the Mediterranean islands of Sardinia (Catzeflis et al., 1985), Ibiza and Pantelleria (Cosson et al., 2005). Among the several subspecies recognized, the deepest genetic (karyotypic, isozyme, and mitochondrial (mt) DNA) and morphological divergence separates western (Moroccan *Crocidura russula yebalensis* and mainland European *Crocidura russula russula*) from eastern lineages (*Crocidura russula agilis* from Tunisia, *Crocidura russula ichnusae* from Sardinia, *Crocidura russula cossyrensis* from Pantelleria and

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Crocidura russula ibicensis from Ibiza, Cosson et al., 2005; Lo Brutto et al., 2004; Sarà and Vogel, 1996; Vogel et al., 2003, 2004). Although an Iberian origin for the species has been proposed based on fossil reports (Dobson, 1998; Dobson and Wright, 2000) genetic evidence collected so far, points to an African origin followed by northwards expansion of the eastern and western lineages (Cosson et al., 2005; Lo Brutto et al., 2004).

This colonization ability is of particular interest given the synanthropic nature of the species. Particularly in cold climates (which might have prevailed at the time of colonization), *C. russula* favours human-inhabited areas because its winter survival depends on heat and nutritive resources generated by human activities (Genoud and Hausser, 1979). As a result, it has been hypothesized that *C. russula* colonized mainland Europe (as well as several Mediterranean islands) following accidental transport by humans (Lo Brutto et al., 2004; Poitevin et al., 1986; Vogel et al., 2003). On the basis of first fossil appearance of *C. russula* in France, European colonization has been suggested to be as recent as 4–5.5 thousand years (Kyr) ago (Poitevin et al., 1986). In contrast, a recent analysis of a mitochondrial gene (cytochrome *b* *Cytb*, Cosson et al., 2005) suggests a much earlier colonization event, approximately 60 Kyr ago. This predates the first solid evidence of human exchange across the Strait of Gibraltar (around 25 Kyr ago Bouzouggar et al., 2002; although modern humans are thought to have colonized Europe from the Levant 40 Kyr ago, Jobling et al., 2004), and therefore throws into question the hypothesis of human transport. Based on these same *Cytb* data, Cosson et al. (2005) also suggest a very ancient separation (2.21 Myr) of eastern and western lineages of *C. russula*. This is in strong contrast with the 0.5 Myr suggested by Lo Brutto et al. (2004) on the basis of another mitochondrial gene (12S rRNA).

Given these controversial claims, investigation of other genomic regions with different inheritance patterns and mutation rates is warranted, to obtain a more accurate picture of the species' evolutionary history. Since the early 1990s mtDNA has been extensively used in phylogeography due to its transmission without recombination (but see Hagelberg, 2003; Rokas et al., 2003), high mutation rate, and the relative technical ease of its use thanks to the availability of universal primers for PCR amplification (e.g. Kocher et al., 1989). Unfortunately mtDNA only provides information about the female germ line and the rapid evolution of mtDNA makes it prone to mutational saturation (homoplasy) over long evolutionary timescales. In contrast, the mammalian Y chromosome has strict paternal inheritance and a slow mutation rate relative to mtDNA (Schaffner, 2004). Therefore, studying both mtDNA and the Y chromosome should enable comparative analysis firstly of genes with different patterns of inheritance and secondly of recent and ancient evolutionary history. The utility of the Y chromosome may unfortunately be restricted due to low levels of variation (e.g. Hellborg and Ellegren, 2004), and more variable nuclear regions are therefore required. Since the X chromosome is biparentally inherited but haploid in males (and therefore easily accessible to genotyping), it provides an opportunity to examine information about both male and female lineages. Moreover, whilst the X has a low mutation rate relative to mtDNA and Y (Schaffner, 2004), it is potentially more variable than the Y due to greater copy number per breeding pair (3X versus 1Y), making it a potentially informative marker for population genetic studies.

In the present study, we perform comparative analyses of mtDNA, X, and Y chromosome markers to provide a more complete evolutionary history of the greater white-toothed shrew. First, we re-evaluate the divergence between eastern and western lineages. Second, we rigorously test the hypothesis of Moroccan ancestry for the European population and examine the timing and dynamics of European colonization and population expansion.

2. Methods

2.1. Sampling, DNA extraction, and locus information

We analyzed a total of 53 male samples of *C. russula* collected between 1977 and 2002 from 19 localities throughout the species' range (Table 1 and Fig. 1). Four individuals of a sister species, *Crocidura leucodon*, were also analyzed as well as one individual of *Crocidura canariensis*, which is more distantly related (Vogel et al., 2003). Total DNA was extracted from frozen toe clips (following collection described in Ehinger et al., 2002) or ethanol-stored soft tissues (Vogel et al., 2003) using a salt/chloroform procedure modified from Miller et al. (1988) by adding one step of chloroform/isoamylalcohol (24:1) purification.

A combined total of over 5 kb of DNA was amplified and sequenced from the Y chromosome (2129 bp including 4 introns *UTY11*, *DBY4*, *DBY8*, *DBY14*, and one coding region, *sry HMG box*), X chromosome (1169 bp from introns *AMGX4* and *ZFX6*) and mtDNA (2240 bp from the control region *CR*, cytochrome oxidase II *COXII* and *Cytb*) using PCR primers detailed in Table 2. PCRs generally contained ~40 ng DNA, 0.2 μM each primer, 0.2 mM dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$, and 1.25 U *Taq* polymerase (Qiagen) in a 50 μl total volume, with the exception of *AMGX4*, with which 2.5 mM $MgCl_2$ and 1× Q solution (Qiagen) were added. PCRs were performed in a PE9700 (Perkin Elmer) thermocycler with cycling conditions as follows: initial denaturation at 95 °C for 5 min, followed by 30–35 cycles of 95 °C for 30–45 s, annealing (T_n, Table 2) for 30–60 s and

Table 1
Sampling information

Species	Sample location	ID codes[a]	Haplotypes		
			Y	X	mtDNA
C. canariensis	Fuerteventura, Spain	IZEA4285	H12	H14	H32
C. leucodon	Brigerbad, Valais, Switzerland	IZEA7526	H11	H13	—
C. leucodon	Réchy, Valais, Switzerland	IZEA7552	H11	H13	H29
C. leucodon	Visp, Valais, Switzerland	IZEA7553	H11	H13	H30
C. leucodon	Quartino, Tessin, Switzerland	IZEA5455	H11	H13	H31
C. russula russula	Sion, Valais, Switzerland	IZEA5564	H2	H1	H17
C. russula russula	Sion, Valais, Switzerland	IZEA7518	H2	—	H16
C. russula russula	Bassins, Vaud, Switzerland	PF10129	H2	H1	H11
C. russula russula	Tartegnin, Vaud, Switzerland	PF10245	H2	H1	H14
C. russula russula	Auvernier, Neuchâtel, Switzerland	PF11003	H2	H1	H15
C. russula russula	Auvernier, Neuchâtel, Switzerland	PF11006	H2	H1	—
C. russula russula	Haut-Geneveys, Neuchâtel, Switzerland	PF11018	H2	H1	H13
C. russula russula	Huemoz, Valais, Switzerland	PF11091	H2	—	—
C. russula russula	Les Paccots, Fribourg, Switzerland	PF11184	H2	H1	—
C. russula russula	Châtel-St-Denis, Fribourg, Switzerland	PF11187	H2	H1	—
C. russula russula	Châtel-St-Denis, Fribourg, Switzerland	PF11195	H2	—	—
C. russula russula	St-Cergue, Vaud, Switzerland	PF11231	H2	H1	H12
C. russula russula	Bonn, Germany	IZEA5461	H6	H1	H3
C. russula russula	Candelario, Spain	IZEA6144	H6	—	—
C. russula russula	Candelario, Spain	IZEA6145	H2	—	—
C. russula russula	Candelario, Spain	IZEA5931	H2	—	—
C. russula russula	Candelario, Spain	IZEA5935	H2	—	—
C. russula russula	Candelario, Spain	IZEA5936	H2	H1	H1
C. russula russula	Garsola, Spain	IZEA1685	H2	H1	H2
C. russula russula	Roscoff l′Aber, France	IZEA5547	H2	H2	H1
C. russula russula	Roscoff l′Aber, France	IZEA5550	H6	H1	H1
C. russula russula	Roscoff l′Aber, France	IZEA5545	H2	H1	H8
C. russula russula	Roscoff l′Aber, France	IZEA5548	H2	H1	H1
C. russula russula	Roscoff l′Aber, France	IZEA5551	H2	H8	H7
C. russula russula	Goudargue, France	IZEA5802	H2	—	—
C. russula russula	Goudargue, France	IZEA5790	H2	H1	H9
C. russula russula	St Mortan, Ardèche, France	IZEA5785	H2	H4	H4
C. russula russula	St Mortan, Ardèche, France	IZEA5786	H2	H4	H5
C. russula yebalensis	Moulay-Bousselham, Morocco	IZEA2646	H1	—	H19
C. russula yebalensis	Moulay-Bousselham, Morocco	IZEA2647	H2	H6	H10
C. russula yebalensis	Moulay-Bousselham, Morocco	IZEA2642	H1	H3	H18
C. russula yebalensis	Moulay-Bousselham, Morocco	IZEA2643	H3	H7	H20
C. russula yebalensis	Marakesch, Morocco	IZEA5059	H3	—	H21
C. russula yebalensis	Marakesch, Morocco	IZEA5060	H2	—	—
C. russula yebalensis	Oukaimeden, Asni, Morocco	IZEA5061	H3	—	H22
C. russula yebalensis	Oukaimeden, Asni, Morocco	IZEA5062	H3	—	—
C. russula yebalensis	Oukaimeden, Asni, Morocco	IZEA5063	H3	—	H25
C. russula yebalensis	Oukaimeden, Asni, Morocco	IZEA2622	H3	H10	H24
C. russula yebalensis	Oukaimeden, Asni, Morocco	IZEA2626	H3	H9	H23
C. russula yebalensis	Imlil, Asni, Morocco	IZEA2631	H4	—	—
C. russula yebalensis	Imlil, Asni, Morocco	IZEA2633	H3	H9	H23
C. russula yebalensis	Imlil, Asni, Morocco	IZEA2636	H1	—	—
C. russula yebalensis	Skhirat, Rabat, Morocco	IZEA2639	H5	H7	—
C. russula yebalensis	Skhirat, Rabat, Morocco	IZEA2641	H5	H5	H26
C. russula yebalensis	Casablanca, Morocco	IZEA5073	H3	—	—
C. russula russula	Unahis da Serra, Portugal	IZEA5917	H7	—	H6
C. russula russula	Unahis da Serra, Portugal	IZEA5922	H2	—	—
C. russula russula	Unahis da Serra, Portugal	IZEA5923	H2	—	—
C. russula russula	Unahis da Serra, Portugal	IZEA5926	H2	—	—
C. russula ichnusae	Gallura, Sardaigne, Italy	IZEA5678	H10	H11	H27
C. russula agilis	Ain Drahan, Tunisia	IZEA4011	H9	—	—
C. russula agilis	Ain Drahan, Tunisia	IZEA3897	H8	H12	H28
C. russula agilis	Ain Drahan, Tunisia	IZEA3898	H8	H12	—

[a] ID codes: "IZEA" refers to samples maintained in the University of Lausanne collection, whereas those designated "PF" were donated from the collection of Pierre Fontanillas.

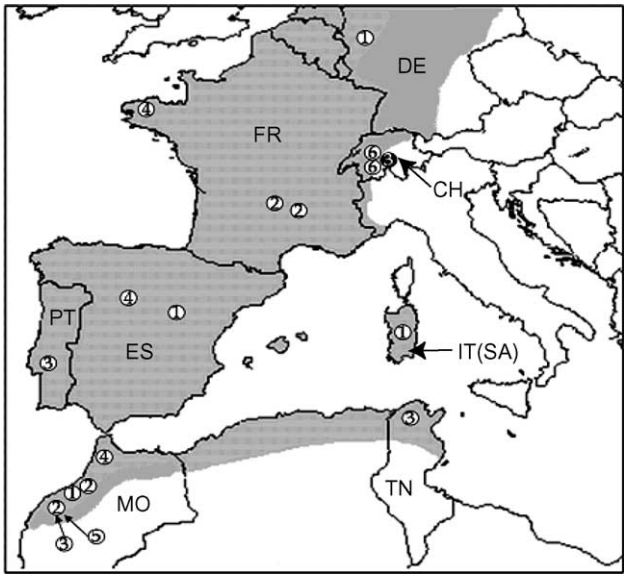


Fig. 1. Sampling information: numbers refer to the number of individuals from each location (white circle = *C. russula*, black circle = *C. leucodon*). The grey area indicates the geographical range of *C. russula*. Country codes, are as follows: CH Switzerland, FR France, PT Portugal, DE Germany, ES Spain, MO Morocco, IT Italy = Sardinia, TN Tunisia.

72 °C for 60–90 s, and a final extension of 72 °C for 10 min. In the case of a touchdown program (Table 2), annealing temperature was decreased from 55 to 45 °C (TD55-45) or from 60 to 50 °C (TD60-50) by 0.5 °C/cycle in the first 20 cycles, and followed by 20 cycles at the lower annealing temperature. Internal PCR primers were designed for *UTY11* and *DBY14* and nested amplifications performed with 1 μl of the product of the first PCR to increase the quantity of PCR product and specificity of the reaction. Specificity of Y chromosome primers was verified by absence of amplification products in females.

PCR products were purified using the QIAQuick PCR purification kit (Qiagen), and eluted in a 30 μl dH_2O. Sequencing was performed in both directions for all loci in a 7.5 μl volume containing approximately 100 ng of purified DNA, 1 μM primer and 3.5 μl of BigDye Terminator Kit v3.1 (Perkin Elmer). The sequencing reaction consisted of 28 cycles of 96 °C for 15 s, 50 °C for 15 s, and 60 °C for 2 min. Products were precipitated with ethanol and analyzed on an ABI Prism 3100 (Applied Biosystems). Sequences were aligned using ClustalW (available at http://npsa-pbil.ibcp.fr) using a gap opening penalty of 10 and a gap extension penalty of 0.1, and then checked by eye. Haplotypes were identified in MacClade 3.08 (Sinauer Associates, Sunderland, MA). Alignment gaps, which were found for all loci except *Cytb* and *COXII*, were excluded in analyses. GenBank accession numbers for the respective loci are given in Table 2 and the alignments generated in this study have been submitted to the TreeBASE database (http://www.treebase.org, submission number SN2362).

2.2. Phylogenetic analysis

Phylogenetic analyses were performed for mtDNA, X, and Y separately. We used an hierarchical likelihood ratio test implemented in MODELTEST v3.06 (Posada and Crandall, 1998) to select the appropriate substitution models. Since different models were selected for each of the three mtDNA regions (*CR*, *Cytb*, and *COXII*), specific substitution models were applied to each region (= partition, Table 3). X and Y were not partitioned further since the chosen substitution models were appropriate to all loci situated on the same chromosome (Table 3). We chose to perform a Bayesian phylogenetic analysis since it permits the combination of information from heterogenous data partitions evolving under different evolutionary models (as is the case with our mtDNA data, Table 3). Bayesian approaches were chosen over maximum likelihood (ML) methods due to computational efficiency (Larget and Simon, 1999). We used a Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) (Hastings, 1970; Metropolis et al., 1953) sampling regime, starting from a random topology, to move through possible trees and estimate the posterior probability distribution, implemented in MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Four chains were run, three of which were "heated" with $\beta = 0.2$, (where heat $= 1/(1+\beta(\mathrm{ID}-1))$, and ID is 1, 2, 3 or 4). We ran 1,000,000 and 5,000,000 generations of MCMCMC, with a burnin of 20,000 generations, and sampled every 100th or 1000th generation. Fifty percent majority rule consensus trees were then constructed from saved trees and the posterior probability calculated for the nodes. Gaps were excluded from all analyses and in each case *C. canariensis* was used as outgroup. Bayesian parameters and tree files are included in our TreeBASE submission.

Phylogenetic trees were also constructed using maximum parsimony and neighbour-joining (NJ) criteria with PAUP*4.0b10 (Swofford, 2002) and support obtained after 1000 bootstrap replicates. For the NJ analyses substitution models were the same as in Table 3, but the HKY85 model (chosen using MODELTEST) was used for mtDNA when the three regions were analyzed together. Since concordant topology was found for all analyses, only the Bayesian results are presented.

2.3. Genetic diversity within and between populations

The number of segregating sites (S), haplotypes (H), haplotype diversity (Hd) and observed nucleotide diversity (π) were calculated for Moroccan and European populations using DNAsp 4.0 (Rozas et al., 2003) in order to test the hypothesis of Moroccan ancestry (Table 4). We predict lower nucleotide diversity in Europe than in Morocco if the Moroccan population is ancestral. Mean within-group divergence was calculated

Table 2
Details of loci employed and PCR primers used

	Locus	Size	Accession	Forward primer		Reverse primer		T_n
mtDNA	*CR*	591	AY918341–AY918372	L16517[a]	CATCTGGTTCTTACTTCAGG	sH651[b]	AAGGCTAGGACCAAACCT	45
	COXII	745	AY918309–AY918340	L6296[c]	TGGAAGTGTCGAGTTGTGG	H7049[c]	TAGGCTTACAAGATGGCAC	55
	Cytb	904	AY918373–AY918404	L14841[d]	AAAAAGCTTCCATCCAACATCTCAG CATGATGAAA	H15915[b]	AACTGCAGTCATCTCCGGTTT ACAAGAC	50
Y	*SRY*	159	AY918439–AY918449	SRY-F1[e]	CATGGTGTGGGCTCGCAATC	SRY-R1[e]	CTGCCTGTAGTCTCTGTGCC	TD60-50
	DBY14	734	AY918427–AY918438	DBY14-F[f]	CAAGAAGTGCCTTCTTGGTTG	DBY14-R[f]	GGCTCCAAATCCTCCACTG	TD60-50
				DBY14-NF1[c]	TGAACATCGGTATCGGGGTAGC	DBY14-NR1[c]	TGAATGGGTGGAGGAGCAG	65
	DBY4	285	AY918405–AY918414	DBY4-F[f]	TGATGGTATTGGYRRTCGTGA	DBY4-R[f]	CGGTTGCCTCTACTGGTATA	TD55-45
	DBY8	175	AY918415–AY918426	DBY8-F[f]	CCCCAACAAGAGAATTGGCT	DBY8-R[f]	CAGCACCACCATAKACTACA	TD60-50
	UTY11	776	AY918450–AY918461	UTY11-F[f]	CATCAATTTTGTAYMAATCCAAAA	UTY11-R[f]	TGGTAGAGAAAAGTCCAAGA	TD55-45
				UTY11-NF1[c]	CTGTAACTGTAATACGTGGCCTTGC	UTY11-NR1[c]	AGATGACTATTGGGACTGG	50
X	*ZFX 6*	825	AY918462–AY918475	ZF6-F3[c]	AAGACCTGATTCCAGGCAGTACC	ZF6-R1[c]	GCTTGTGGCTCTCCAKGTG	57
	AMGX4	344	AY918297–AY918308	AMG4-F1[c]	GAAGTGGTACCAGAGCATG	AMG4-R1[c]	TGGTGGTGCAGCCATCCAC	TD55-45

Size (in bp) is the length of sequence analyzed for *C. russula*. Accession is GenBank Accession Numbers. T_n is annealing temperature used in the PCR (where "TD" represents a touchdown procedure).

[a] Fumagalli et al. (1996).
[b] Kocher et al. (1989).
[c] This study.
[d] Irwin et al. (1991).
[e] Matsubara et al. (2001).
[f] Hellborg and Ellegren (2003).

Table 3
Model parameters for Bayesian phylogenetic analyses

	Locus	Partition[a]	Model[b]	Nst[c]	α[d]	(A,C,G,T)[e]	(A–C,A–G,A–T,C–G,C–T,G–T)[f]	T_i/T_v[g]
mtDNA	*COXII*	1–745	TrN+G	6	0.1694	(0.33,0.22,0.13,0.32)	(1,8.6,1,1,19.9,1)	See[h]
	Cytb	746–1648	TrN+G	6	0.2297	(0.29,0.27,0.13,0.31)	(1,5.9,1,1,10.9,1)	See[h]
	CR	1649–2287	HKG+G	2	0.1817	(0.30,0.16,0.20,0.34)	(1,1,1,1,1,1)	2.87
	Y (combined)	1–2141	HKY+G	2	0.2883	(0.31,0.18,0.19,0.32)	(1,2.3,0.6,0.6,2.3,1)	1.37
	X (combined)	1–1176	HKY	2	Equal*	(0.31,0.19,0.18,0.32)	(1,2,0.6,0.6,1.3,1)	1.01

[a] Partition in alignment, with gaps included. The number of sites excluding gaps was: 2200 bp for mtDNA, 2014 bp for the Y chromosome and 113 bp for X.
[b] Substitution model chosen using MODELTEST (Posada and Crandall, 1998); TrN Tamura and Nei (1993), +G with gamma distribution shape parameter, HKY (Hasegawa et al., 1985).
[c] Number of substitution types incorporated in Bayesian analysis.
[d] Shape parameter of the gamma distribution, where Equal* refers to equal rates for all sites.
[e] Base frequencies.
[f] Substitution frequencies.
[g] Transition/transversion ratio estimated using MODELTEST.
[h] Empirical values for each substitution type given in footnote f were used for *COXII* and *Cytb*, because of the large difference between the rate of A–G and C–T transitions.

Table 4
Genetic diversity within Morocco and Europe for mtDNA, X, and Y chromosome loci

	X		Y		mtDNA	
	Morocco	Europe	Morocco	Europe	Morocco	Europe
L	1169	1168	2129	2129	2239	2239
n	8	20	17	32	11	19
S	13	5	7	3	37	37
H	6	4	5	3	10	16
Hd	0.929 (0.084)	0.363 (0.131)	0.699 (0.102)	0.232 (0.094)	0.982 (0.046)	0.965 (0.036)
$\pi \times 10^{-3}$	3.82 (0.53)	0.5 (0.24)	1.26 (0.22)	0.18 (0.08)	5.13 (0.60)	2.96 (0.35)
$p \times 10^{-3}$	0.38 (0.14)	0.05 (0.02)	0.13 (0.06)	0.01 (0.01)	0.51 (0.08)	0.30 (0.06)

L, sites excluding gaps; n, number of individuals; S, number of segregating sites; H, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity per site; p, percentage within population p-distances. Associated standard errors calculated after 500 bootstrap replicates are given in parentheses where applicable.

for Moroccan and European populations (Table 4) and net sequence divergence (which corrects for within phylogroup diversity, Avise and Walker, 1998) was estimated between Morocco, Europe, the eastern lineage (Tunisia plus Sardinia) and *C. leucodon* (Table 5, note in this case *C. leucodon* was used as an outgroup in preference to *C. canariensis* since four samples were available for the former as opposed to one for the latter). In both cases simple p-distances were used to estimate divergence, but sites with multiple hits were excluded, and standard errors were calculated after 500 bootstrap replicates using MEGA v2.1. (Kumar et al., 2000).

2.4. Divergence time between eastern and western lineages

Divergence time between eastern and western lineages was estimated from mtDNA *CR* alone since an estimate has recently been published based on *Cytb* sequences (Cosson et al., 2005). Divergence was estimated from $T = P/2\mu$, where P is the net p-distance (Avise and Walker, 1998; Table 5) between Moroccan and Tunisian populations and μ is the mutation rate per base (12.6×10^{-9} subs/site/yr, for mammalian mtDNA *CR*, Pesole et al., 1999). We assume an average generation time of one year for shrews, given that their life cycle is mostly annual with fewer than 5% of individuals surviving a second winter (Jeanmaire-Besançon, 1986).

Table 5
Divergence between populations

	Europe	Morocco	Tunisia	*C. leucodon*
mtDNA				
Europe ($n = 22$)	—	0.085	0.465	0.640
Morocco ($n = 11$)	0.304	—	0.472	0.635
Tunisia ($n = 1$)	5.802	5.806	—	0.576
C. leucodon ($n = 3$)	11.972	12.005	10.344	—
Y				
Europe ($n = 33$)	—	0.030	0.293	0.522
Morocco ($n = 16$)	0.043	—	0.298	0.526
Tunisia ($n = 3$)	1.509	1.559	—	0.505
C. leucodon ($n = 4$)	4.600	4.647	4.588	—
X				
Europe ($n = 20$)	—	0.017	0.237	0.700
Morocco ($n = 8$)	0.033	—	0.206	0.677
Tunisia ($n = 2$)	0.677	0.612	—	0.706
C. leucodon ($n = 4$)	4.278	4.195	4.547	—

Average percentage net p-distances (lower diagonal) and associated standard errors calculated after 500 bootstrap replicates (upper diagonal). The number of individuals analyzed for each population is denoted as "n." The number of nucleotides included in the analyses was 2222 bp for mtDNA, 2020 bp, for Y and 1167 bp for X.

2.5. Mismatch distributions

We computed mtDNA mismatch distributions (the distribution of pairwise differences) from European and Moroccan populations separately, to test the hypothesis of Moroccan ancestry and estimate the time since European colonization (corresponding to the onset of population expansion). If the Moroccan population is ancestral we expect to find a more ancient signature (if any) of population expansion. It would be interesting to repeat this analysis with X and Y chromosome loci in order to differentiate between population expansion (which affects the whole genome) and selection (which affects the distribution of pairwise differences in a similar way, but should be locus-specific), but polymorphism is too low at these loci for this analysis to be informative (Pereira et al., 2001).

All three mtDNA regions were grouped together for this analysis in order to sample the maximum number of substitutions. Mismatch distributions were computed using Arlequin v2.000 (Schneider et al., 2000). The time since expansion, t, was estimated from $\tau = 2ut$ (where τ is the time since expansion in units of $1/2u$ generations), using a generalized least squares approach adapted from Rogers (1995), as described in Schneider and Excoffier (1999). The mutation rate estimated for the three mtDNA regions combined, $u = 3.56 \times 10^{-5}$ substitutions per generation, was obtained from $u = D/2T$, where $D = 160.1$ is the average number of substitutions separating Moroccan and Tunisian populations and $T \approx 2.25$ Myr is the time since divergence of these populations estimated by us from *CR* and from *Cytb* by Cosson et al. (2005). Female population size before and after expansion (N_0 and N_1) was then estimated from $\theta_0 = 2uN_0$ and $\theta_1 = 2uN_1$ respectively, where θ is the expected nucleotide diversity. Finally Tajima's D (Tajima, 1989a,b) and Fu's Fs (Fu and Li, 1993; Fu, 1997) were also calculated from the mismatch distribution and their significance tested by 1000 randomization replicates in Arlequin v2.000. These statistics are sensitive to an excess of rare alleles which can be explained by selection as well as population expansion.

3. Results

3.1. Phylogenetic analyses

Majority rule consensus trees for mtDNA, X, and Y, based on 1,000,000 MCMCMC generations, sampling every 100th generation, are shown, respectively, in (Figs. 2A–C). Posterior probabilities are high for the major clades, with good support for the monophyly of *C. russula* and separation of eastern (Tunisia plus Italy (Sardinia)) and western (Morocco plus continental Europe) lineages for all three trees. The same basic topologies were obtained when 5,000,000 generations and a sample frequency of every 1000 generations was used. The sole exception was that the placement of mtDNA haplotype 18 (H18, Fig. 2A), was basal to the western clade. This reduced the posterior probability of the node leading to the Moroccan and European clades to 0.51 instead of 1.00. Monophyly of *C. russula*, which has been described previously for mtDNA (Cosson et al., 2005; Vogel et al., 2003), was confirmed by all three loci.

A large divergence, which is always well supported, is seen between the eastern and western lineages for all three loci, although the branch separating these two clades is shorter (relative to outgroup taxa) for X than for Y or mtDNA. This potentially relates to differences between the X and the sex-specific loci in terms of copy number, recombination rate, mutation rate, and perhaps selection.

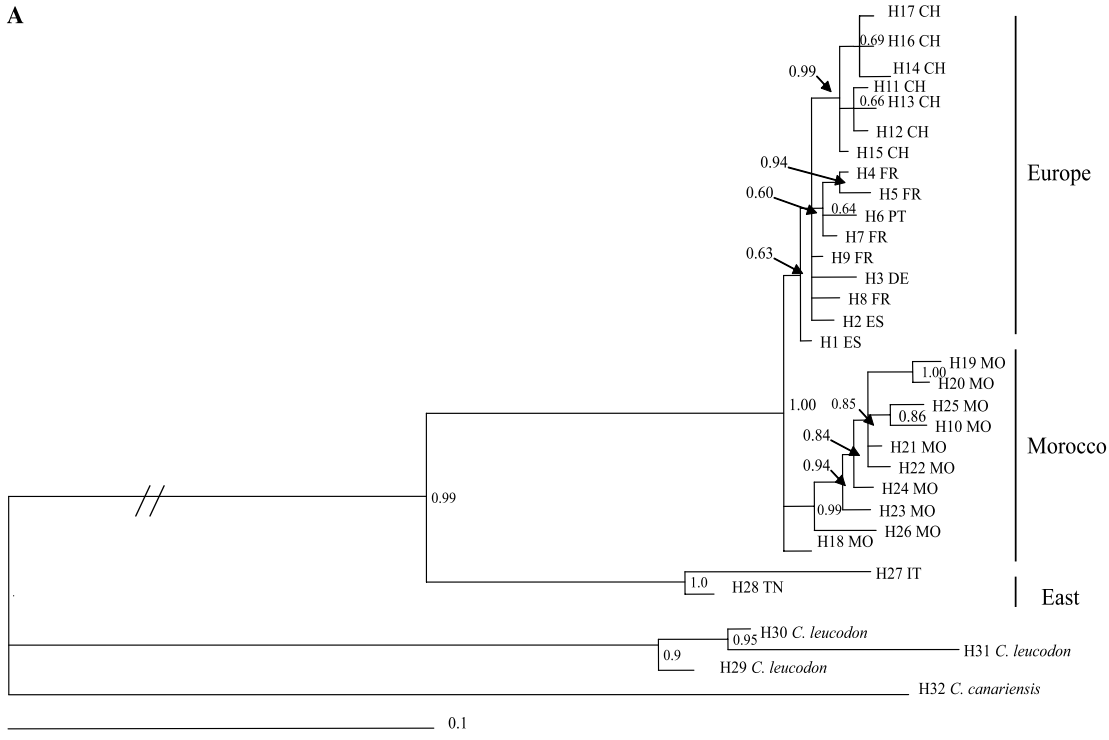
The western clade is unresolved for all three loci. There is some suggestion of separation into European and Moroccan clades with mtDNA but this is not well supported (Fig. 2A). In addition it is not clear from the mtDNA tree whether or not Moroccan haplotypes are basal in the western clade. For the X and Y chromosomes (Figs. 2B and C), there is no distinct separation of Moroccan and European haplotypes. For the X chromosome, two Moroccan haplotypes are basal to all other western haplotypes (Fig. 2B), which could be suggestive of Moroccan ancestry (but note the low posterior probability of 0.77), but for the Y chromosome a Portuguese haplotype is basal to the western clade (Fig. 2B). It is therefore not clear from the phylogenetic analyses whether the Moroccan population is ancestral to Europe.

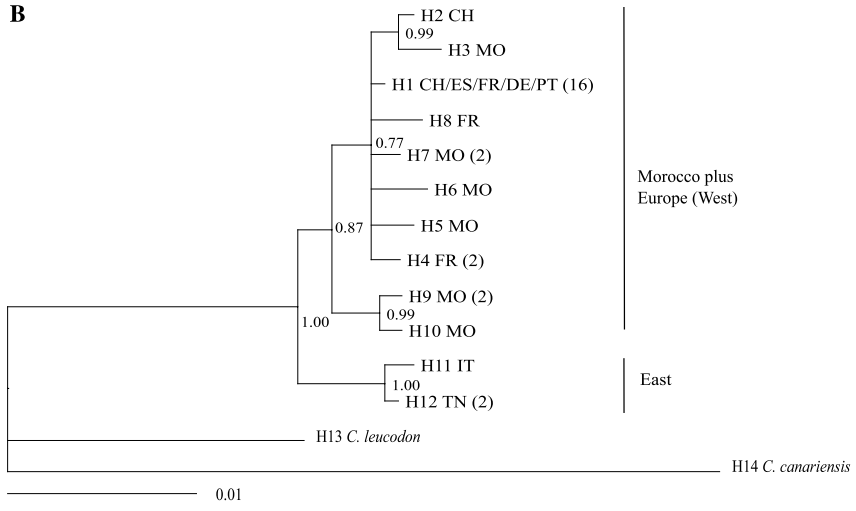
3.2. Genetic diversity among and within populations

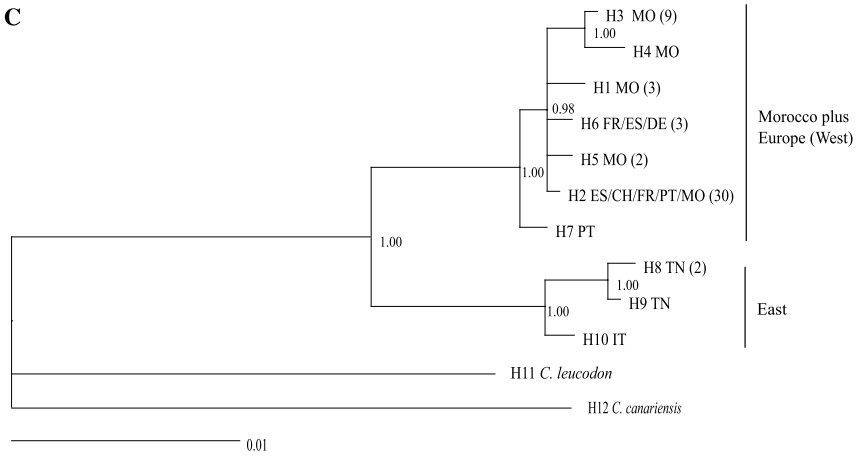
Divergence between western and eastern lineages is substantial relative to that between Moroccan and European populations (Table 5). The time since divergence of the eastern and western lineages (represented by Tunisian and Moroccan individuals, respectively) was estimated from mtDNA *CR* sequences as 2.25 Myr (95% CI 1.77–2.73, where the net p-distance = 0.0569, S.E. 0.00895). The observation of greater nucleotide diversity in the Moroccan population than in Europe (Table 4), as well as low values of divergence between Morocco and Europe relative to Tunisia (Table 4) is consistent with Moroccan origins for the European population of *C. russula*.

3.3. Mismatch distribution analyses

Unimodal mismatch distributions for both Morocco and Europe (Figs. 3A and B) are consistent with rapid expansion (and/or selection) of both populations. Goodness-of-fit tests of observed compared to simulated data


A
H17 CH
0.69 H16 CH
H14 CH
0.99
H11 CH
0.66 H13 CH
H12 CH
H15 CH
0.94
H4 FR
H5 FR
0.60
0.64 H6 PT
H7 FR
0.63
H9 FR
H3 DE
H8 FR
H2 ES
H1 ES
Europe
1.00 H19 MO
H20 MO
1.00 0.85 H25 MO
0.86 H10 MO
0.84
H21 MO
0.94
H22 MO
H24 MO
0.99 H23 MO
H26 MO
H18 MO
Morocco
0.99
H27 IT
1.0 H28 TN
East
0.95 H30 C. leucodon
H31 C. leucodon
0.9 H29 C. leucodon
H32 C. canariensis
0.1


B
H2 CH
0.99
H3 MO
H1 CH/ES/FR/DE/PT (16)
H8 FR
0.77
H7 MO (2)
H6 MO
0.87
H5 MO
H4 FR (2)
Morocco plus
Europe (West)
H9 MO (2)
1.00
0.99
H10 MO
H11 IT
1.00
H12 TN (2)
East
H13 C. leucodon
H14 C. canariensis
0.01


C
H3 MO (9)
1.00
H4 MO
H1 MO (3)
0.98
H6 FR/ES/DE (3)
Morocco plus
Europe (West)
H5 MO (2)
1.00
H2 ES/CH/FR/PT/MO (30)
H7 PT
1.00
H8 TN (2)
1.00
H9 TN
East
1.00
H10 IT
H11 C. leucodon
H12 C. canariensis
0.01

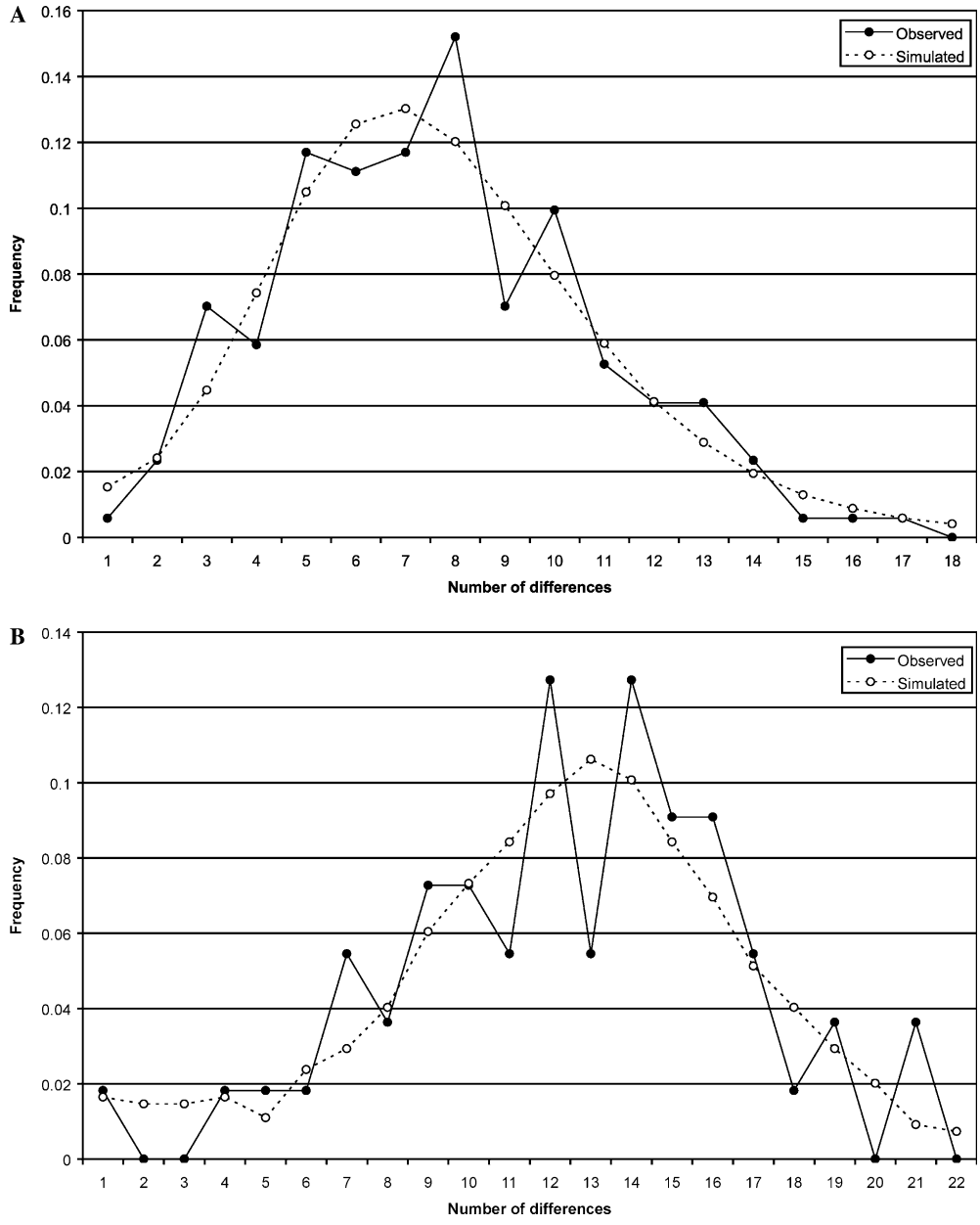


Fig. 3. mtDNA mismatch distributions for: (A) European and (B) Moroccan populations of *C. russula*. The observed pairwise distribution is shown as a solid black line and the dashed line refers to the simulated distribution expected under population expansion. Both analyses are based on 37 segregating sites. For Europe, the observed mean number of pairwise differences, $m = 6.6$ (95% CI 4.2–10.4), and the variance, $v = 9.94$, whereas for Morocco $m = 11.5$ (95% CI 6.7–16.6) and $v = 17.5$. The greater variance and mean number of pairwise differences seen for Morocco compared to Europe suggests a more ancient population expansion in the former.

were non significant (Europe SSD 0.004, $p = 0.72$, Morocco, SSD 0.008, $p = 0.84$), therefore the null hypothesis of population expansion could not be rejected. For Europe, the mean and variance of the number of pairwise differences ($m = 6.6$, 95% CI 4.2–10.4, $v = 9.94$, Fig. 3A), are substantially lower than for Morocco ($m = 11.5$, 95% CI 6.7–16.6, $v = 17.5$, Fig. 3B), which indicates a more ancient population expansion in Morocco than in Europe. For Europe the time since expansion, estimated from $\tau = 5.05$ (95% CI 2.67–10.80), corresponds to 70,955 years (95% CI 37,556–151,742), suggesting that colonization occurred in excess of 38 Kyr

Fig. 2. Majority rule consensus trees for: (A) mtDNA, (B) X chromosome, and (C) Y chromosome. Trees are based on 1,000,000 MCMCMC generations, sampling every 100th generation after a burnin of 20,000 generations. Substitution model parameters used for the three partitions are detailed in Table 3. Numbers on branches indicate posterior probability support for clades. Country codes are as for Fig. 1. For the mtDNA tree, the branch separating *C. russula* from *C. leucodon* and *C. canariensis* has been collapsed to display the *C. russula* clade with greater clarity.

ago. A greater value of τ for Morocco ($\tau = 11.89$, 95% CI 6.58–16.68) corresponds to 166,994 years (95% CI 92,416–234,270) since expansion. The number of individuals was estimated before and after expansion for both Europe ($N_0 = 28{,}960$, 95% CI 0–106,868, $N_1 = 1{,}022{,}640$, 95% CI 301,671–100,320,393) and Morocco ($N_0 = 10{,}769$, 95% CI 0–72,308, $N_1 = 1{,}206{,}154$, 95% CI 275,385–128,860,000), but clearly the amount of error associated with these values is large. Finally, corresponding tests of neutral equilibrium were significant for Europe (Fu's $Fs = -11.7$, $p < 0.0001$, Tajima's $D = -1.5$, $p = 0.05$), consistent with population expansion and/or selection, but for Morocco D is not significant ($D = -0.42$, $p = 0.30$) and Fs is only just significant ($Fs = -3.74$, $p = 0.04$). This is again consistent with a weaker (and therefore older) signal of population expansion in Morocco than in Europe.

Taken together, these results indicate a large divergence between eastern and western lineages, colonization of Europe from Moroccan ancestors and subsequent population expansion of European shrews in excess of 38 Kyr ago.

4. Discussion

4.1. Eastern and western phylogroups of C. russula are highly divergent

Consistent with previous reports based on morphological, chromosomal and molecular data (Cosson et al., 2005; Lo Brutto et al., 2004; Sarà and Vogel, 1996; Vogel et al., 2003, 2004), our analyses of mtDNA, X, and Y chromosome loci consistently support the deep separation of eastern (Tunisia plus Sardinia) and western (Morocco plus mainland Europe) lineages of *C. russula* (Table 5 and Fig. 2), which indicates that this is a genome-wide result rather than just a gene tree. An investigation of geographically intermediate individuals is required to see if this still holds true at a local scale, since a potential hybrid zone exists in Algeria (Sarà and Vogel, 1996). Divergence time estimates between Moroccan and Tunisian populations based on mtDNA *CR* are in the order of 2.25 million years (1.8–2.7 Myr), in excellent agreement with the 2.21 Myr calculated from *Cytb* by Cosson et al. (2005). Both estimates are much greater than the previous approximation of 0.5 Myr (Lo Brutto et al., 2004). This latter estimate was calculated based on an mtDNA mutation rate average of 2% divergence per million years (Brown et al., 1979) applied to a small number of differences in a very short segment of the 12S rRNA gene. Since no confidence intervals were given, it is unclear whether this estimate overlaps with our own and that of Cosson et al. (2005).

Evidence of reduced male (but not female) fertility has been found during backcross experiments between individuals from Tunisian and Moroccan populations (Vogel et al., 2004), and perhaps relating to this (although purely speculative), we found two amino acid replacement substitutions, but no synonymous changes, between eastern and western populations in the HMG box of the sex-determining region of the Y chromosome (*sry*). This region is believed to be highly conserved even at the interspecific level (e.g. Sánchez et al., 1996). Given the large differentiation of mtDNA and the sex chromosomes between eastern and western lineages, we therefore support the taxonomic separation of *C. russula* into *C. russula* (Morocco plus mainland Europe) and *Crocidura ichnusae* (Tunisia plus Mediterranean islands). *C. ichnusae* Festa, 1912 is the oldest available name for the eastern lineage and therefore has priority over *C. cossyrensis* Contoli, 1989 (note that no type specimen exists for the other potential synonym, *C. agilis*, Loche, 1867, and it has therefore been referred to as a *species dubium*, Sarà and Vogel, 1996).

4.2. The relationship between Moroccan and European populations of C. russula

The Moroccan and European populations of *C. russula* are often referred to as separate subspecies (*C. r. yebalensis* Cabrera, 1913, and *C. r. russula* Hermann, 1780, respectively) because of the smaller size of Moroccan individuals (Vogel and Maddelena, 1987). Separation of these two populations is suggested but not well supported from mtDNA (Fig. 2A), but they are not differentiated at nuclear loci (Figs. 2B and C). Moreover, the most common Y chromosome haplotype is shared between Morocco and Europe, illustrating the close relationship between these two populations, and questioning their status as separate subspecies.

Because the western clade is poorly resolved for all three loci (Fig. 2) we are unable to conclude from phylogenetic analyses alone whether or not the Moroccan population is ancestral to Europe. Moroccan ancestry of European shrews is nevertheless supported by greater nucleotide diversity in Morocco than in Europe for each of our three genomic regions (Table 4). In addition, our mtDNA mismatch distributions indicate that population expansion in Morocco predates that in Europe (Figs. 3A and B), which would suggest that the Moroccan population is older. We estimate that the Moroccan population expansion began approximately 165 (95% CI 92–234) Kyr ago, whereas European population expansion began approximately 71 (95% CI 38–152) Kyr ago. Even though these confidence intervals overlap, it is clear from the mean and variance of the respective distributions and from corresponding tests of neutral equilibrium (Tajima's D and Fu's Fs are negative and significant for the European population, but only Fs is significant for Morocco) that the Moroccan expansion is the more ancient.

It is possible that directional selection on mtDNA has influenced these results, since both selection and expansion lead to an excess of rare alleles and a corresponding unimodal mismatch distribution (Rogers and Harpending, 1992; Slatkin and Hudson, 1991). To distinguish between these opposing hypotheses, it would be useful to repeat this analysis for nuclear regions (assuming that expansion affects all genomic regions, whereas selection will affect certain genes more than others). Unfortunately this was not possible due to the low number of variable sites on the X and Y chromosomes. However, with evidence from other studies suggesting that the European mainland has been colonized from the ancestral Moroccan population (e.g. Cosson et al., 2005), and given the nucleotide diversity data presented here, we favour the hypothesis of expansion rather than selection.

4.3. Crossing the Gibraltar Strait: the when and how of European colonization

Despite similar habitats in the Maghreb and the European Mediterranean, only 17 non-flying terrestrial mammal species are common to both regions (Dobson, 1998). For a small terrestrial species, the Gibraltar Strait is an important barrier to dispersal, being 14 km wide at its narrowest point and (currently) exceeding 200 m in depth. It is therefore interesting to evaluate the timing and dynamics of colonization of European shrews from Morocco. Moroccan shrews could have colonized Europe either via a land bridge connecting the two continents, or after the opening of the Gibraltar Strait, either by rafting on vegetation, or in recent times via anthropogenic means.

Geological evidence indicates that Morocco and the Iberian Peninsula have been connected by a land bridge only twice (Blondel and Aronoson, 1999); 16–14 Myr ago during the Betic crisis, and ~5.59–5.33 Myr ago, during the Messinian salinity crisis (in which the Messinian sea gradually desiccated making dispersal possible throughout the whole of the Mediterranean region, Duggen et al., 2003; Krijgsman et al., 1999). Dispersal during the latter period is believed to account for large divergence values between Iberian and Moroccan species such as *Salamandra* sp. (Steinfartz et al., 2000; Veith et al., 2004) and *Buthus* scorpions (Gantenbein and Largiader, 2003).

In contrast, divergence values between European and Moroccan shrews (Table 5) are too small to be consistent with dispersal via either of these land bridges, and the date obtained from our mtDNA mismatch distribution suggests that Europe was colonized as recently as 38 Kyr ago (the lower 95% confidence interval). Our dates for European colonization correspond to the Würm Glacial, ~80–10 Kyr ago. During this time period, sea levels in the Gibraltar Strait were approximately 120 m lower than they are today, revealing several small islands in the channel (Flemming et al., 2003), which could have facilitated traversal by shrews. Dispersal would have been possible either via rafting on vegetation (as proposed for the ribbed salamander *Pleurodeles waltl*; Busack, 1986) or by anthropogenic means. Accidental transport by humans, which is believed to be an important mechanism for transferring species (e.g. the wood mouse, *Apodemus sylvaticus*, Michaux et al., 2003), has been proposed to explain the presence of *C. russula* on both sides of the Gibraltar Strait, and on several Mediterranean islands (Lo Brutto et al., 2004; Vogel et al., 1990). Our lower confidence limit (38 Kyr) predates the first solid archaeological evidence of humans crossing the Gibraltar Strait (25 Kyr). Nevertheless, given that the Iberian coastline is visible from Morocco, and that modern humans had the technical capabilities to build boats and colonize Australasia by 40 Kyr ago (Jobling et al., 2004), human transport cannot be excluded.

Although our estimate of colonization time is fairly consistent with a recent estimate based on *Cytb* divergence (60 Kyr ago, Cosson et al., 2005), it is considerably earlier than some previous reports suggest (Lo Brutto et al., 2004; Poitevin et al., 1986). The very low levels of variation found between Moroccan and European Y chromosomes might imply that colonization was more recent than our mtDNA estimate indicates. This can potentially be explained by slow mutation rate of the Y chromosome (compared to mtDNA) and low copy number (compared to X), which mean that it is slow to recover variation following a bottleneck. In contrast, it is possible that our date is actually an *underestimate*, since rapid population expansion can lead to erroneous conclusion of very recent ancestry (Nichols, 2001). This is unlikely however since one would expect greater recovery of variation than we observe on the sex chromosomes if the founder event was ancient.

Whether the shrews arrived in Europe independently or via human transport, it is likely that only a small number of founding individuals reached the Iberian Peninsula, and that the Mediterranean Sea prevented reinforcement of the European population (Hewitt, 1996). This bottleneck is supported by the strong signature of rapid population expansion from mtDNA, and the particularly low levels of variation observed on the Y chromosome of European shrews. Colonization by only a small number of founders would have been sufficient to initiate rapid expansion (Vogel, 1999) due to the prolific nature of this annual species (Cantoni and Vogel, 1989). Finally, expansion of the species' range would have been influenced by climatic conditions during the Last Glacial Maximum, (23–18 Kyr) and the Younger Dryas (10.5–9.5 Kyr, Hewitt, 1996). This is illustrated by the dependency for

today's European populations of *C. russula* on artificial heat sources (such as compost piles) for over-winter survival (Genoud and Hausser, 1979).

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