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Pliocene and Pleistocene diversification and multiple refugia in a Eurasian shrew (*Crocidura suaveolens* group)

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Abstract

We sequenced 998 base pairs (bp) of mitochondrial DNA cytochrome *b* and 799 bp of nuclear gene BRCA1 in the Lesser white-toothed shrew (*Crocidura suaveolens* group) over its geographic range from Portugal to Japan. The aims of the study were to identify the main clades within the group and respective refugia resulting from Pleistocene glaciations. Analyses revealed the Asian lesser white-toothed shrew (*C. shantungensis*) as the basal clade, followed by a major branch of *C. suaveolens*, subdivided sensu stricto into six clades, which split-up in the Upper Pliocene and Lower Pleistocene (1.9–0.9 Myr). The largest clade, occurring over a huge range from east Europe to Mongolia, shows evidence of population expansion after a bottleneck. West European clades originated from Iberian and Italo-Balkanic refugia. In the Near East, three clades evolved in an apparent hotspot of refugia (west Turkey, south-west and south-east of the Caucasus). Most clades include specimens of different morphotypes and the validity of many taxa in the *C. suaveolens* group has to be re-evaluated.
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Keywords: *Crocidura suaveolens*; Cytochrome *b* gene; BRCA1 gene; Phylogeography; Colonisation routes; Glacial refugia; Eurasia

1. Introduction

It is now generally accepted that Quaternary climatic fluctuations profoundly shaped the genetic diversity of terrestrial biota throughout the Holarctic (Hewitt, 2000). Although different species may have had different refugia, colonisation routes (depending on migration abilities), resilience to environmental change, and stochastic events in their histories, such as local extinction (Hewitt, 2003; Michaux et al., 2005), accumulated data reveal a general picture in Europe involving southern refugia and northward colonisation routes. Locally, mountains and seas acted as significant barriers, isolating populations in different glacial refugia and constraining post-glacial migration routes (Hewitt, 1999; Taberlet et al., 1998). Temperate species, which presently occupy central and northern Europe, mainly derive from Mediterranean refugium populations that underwent range expansion in the late glacial and early post-glacial periods (Hewitt, 1996), with a western form deriving from an Iberian refugium and an eastern form from the Italo-Balkanic refugium (Dumolin-Lapegue et al., 1997; Ferris et al., 1993, 1998; Santucci et al., 1998; Thorpe, 1984). Several authors, however, suggest an additional mode of colonisation of central and northern Europe by non-Mediterranean populations, coming from one or more eastern refugia: Caucasus, southern Ural, central Europe, and western Asia (Bilton et al., 1998; Cooper et al., 1995; Michaux et al., 2004; Nesbo et al., 1999; Palme and Vendramin, 2002; Seddon et al., 2002). A few other species (Brunhoff et al., 2003; Taberlet et al., 1998) exhibit mixed

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patterns, where northern Europe has been colonised both from Mediterranean and non-Mediterranean refugia.

Notwithstanding some holartic species (Brunhoff et al., 2003) and scant data for temperate species (Ohdachi et al., 2001; Yasuda et al., in press), there are no well-documented phylogeographies encompassing the whole Palaearctic, in spite of the fact that such data may highlight the interrelationships between the Quaternary histories of Asian and European biota and improve our general knowledge of Quaternary refugia in central and eastern Asia. Moreover, such information is relevant for understanding and conserving the current biological diversity in that part of the world. This paper supplies such data by analysing numerous specimens of shrews of the *Crocidura suaveolens* group with a large distribution throughout the temperate Palaearctic, from Spain to China and Japan (Corbet, 1978; Hutterer, 1981). Within this large area, populations exhibit different morphotypes, which have been interpreted as different species (see Section 2.1 for more details) or have even been assigned as subspecies of other species, e.g., of the Greater white-toothed shrew (*C. russula*). The common characteristic of all these populations is, however, a similar karyotype with a constant number of chromosomes ($2N = 40$, $NF = 50$). For this reason, and based on isoenzyme variations, Catzeflis et al. (1985) included all European populations within the same species. Initially, mitochondrial DNA data (Vogel et al., 2003) confirmed this view, showing that the morphologically distinct taxon *Crocidura gueldenstaedtii* is actually situated within the *C. suaveolens* clades from southern and western Europe. Other authors still continue to defend the classic view with the distinction of several species based on geographic morphological variation, but also on molecular data (Bannikova et al., 1993, 2005). Several molecular studies confirm the existence of *C. sibirica* from central Asia (Han et al., 2002; Motokawa et al., 2000) and *C. shantungensis* from east China, Korea, Cheju, and Tsushima Island, which are distinct from a central European sample of *C. suaveolens* (Ohdachi et al., 2004).

The taxonomic assignment of the sampled populations was done according to Zaitsev (1993), and completed by more recent morphological interpretations (Jiang and Hoffmann, 2001; Motokawa et al., 2000). The phylogeographic structure within the *C. suaveolens* group was first characterised, based on the mtDNA cytochrome *b* gene (*cyt-b*), which permits, according to Bradley and Baker (2001), good resolution of the intra and peri-specific levels. The congruence between genetic data and classical morphological interpretations was then examined. Considering that the mitochondrial genome of a taxon can introgress into another closely related taxon (Arntzen and Wallis, 1991; Ruedi et al., 1997; Tegelstrom, 1987), a fragment of the nuclear gene Breast Cancer Susceptibility 1 (BRCA1) was analysed in certain samples. Using such data from both mitochondrial and nuclear genomes, and considering the molecular clock, the colonisation and differentiation of the populations through time and space was reconstructed.

2. Materials and methods

2.1. Origin of the material

Shrews were collected throughout a large part of Eurasia (Fig. 1 and Table 1). The set of samples included material from the following collections: St. Petersburg (ZISP), Russia; Montpellier (JFC), France; Prague (DZCU), Czech Republic; Lausanne (IZEA), Switzerland. Some sequences were taken from Vogel et al. (2003) and Ohdachi et al. (2004) (Table 1).

For clarification, the following nomenclature was employed in our study:

- *C. suaveolens* Pallas, 1811 (type locality near Sevastopol, Ukraine), from Portugal to East Asia (Zaitsev, 1993).
- *C. shantungensis* Miller, 1901; from East China, Korea, Cheju and Tsushima Island (Jiang and Hoffmann, 2001).
- *C. sibirica* Dukelski, 1930; (type locality Minusinsk, Russia) limited to Siberia and China (Corbet, 1978; Jiang and Hoffmann, 2001; Yudin, 1989).
- *C. caspica* Thomas, 1907; from south of the Caspian Sea (Zaitsev, 1993; Tembotova, 1999).
- *C. gueldenstaedtii* Pallas, 1811, with the type locality of Dusheti, Georgia (Grafodatsky et al., 1988; Tembotova, 1999; Zaitsev, 1993).
- *C.* (*russula*) *monacha* Thomas, 1906; from central and east Turkey, and to which were also assigned shrews from Lebanon and Israel (Atallah, 1977).

2.2. DNA extraction and amplification

Most of the IZEA samples were first frozen in the field in liquid nitrogen, then kept for several years at −70 °C, and finally stored in ethanol until DNA extraction. Those of the other collections were directly stored in ethanol. DNA extraction was carried out using the QIA Amp DNA Mini Kit (Qiagen). Double-stranded DNA amplifications of cytochrome *b* gene (*cyt-b*) were performed with different combinations of primer pairs L14841/C4, C8/C4, C1/C2, C6/C7, C3/H15915, C5/H15915, and L14841/H15915 (see Table 2 and Irwin et al., 1991). Amplification of the BRCA1 nuclear gene was performed using the primer pairs B1f/B1r, Bf2/Br, Bf/Br2, Bf/Br3, Bf3/Br4, Bf4/B1r, B1f/Bdr, Bfa/Br11, Bf11/ Br12, Bf12/ Br13, Bf13/ Br14, Bf14/ Br15, Bf15/ Br16, Bf16/ Br17, and Bf17/ Br18 (Table 2). Amplification conditions for the *cyt-b* consisted of 35 thermal cycles of 30 s denaturation (60 s for the set L14841/H15915) at 94 °C, 45 s annealing (60 s, L14841/H15915) at 50 °C and 60 s (120 s, L14841/H15915) extension at 72 °C. Amplification conditions for the BRCA1 locus consisted of 40 cycles of 45 s denaturation (60 s, BRCA1f/BRCA1r) at 94 °C, 45 s annealing (60 s, BRCA1f/BRCA1r) at 50 °C and 90 s extension (120 s, BRCA1f/BRCA1r) at 72 °C.

PCR products were then electrophoresed on a 1% agarose gel, visualised with ethidium bromide staining to verify

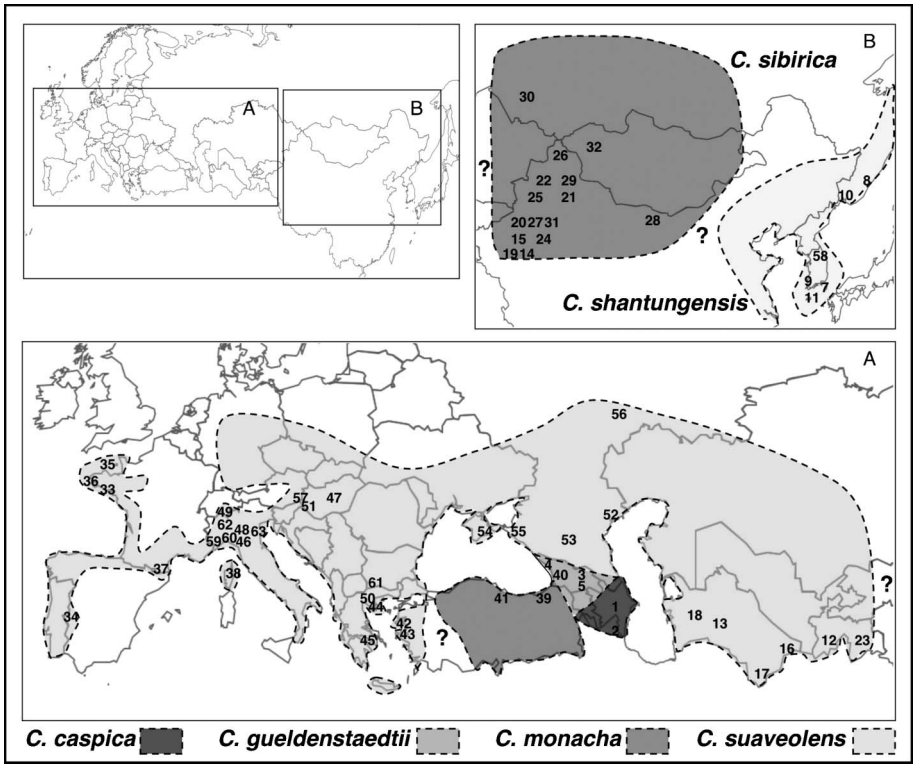

Fig. 1. Locations of samples (A: in western Eurasia, and B: in eastern Eurasia), and currently known repartition of different morphospecies.

PCR quality, and purified by centrifugal dialysis using the QIAquick PCR Purification Kit (Qiagen), according to manufacturer's instructions.

Cycle sequencing was performed in 10 µl total volume containing 1–3 µl of amplified DNA, 1 µl of 10 µM primer, 4 µl ABI PRISM Dye Terminator 1 (Perkin-Elmer). Sequence reactions were visualised on an ABI 3100 genetic analyser (Applied Biosystems).

2.3. Phylogenetic methods

Nucleotide sequences of *cyt-b* and BRCA1 genes were edited with Sequence Navigator (Parker, 1997) and aligned by eye. Three methods of phylogenetic analyses were carried out for the two genes, using PAUP*version 4.0b8 PPC (Swofford, 1998). Tests were conducted on the total fragments (998 bp for *cyt-b*, 799 bp for BRCA1), all codon positions were used, and trees were rooted using sequences of *C. nigripes* (DQ059024 for *cyt-b*, and DQ059022 for BRCA1) and *C. brunnea* (DQ059025 for *cyt-b*, and DQ059021 for BRCA1). A Neighbour Joining (NJ) tree was constructed using Kimura two-parameter genetic distances (K2P; Kimura, 1980). The Parsimony analyses (MP) were performed using the following options: heuristic search, stepwise-addition of sequences, 10 replicates of random addition of taxa, and TBR branch swapping (Swofford, 1998), all codon positions were equally weighted, and as we observed a deletion of three base pairs in some individuals for BRCA1, we treated it as a fifth state. For ML analyses, likelihood ratio tests, implemented with the computer program Modeltest 3.06, were first used to choose the substitution model that best fitted the data, according to the protocol of Posada and Crandall (1998). For *cyt-b*, the TVM+G model was selected with base frequencies (A = 0.2954, C = 0.2710, G = 0.1177, T = 0.3159) estimated from the data, an unequal distribution of rates at variable sites ($\alpha = 0.2193$) and five different substitution types (rate [A–C] = 0.3102, rate [A–G] = [C–T] = 11.4897, rate [A–T] = 0.8589, rate [C–G] = 0.4558, rate [G–T] = 1.0000). For BRCA1, the K81uf model was selected with a proportion of invariable sites (0), and base frequencies (A = 0.3828, C = 0.1724, G = 0.2070, and T = 0.2092) estimated from the data. Maximum likelihood analyses were then performed, assuming this model, using the heuristic search option with a stepwise-addition of sequences. NJ, MP, and ML results were compared for congruence of tree topologies. Bootstrap support values were obtained with 1000 pseudoreplicates for MP, NJ, and ML (for BRCA1) analysis, and 10 random replicates of stepwise-addition sequences. Then, for *cyt-b*, Bayesian analyses were conducted using MrBayes version 3.0 b4 (Huelsenbeck et al., 2001), which performs Metropolis-coupled Markov chain Monte Carlo analysis. A GTR model was used, with an among-site rate variation following a gamma distribution. The Markov chain was run for 1,000,000 generations and sampled once every 100 generations; burning was set at 100,000 generations. To assure convergence in the Bayesian analyses, two independent runs were performed and compared.

Table 1
Species sequenced and used in the present study, geographic origin of samples, identification codes (* = type locality), location on map (Fig. 1), accession number of *cyt-b* sequences, and BRCA1 alleles of analysed samples

Species	Sample location	Collection codes	Identification codes	Number on the map	Accession number (*cyt-b*)	BRCA1 allele(s)
C. caspica	Azerbaijan	IZEA 7793	AZ1	1	AY843487	A12
C. caspica	Gilan, IR	DZCU IRA87	IR1	2	DQ059023	A12
C. gueldenstaedtii	Dusheti, GE	IZEA 7795	GE1	3	AY843488	
C. gueldenstaedtii	Sukhumi, GE	IZEA 2854	GE2	4	AY843496	
C. gueldenstaedtii	Dusheti, GE	IZEA 2687*	GE3	3	AY843497	
C. gueldenstaedtii	Shulaveri, GE	ZISP N	GE4	5	AY843500	A13
C. monacha	Altindere, TR	IZEA 6026*	TR1	39	AY843499	
C. monacha	Rize, TR	IZEA 6029	TR2	40	AY843498	
C. monacha	Cakalli, TR	IZEA 6014	TR3	41	AY843502	A13
C. shantungensis	Tsushima island, JP	IZEA 7510	JP2	7	AY843447	A11
C. shantungensis	Putjatin island, RU	GenBank	RU2	8	AB077080	
C. shantungensis	Kyungju, KR	GenBank	KR1	9	AB077079	
C. shantungensis	Popov Island, RU	GenBank	RU3	10	AB077082	
C. shantungensis	Cheju island, KR	GenBank	KR2	11	AB077077	
C. shantungensis	Putjatin island, RU	GenBank	RU4	8	AB077081	
C. shantungensis	Cheju island, KR	GenBank	KR3	11	AB077078	
C. shantungensis	Ullung island KR	GenBank	KR4	58	AB077076	
C. shantungensis	Ullung island KR	GenBank	KR5	58	AB077075	
C. shantungensis	Popov Island, RU	GenBank	RU5	10	AB077278	
C. suaveolens	Pengikend, TJ	ISP T-77221	TJ1	12	AY843478	
C. suaveolens	Firusa, TM	ZISP T-73760	TM1	13	AY843481	
C. sibirica	Qiemo, CN	IZEA 7504	CN1	14	AY843470	
C. sibirica	Minfeng, CN	IZEA 7497	CN2	15	AY843468	
C. suaveolens	Kerki, TM	ZISP T-73755	TM2	16	AY843480	
C. suaveolens	Badchiz, TM	ZISP T-73756	TM3	17	AY843479	
C. suaveolens	TM	IZEA 3199	TM4	18	AY843466	
C. sibirica	Hotan, CN	IZEA 7491	CN3	19	AY843471	
C. sibirica	Aksu Tomor Peak, CN	IZEA 7484	CN4	20	AY843463	
C. sibirica	Turpan, CN	IZEA 7508	CN5	21	AY843465	A1/A2
C. sibirica	Fukang station, CN	IZEA 7431	CN6	22	AY843473	
C. suaveolens	Ikanderkul, TJ	ZISP T-77220	TJ2	23	AY843482	
C. sibirica	Bohu, CN	IZEA 7474	CN7	24	AY843469	
C. sibirica	Bogda Shan, CN	IZEA 7443	CN8	25	AY843472	
C. sibirica	Fuhai, CN	IZEA 7454	CN9	26	AY843464	A1/A3
C. sibirica	Baiyanggou, CN	IZEA 7466	CN10	27	AY843462	
C. sibirica	Gobi desert, CN	IZEA 7980	CN11	28	AY843474	A2
C. sibirica	Qiemo, CN	GenBank	CN12	14	AB077084	
C. sibirica	Qiemo, CN	GenBank	CN13	14	AB077083	
C. sibirica	Mosuowan, CN	GenBank	CN14	29	AB077086	
C. sibirica	Novosibirsk, RU	GenBank	RU6	30	AB077279	
C. sibirica	Korla, CN	GenBank	CN15	31	AB077085	
C. sibirica	Sharg vil. MN	GenBank	MN1	32	AB077088	
C. sibirica	Mosuowan, CN	GenBank	CN16	29	AB077087	
C. sibirica	Novosibirsk, RU	Coll. Panov N58572	RU7	30	AY843485	A1
C. sibirica	Novosibirsk, RU	Coll. Graphodatsky	RU8	30	AY843486	A1/A2
C. sibirica	Novosibirsk, RU	Coll. Bannikova	RU9	30	AY843484	A1/A2
C. sibirica	Novosibirsk, RU	GenBank	RU10	30	AB077089	
C. suaveolens	Hoedic, FR	JFC Hoedic	FR1	33	AY843490	
C. suaveolens	Candelario, ES	IZEA 5927	ES1	34	AY843492	
C. suaveolens	Sark, GB	JFC Sark	GB1	35	AY843489	
C. suaveolens	Triélen, FR	JFC Triélen	FR2	36	AY850035	
C. suaveolens	Figuerasse, ES	IZEA 3191	ES2	37	AY843491	A10
C. suaveolens	Bonifacio, Corse, FR	IZEA CO2	FR3	38	AY843501	
C. suaveolens	Lesvos, GR	IZEA 3930	GR1	42	AY843460	A8
C. suaveolens	Vukarikisilka,TR	IZEA 6005	TR4	43	AY843461	A7
C. suaveolens	Epanomi Thessalonichi, GR	IZEA 3916	GR2	44	AY843448	A4
C. suaveolens	Athina, GR	IZEA 1354	GR3	45	AY843455	
C. suaveolens	Fivizano, IT	IZEA 489	IT1	46	AY843450	
C. suaveolens	Hungary	JFC H15	HU1	47	AY843454	
C. suaveolens	San Nicolo, IT	IZEA 7509	IT2	48	AY843453	A5
C. suaveolens	Gordevio, CH	IZEA 3198	CH1	49	AY843452	A5

(*continued on next page*)

Table 1 (*continued*)

Species	Sample location	Collection codes	Identification codes	Number on the map	Accession number (*cyt-b*)	BRCA1 allele(s)
C. suaveolens	Thessaloniki, GR	IZEA 1352	GR4	50	AY843449	
C. suaveolens	Fülophasa, HU	IZEA 6732	HU2	51	AY843451	A6
C. suaveolens	Astrakhanskaya reg., RU	ZISP T-72688	RU11	52	AY843477	A1
C. suaveolens	Stavropol, RU	IZEA 4200	RU12	53	AY843467	A1
C. suaveolens	Sevastopol, UA	IZEA 7796*	UA1	54	AY843475	A1
C. suaveolens	Krasnodarsskyi, RU	ZISP T-73479	RU13	55	AY843476	
C. suaveolens	Kazan, RU	ZISP T-85944	RU14	56	AY843483	A1
C. suaveolens	Wien, AU	GenBank	AU1	57	AB077280	
C. suaveolens	Fraitusa, IT	IZEA 7977	IT3	59	AY843494	
C. suaveolens	Fraitusa, IT	IZEA 7979	IT4	59	AY843493	A9
C. suaveolens	Ventimiglia, IT	IZEA 7944	IT5	60	AY843495	
C. suaveolens	Sandanski, BG	IZEA 7975	BG1	61	AY843458	A4
C. suaveolens	Vercelli, IT	IZEA 7945	IT6	62	AY843459	
C. suaveolens	Latisana, IT	IZEA 7978	IT7	63	AY843457	
C. suaveolens	Latisana, IT	IZEA 7976	IT8	63	AY843456	A5
C. nigripes	Sulavesi, ID	IZEA 4400	ID1	—	DQ059024	ID1
C. brunnea	Java, ID	IZEA 4549	ID2	—	DQ059025	ID2

Abbreviations of countries are: Austria (AU), Azerbaijan (AZ), Bulgaria (BG), Japan (JP), China (CN), England (GB), France (FR), Georgia (GE), Greece (GR), Hungary (HU), Indonesia (ID), Iran (IR), Italy (IT), Mongolia (MN), Russia (RU), South Korea (KR), Spain (ES), Switzerland (CH), Tajikistan (TJ), Turkmenistan (TM), Turkey (TR), and Ukraine (UA). Main collections are mentioned in the text; sequences taken from GenBank are mentioned under collection codes.

Table 2
Primers designed in our laboratory, location on mitochondrial genome for the *cyt-b* primers, and on the whole amplified fragment for BRCA1 primers

Primer (abbreviation)	Primer sequence	Location
Cytb1 (C1)	5′-tta ttc gca gta ata gca aca gc-3′	15138–15160
Cytb2 (C2)	5′-ata tgg ggtggt gtg ttg agg-3′	15563–15583
Cytb3 (C3)	5′-tat tct ccc cag aca tat tag g-3′	15511–15532
Cytb4 (C4)	5′-aac tgt tgc tcc tca gaa tga tat ttg acc tca-3′	15183–15216
Cytb5 (C5)	5′-tat ttt ccc cag ata tgt tag g-3′	15511–15532
Cytb6 (C6)	5′-ctt gaa aca tga aac att gg-3′	15108–15127
Cytb7 (C7)	5′-aat aga aaa tat cat tct gg-3′	15588–15607
BRCA1f (Bf)	5′-tga gaa cag cac ttt att act cac-3′	0–24
BRCA1r (Br)	5′-att cta gtt cca tat tgc tta tac tg-3′	913–938
BRCA1f2 (Bf2)	5′-aag tag aag agt cct ctc c-3′	472–490
BRCA1r2 (Br2)	5′-gca gtt caa gtt agg gaa gc-3′	552–571
BRCA1f3 (Bf3)	5′-tga ttg gtt ttc tag aag tg-3′	295–314
BRCA1r3 (Br3)	5′-atc atg gaa atc atc aga ag-3′	327–346
BRCA1f4 (Bf4)	5′-ctt gaa ctg cac aac tga ag-3′	562–581
BRCA1r4 (Br4)	5′-ttg tga agg ggt gtt tgt gt-3′	622–641
BRCA1dr (Bdr)	5′-ctc gat ttt ctt ttc agg aga-3′	728–748
BRCA1fa (Bfa)	5′-gta ata aaa gta aac agt ctg gc-3′	60–82
BRCA1f11 (Bf11)	5′-aga gca aaa ggc aga tct g-3′	154–172
BRCA1r11 (Br11)	5′-ggt ttt ctt ctt cca tat agg-3′	184–204
BRCA1f12 (Bf12)	5′-atg act tgg atg act tcg ac-3′	254–273
BRCA1r12 (Br12)	5′-cca atc att aac ttt ctg c-3′	283–301
BRCA1f13 (Bf13)	5′-gca ggg tct aat tca aat ac 3′	347–366
BRCA1r13 (Br13)	5′-gct gca ctt ggg att tct tc 3′	380–399
BRCA1f14 (Bf14)	5′-cca gtg atc agt gtg atg c 3′	441–459
BRCA1r14 (Br14)	5′-gtt tgg aga gga ctc ttc tac 3′	474–494
BRCA1f15 (Bf15)	5′-gag gaa agc aag ctt ccc ta 3′	541–560
BRCA1r15 (Br15)	5′-g tta cat ctt cag ttg tgc 3′	570–587
BRCA1f16 (Bf16)	5′-cac ccc ttc aca aat aaa tta 3′	629–649
BRCA1r16 (Br16)	5′-ct cag gac caa ggc ttg atg 3′	669–688
BRCA1f17 (Bf17)	5′-gat tgt tca aaa gtc tcc tg 3′	715–734
BRCA1r17 (Br17)	5′-cat ttt gat cca ttt ggt tg-3′	754–773
BRCA1r18 (Br18)	5′-ga tga tgc tgt tga gtt agc-3′	848–867

2.4. Molecular clock

Estimation of divergence time from the molecular data was performed, according to the calibration developed for the Soricidae by Fumagalli et al. (1999), based on an estimate of 20 million years for the split between Crocidurinae and Soricinae. It was developed considering the *cyt-b* sequence divergence based on third-position transversions. As proposed by Edwards (1997), the genetic distance between two different populations was corrected for ancestral mtDNA polymorphism using the formula: $P_{net} = P_{AB} - 1/2(P_A + P_B)$, where P_{net} is the corrected distance between the isolated populations A and B, P_{AB} is the mean genetic distances in pairwise comparisons of individuals from A vs. B, and P_A and P_B are the mean genetic distance among individuals within these populations.

To identify whether there was heterogeneity in the rates of *cyt-b* substitutions among different clades, relative-rate tests were conducted between each of them. The relative rate tests were performed with RRTREE, version 1.0 (Robinson et al., 1998), which improves the test of Wu and Li (1985) by taking into account taxonomic sampling and phylogenetic relationships. Relative-rate tests were performed on the proportions of synonymous (K_s), non-synonymous (K_a) substitutions, and synonymous transversions (B4).

2.5. Nucleotide diversity and genetic structure

Nucleotide diversity (π) was estimated using the DnaSP program version 4.10.3 (Rozas et al., 2005). The population genetic structure was determined by an analysis of molecular variance (AMOVA), available in Arlequin version 2.0 (Schneider, 2000). This analysis was performed at two

different hierarchical levels: among clades and within clades.

2.6. Expansion time

To test the hypothesis of recent population growth from low-diversity founder populations within the different clades, several tests were performed. Three methods were implemented in DnaSP program version 4.10.3 (Rozas et al., 2005) and one in Arlequin version 2.0 (Schneider, 2000). The first method, Ramos-Onsins and Rozas (2002) R_2 statistic, is based on the difference between the number of singleton mutations and the average number of nucleotide differences. Lower values of R_2 are expected under a scenario of recent population growth. The second method, Fu's (1997) F_S statistic, tests the probability of having no fewer than the number of observed alleles in the sample given that θ (heterozygosity per sites) $= \pi$. This statistic tends to be negative when there is an excess of recent mutations (or rare alleles). The third method, Tajima's (1989) D statistic, tests the null hypothesis that two estimates of the neutral mutation parameter, one derived from the average number of pairwise nucleotide differences and the other based on the number of segregating sites in the sample, are equal. In the fourth test, pairwise mismatch distributions among individuals were plotted and tested for goodness-of-fit to a model of sudden expansion using parametric bootstrapping with 1000 replicates (Schneider and Excoffier, 1999). Expansion time after the bottleneck was estimated from the mismatch distribution (τ) (Rogers, 1995) and uncorrected (p) distances. Evolutionary rate for uncorrected (p) distance was estimated using the molecular clock developed by Fumagalli et al. (1999).

3. Results

3.1. Cytochrome b gene

The 76 sequences of 998 bp used in this study showed 315 variable sites, of which 246 were parsimony-informative. No insertions or deletions were observed. As the three phylogenetic methods gave identical arrangements of the main branches, the relationship between haplotypes is given only for the ML analysis in Fig. 2.

Seven major clades strongly supported by bootstrap values (all of 100%) were observed:

Clade I: is the most basal, and comprises all haplotypes from Eastern Asia, corresponding to the morphotype *C. shantungensis*.

Clade II: includes all haplotypes from Russia and central Asia, which correspond to two different morphotypes *C. suaveolens*, including a specimen of the type locality in the Ukraine, and *C. sibirica* (Fig. 4).

Clade III: includes two specimens of *C. caspica* and one specimen of *C. gueldenstaedtii* from Georgia. This clade is closely related with clade II (central Asia and Russia), with which they formed a well-distinct group (bootstrap value >88%).

Clade IV: comprises haplotypes of the morphotype *C. suaveolens* from West Europe (France, Spain, England, and Liguria in Italy). It is the most basal clade of European *C. suaveolens*. It includes several sub-species, e.g., *C. suaveolens cantabra* Cabrera, 1908; described from Spain.

Clade V: includes all haplotypes of the morphotype *C. monacha* from north and east Turkey, the haplotype of *C. suaveolens* from Corsica and, with one exception, all haplotypes of the morphotype *C. gueldenstaedtii* from Georgia. Thus, it is not possible to dissociate *C. gueldenstaedtii* from *C. monacha*. This clade and the two remaining formed a highly supported clade (bootstrap values >97%).

Clade VI: includes the haplotypes of the morphotype *C. suaveolens* from Lesvos (Greece) and west Turkey, which has been considered as the nominal sub-species (Corbet, 1978).

Clade VII: comprises all haplotypes of the morphotype *C. suaveolens* from South and Central Europe (Greece, Italy, Switzerland, Austria, Bulgaria, and Hungary). It includes several sub-species, such as *C. s. mimula* Miller, 1901; from Switzerland, *C. s. balcanica* Ondrias, 1970; from Greece, and *C. s. italica* Cavazza, 1912; from Italy.

The AMOVA shows that the majority of the total mtDNA variation (89.71%) is distributed among the seven clades, whereas a low percentage of this variation (10.29%) is observed within clades. Mean pairwise corrected K2P distances between clades (Edwards, 1997) range from 3.7 to 10.8% (Table 3).

Within clades, K2P distances are less than 2.5%, and nucleotide diversities range from 0.004 to 0.012 (Table 3). In spite of having by far the largest sample size, nucleotide diversity in clade II was similar or even smaller than those found in the other clades.

3.2. Molecular clock

The calibration developed by Fumagalli et al. (1999) for the Soricidae helped to estimate the divergence times between the main lineages within the *C. suaveolens* group, although it could not be used directly because of the low numbers of third-position transversions observed. Therefore, we estimated the correlation between the third position transversions and maximum likelihood distances (ML distances) inferred from a ML tree constrained to clock-like evolution after adding published sequences of the main lineages of *Crocidura* species in Europe (Vogel et al., 2003). No evidence of saturation was detected between the main lineages observed within the *Crocidura* species in Europe, suggesting that all substitutions may be included in such analyses without degradation of the phylogenetic signal.

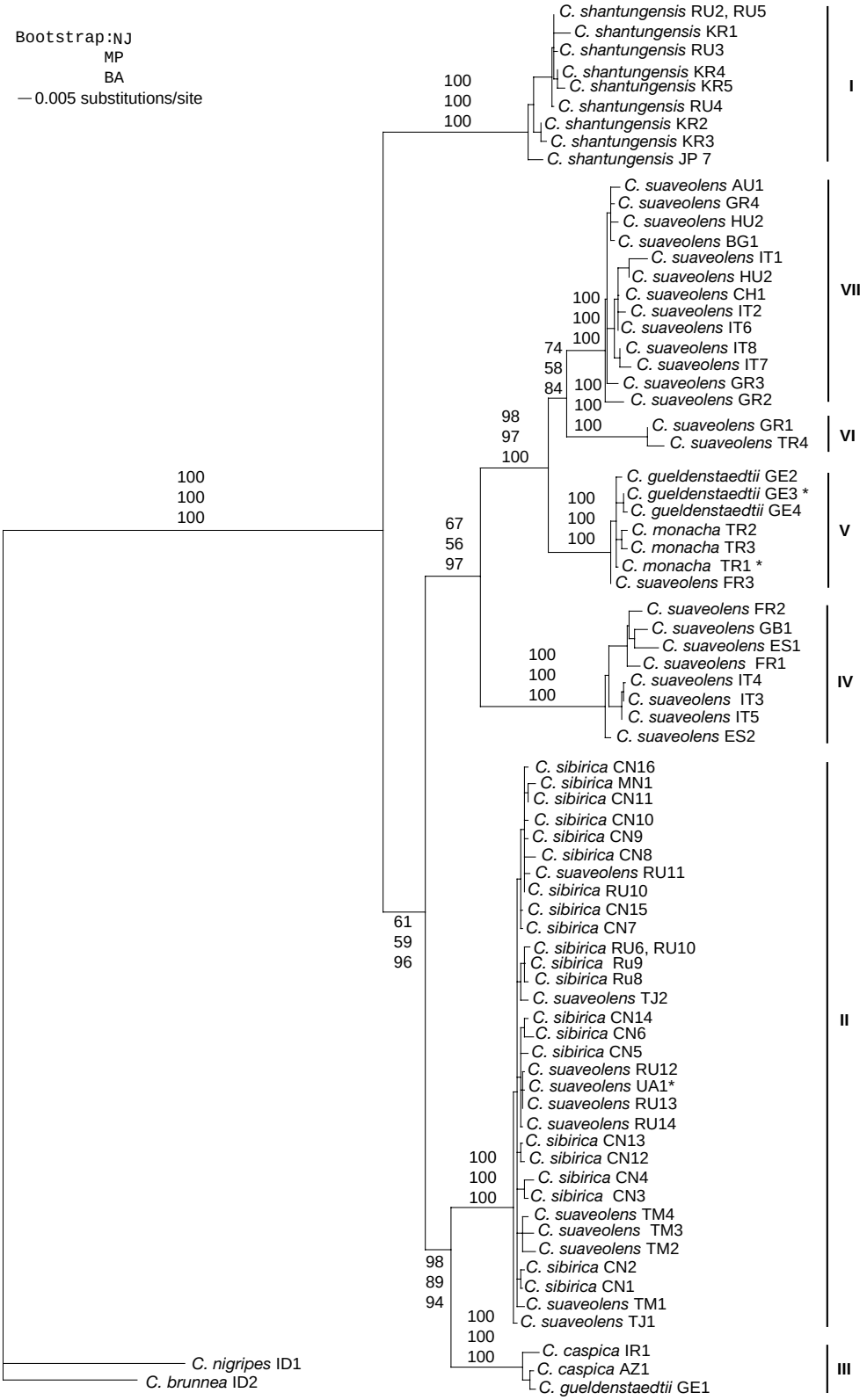

Fig. 2. Phylogeny of the 998 bp *cyt-b* fragment analysed with maximum likelihood, using the TVM + G model of substitution and TBR branch swapping. Values in branches are bootstrap indices of support for the major branches for Bayesian (BA), parsimony (MP), and distance (NJ) analyses (percentage of 1000 replications for each of the 10 random orders of stepwise addition of sequences). Codes are as in Table 1.

A good correlation was observed ($r = 0.88$, $P < 0.001$) giving a rate of 0.057 ML distance per million years, with a 95% confidence interval of 0.044–0.070 ML distance.

Relative-rate tests revealed that clade V evolved more rapidly than clades VI and VII ($P < 0.05$). Moreover, there were no significant differences in evolutionary rate between

Table 3
Mean corrected pairwise sequence divergences between the seven clades (Kimura two-parameter distances); diagonally, mean pairwise sequence divergence (K2P) and nucleotide diversity within clades

	I	II	III	IV	V	VI	VII
I	0.009/0.009						
II	0.082	0.007/0.007					
III	0.079	0.047	0.009/0.009				
IV	0.094	0.079	0.084	0.013/0.012			
V	0.102	0.077	0.083	0.078	0.005/0.005		
VI	0.108	0.083	0.083	0.079	0.054	0.005/0.006	
VII	0.092	0.069	0.073	0.059	0.036	0.037	0.024/0.010

any other clades ($P > 0.05$). We, thus, decided to exclude clade V from the molecular clock analysis. Dating of the major splits was then estimated using the calibration of Fumagalli et al. (1999). The oldest split corresponds to the separation between eastern Asian haplotypes (morphotype *C. shantungensis*, clade I) and all the others. This event took place about 1.94 Myr BP [1.58–2.52], i.e., during the Upper Pliocene (Ogg, 2004). The events that separated the European and central Asian clades occurred more recently, around the beginning of the Pleistocene (clades (IV) + (VI) + (VII) vs. (II) + (III), 1.88 Myr BP [1.53–2.42]). Then, later in the Lower Pleistocene, occurred the split between western and eastern European haplotypes (clades (VI) + (VII) vs. (IV), 1.72 Myr BP [1.40–2.23]). The most recent partition was found between clades VI (Italy–Balkans) and VII (west Turkey), with an estimated divergence time situated at the end of the Lower Pleistocene (0.94 Myr BP [0.76–1.21]). Finally, the split between clades II and III is dated at 1.12 Myr BP [0.91–1.45].

3.3. Expansion time

Clade II, comprising Russian and central Asian haplotypes, revealed a signature of population expansion. We observed a non-significant P value for the mismatch distribution test of goodness-of-fit ($P = 0.33$) and a significant P value for R_2, F_S, and Tajima's D statistics ($P < 0.05$). The timing of this expansion was estimated from the mismatch distribution ($\tau = 7.025$ and 95% CI: 4.70–8.24), according to Rogers (1995). Assuming no saturation of uncorrected distances (p) (correlation with third position transversions was $r = 0.86$, $P < 0.01$), distance was 0.0612 per million years (95% CI: 0.054–0.069). With a generation time of 1 year, the population expansion time was estimated at 58,000 years BP (95% CI: 34,000–76,000). Concerning the other clades, Tajima's D and R_2 statistics did not show evidence of population expansion, whereas F_S statistics were significant for clades V and VII. Consequently, we inferred a scenario of non-expansion for the latter.

3.4. BRCA1 gene

A striking result from our mtDNA analysis is the discrepancy between some morphotype assignments and the clustering pattern of their haplotypes. For example, clade II includes haplotypes from *C. sibirica* and *C. suaveolens*, clade III includes haplotypes from *C. caspica* and *C. gueldenstaedtii*, and clade V includes haplotypes from *C. monacha* and *C. gueldenstaedtii* morphotypes. This could result either from the invalidity of the species assignments or from the introgression events in some species of the mtDNA from another species, following hybridisation. To resolve this question, we analysed BRCA1, a nuclear gene. This analysis failed for *C. gueldenstaedtii* sample of clade III, probably due to damaged DNA in this old specimen.

The 15 different sequences of 799 bp showed 44 variable sites, of which 25 are parsimony-informative, and one deletion of three nucleotides (allele A1). In the 10 samples from clade II, three alleles were found (named 1–3; see Table 1) and four heterozygotes detected. Since the four heterozygotes possessed one copy of an allele showing the deletion, we designed a primer on this region of the gene (BRCA1dr) to amplify the other alleles only and to attribute the bases correctly at the polymorphic sites. On the other hand, no heterozygote was detected in the other clades and no polymorphism within clades I, III, and V. In the five analysed samples of clade VII, three different alleles were found (named 4–6). Two alleles were found within clades IV (named 9 and 10) and VI (named 7 and 8). Accession numbers are as follows: alleles A1–A6, AY839147–AY839152; alleles A7–A13, DQ059014–DQ059020. The phylogenetic signal from the BRCA1 gene does not contradict that of *cyt-b* (Fig. 3). But, the relationships between clades are partially unresolved. Samples of clade II, morphotypes *C. sibirica* and *C. suaveolens* from Russia and Ukraine, cluster together and differ from clade VII (morphotype *C. suaveolens* from Switzerland, Italy, Greece, Hungary, and Bulgaria). *C. monacha* and *C. gueldenstaedtii* from clade V share the same allele, as well as the two *C. caspica*. In contrast to *cyt-b*, clade V is closer to clades II and III than those of central Europe, and western Turkey (clades VI and VII), but it is weakly supported by bootstrap values (61% in MP, and 63% in ML).

4. Discussion

4.1. Genetics versus morphology

From our results, *C. sibirica*, although morphologically distinct (Zaitsev, 1993), cannot be confirmed as a valid

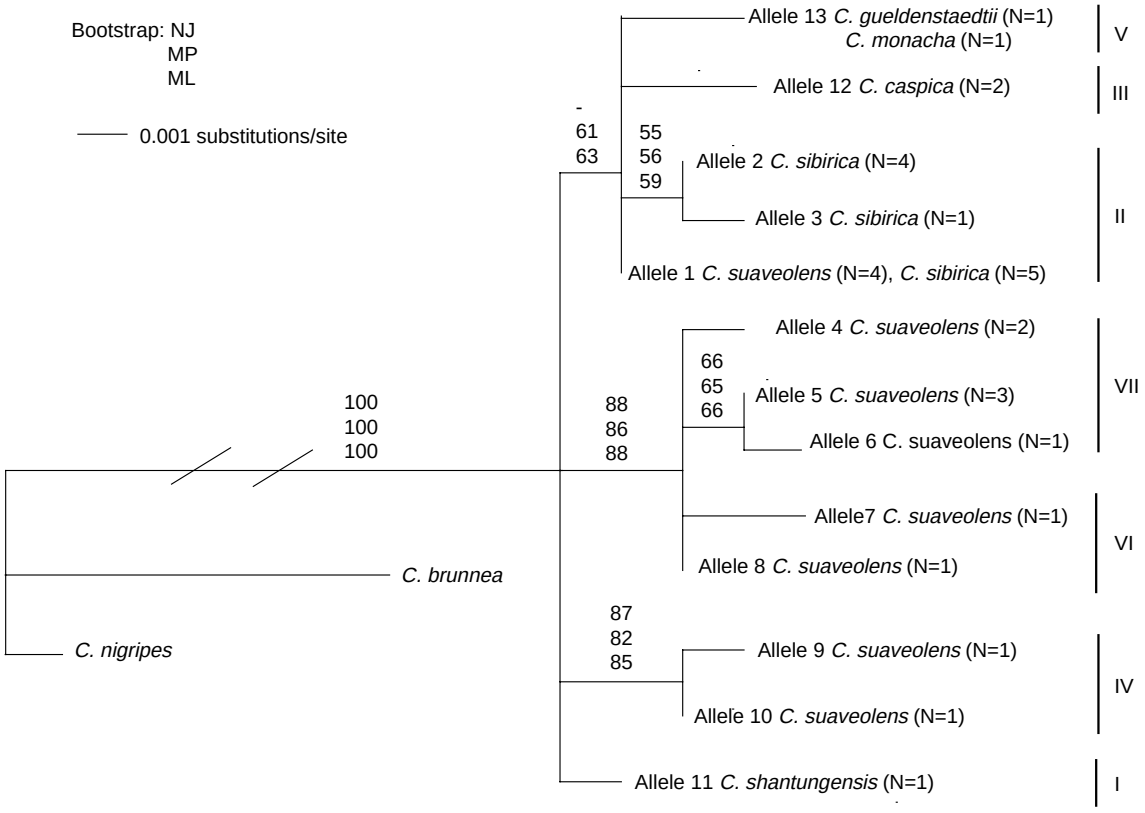

Fig. 3. Phylogeny of the 799 bp BRCA1 fragment analysed with maximum likelihood, using K81uf model of substitution and TBR branch swapping. Values in branches are bootstrap indices of support for distance (NJ), maximum parsimony (MP), and maximum likelihood (ML) analyses (percentage of 1000 replications for each of the 10 random orders of stepwise addition of sequences). Alleles 1–3 belong to clade II, 4–6 to clade VII, 7 and 8 to clade VI, 9 and 10 to clade IV, 11 to clade I, 12 to clade III, and 13 to clade V.

species. Indeed, this taxon is genetically inseparable from *C. suaveolens* from the type locality (near Sevastopol, Ukraine), belonging to the same clade (II). A possible introgression of the mitochondrial genome can be rejected, because nuclear and mitochondrial genes gave the same results. It is interesting to note that Bannikova et al. (1993, 2005), using Short Interspersed Nuclear Elements (SINEs), did not find the same results. In fact, *C. suaveolens* from western Russia (Dagestan, Moscow, and Kalmykia) form a well-defined clade, which is distinct of *C. sibirica* from Siberia (Kemerovo). With regards to our results, we suspect that all their samples do belong to clade II. Thus, the tree based on their non-coding sequences represents an internal structure inside clade II. It would, nevertheless, be interesting to screen our samples with the same SINE markers to clarify the situation.

Concerning *C. caspica*, both nuclear and mitochondrial genes point it as an evolutionarily independent unit within the suaveolens complex, although its taxonomic rank as species or subspecies requires further research. We were not able to amplify the nuclear gene of a sample with a *C. gueldenstaedtii* morphotype and a mtDNA similar to *C. caspica*. Consequently, a possible introgression of the mitochondrial genome of *C. caspica* into *C. gueldenstaedtii* cannot be rejected. The analysis of BRCA1 and *cyt-b* genes of numerous samples from both morphotypes, particularly along their contact zone, should be an urgent priority. Moreover, the nuclear results suggest a closer relationship between clade V (*C. gueldenstaedtii* and *C. monacha*) and clade III (*C. caspica*), and II (*C. sibirica* and *C. suaveolens*), than the mitochondrial analyses, which could be the result of shared ancestral polymorphism and, or, hybridisation between them.

The age of the *C. suaveolens* group, its huge distribution area, and the marked phylogeographic structure here detected may imply instances of consummated or ongoing speciation processes. Therefore, the question arises whether the most basal branches have reached the level of biological species. Such species are easily recognised when living in sympatry. However, in the case of a parapatric distribution, the interruption of gene flow in the contact zone is the only diagnostic criterion on which a decision can be based (Brünner et al., 2002), whereas high genetic distances might just suggest scenarios that need more detailed investigations. A study of four genera of rodents and seven genera of bats (Bradley and Baker, 2001) showed a mean intraspecific divergence of 2.1% (up to 6.3%) for rodents and 3% (up to 8.7%) for bats, and a mean divergence between sister species of 8.1% (2.5–19.2%). For rodents, values of 4.7% between north and southern branches of *Arvicola terrestris* (Taberlet et al., 1998) and 5.2% between eastern and western branches of *Microtus agrestis* (Hellborg et al., 2005) were suspected to

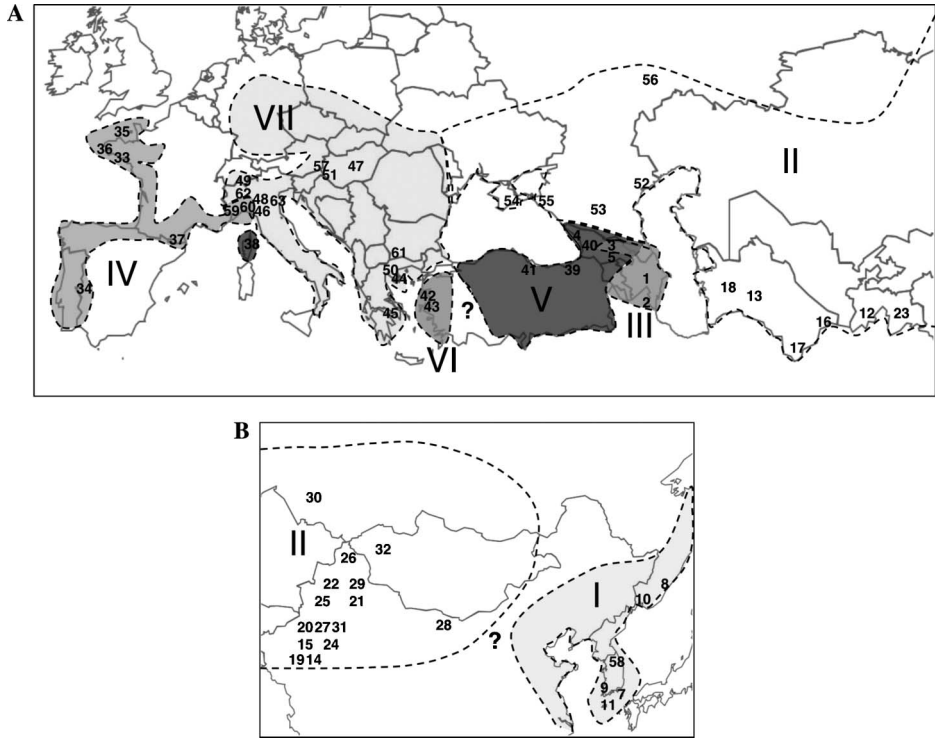

Fig. 4. Repartition of the clades found in our study ((A) II to VII; (B) I, II), with sample locations.

possibly indicate different species. In shrews, eastern and western branches of *Crocidura russula*, with 9% divergences (Vogel et al., 2003), showed male sterility of F1 hybrids in captivity (Vogel et al., 2004) and are, therefore, considered to present two species (Brändli et al., in press; Cosson et al., 2005). The contact zone situated in Algeria is not known and, therefore, the disruption of gene flow is not yet proven.

The K2P distance of 4.7–10.8%, as shown for the *C. suaveolens* group, may be situated within a single species, incipient species or well-separated full species. As morphological taxa (probably strongly conditioned by local selective forces) are not congruent with genetic clades, we consider premature to ascribe distinct species names to the different genetic populations. Their distribution seems to be parapatric and nothing is yet known regarding gene flow at the contact zones. Under these conditions, our interpretation is deliberately conservative. Based on the highest genetic distance between *C. shantungensis* (clade I) and all other clades, we accept the species assignment of this one by previous authors (Jiang and Hoffmann, 2001; Motokawa et al., 2000; Ohdachi et al., 2004). Concerning the other clades (II–VII), we could not exclude the presence of species and, or incipient species. However, the only way to unravel this question is to study gene flow at the contact zones of the respective populations (see Fig. 4).

4.2. Barriers, refugia, and colonisation routes in Eurasia

The current distribution of the *C. suaveolens* group throughout the Palaearctic is roughly limited to the north by the 50th parallel, which corresponds to the transition between the temperate and coniferous forests. This and other considerations, such as altitudinal limits within temperate areas, suggest that the '*C. suaveolens*' range is limited by cold climates. During the Middle Pliocene, i.e., 3.6–2.5 Myr BP, the climate was hotter and wetter than today (Chandler et al., 1994; Combourieu-Nebout et al., 2000; Fauquette and Bertini, 2003; Willis et al., 1999). The distributions of deciduous and coniferous forest biomes were shifted to the north by 10 or so latitudinal degrees. The major part of Eurasia was then covered by deciduous forest, which corresponds approximately to the habitat of the *C. suaveolens* group. Coniferous forests were situated where the tundra and arctic desert are found at present (Haywood et al., 2002; Thompson and Fleming, 1996). Probably, *C. suaveolens* was then continuously distributed in Eurasia, from the Atlantic to the Pacific coast, more to the north than today.

During the Upper Pliocene (2.5–1.84 Myr BP), the climate became colder and drier (Webb and Bartlein, 1992), leading to a rarefaction of the deciduous forest, with favourable habitats located more to the south (40–50° latitude), and limited to low altitude, or near the Pacific and the Atlantic Ocean coast. With the first major glaciations at the beginning of the Pleistocene (1.84 Myr BP), we suppose that the white-toothed shrews of the northern part of eastern Asia (Russia, Mongolia, Xinjiang, and Tibet) became extinct, except the population on the Pacific coast (Fig. 5) that survived all future climatic events (clade I, *C. shantungensis*). During the same event, the population west of the

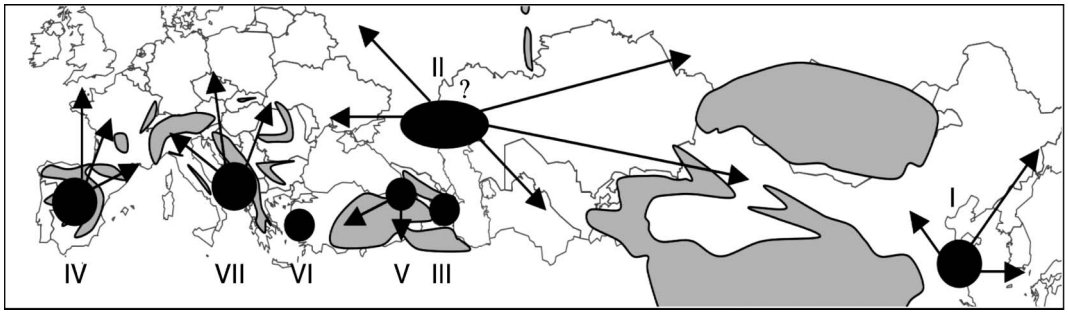

Fig. 5. Potential refuges (black), barriers (grey), and range expansion (arrows) of the seven clades.

Ural Mountains found refugia in southern areas. As southern Eurasia is characterised by some very high plateaus and mountain chains (Himalayas, Pamir, Elburz, Caucasus, and Alps), retracting populations found refugia only in mild lowland habitats, generally situated close to the seashore and isolated from each other by mountain chains. One section (clade II and clade III) probably found refuge east of the Caucasus on the Caspian coast, while the more westerly population (ancestors of clade IV and sister clades) survived in the Mediterranean basin. This split is consistent with the still high divergence between these clades (K2P distance: 6.9–8.4%) and might be situated at 1.9 Myr BP in the Lower Pleistocene.

After a re-colonisation of more northern latitudes, other contractions occurred during less dramatic cold periods allowing survival in different refugia (Fig. 5). Clade II and clade III probably evolved in mild northwestern regions (Black Sea, e.g., Crimea) and southeast of the Caucasus (southern coast of Caspian Sea). These clades are well separated (mean corrected K2P distance: 5.0%), similarly to what was observed in the wild goat (Manceau et al., 1999) and in the yellow-necked fieldmouse (Michaux et al., 2004) in the same region. This event is situated in the Lower Pleistocene, 1.1 Myr BP. A refugium northwest of the Caucasus was suggested by Hewitt (1999) for the European oak (*Quercus robur–petraea* complex) and by Jaarola and Searle (2002) for the field vole (*Microtus agrestis*), or north of the Caspian Sea, as well as Crimea, as hypothesised by Bilton et al. (1998) for small European mammals showing a recent northern distribution. Clade II is the only one showing evidence of population expansion after a bottleneck, which is supported by a low nucleotide diversity (0.007), despite the large sample size ($n = 33$) and more than 4500 km separating the more distant samples. This event dates to the Upper Pleistocene, 58,000 years BP (CI: 34,000–78,000), which is approximately situated during a unstable climatic period in Eurasia (Riss–Würm interglacial, 70,000–20,000 years BP), as shown by Dansgaard et al. (1993).

With regard to the western population, clade IV remained in the Iberian Peninsula, colonising France after the last glaciations and coming in contact with clade VII of an Italo-Balkanic refugium at the level where the Alps join the Mediterranean Sea. After the last glaciations, clade VII expanded over a large area of central Europe, including Germany, Austria, and Hungary. The barrier to the eastern extension is obvious, the Marmara Sea has been, most likely, a barrier to the eastern extension of this clade and restricted clade VI to a western Turkey refugium. The split between clades VI and VII, situated at 938,000 years BP (764,000–1,216,000) corresponds to the Günz glacial period (790,000–950,000 years BP), suggesting a colonisation of western Turkey by animals from central Europe when the sea level was lower. To the south, clade VI was isolated from the clade V by the "Anatolian Diagonal," first described by Davis (1971), a mountain range that extends from northeastern Turkey toward the southwest, dividing Anatolia into two botanical and zoogeographical areas, as found in a phylogeographic work on the horned-nose viper, *Vipera ammodytes* (Ursenbacher, personal communication).

The refugium of clade V was possibly situated on the south coast of the Black Sea or again related to the Mediterranean Sea. In fact, according to Catzeflis et al. (1985) shrews from Israel belong to the same phylogeographic clade as shrews from Turkey. A northern extension of this clade during the Holocene was limited to the east by clades II and III, and in the west by clade VI.

5. Conclusions

Our investigation was based on a large sample covering a broad range within the distribution of the *Crocidura suaveolens* group. The results support the monophyly of the *Crocidura suaveolens* group, which exhibit substantial mitochondrial and nuclear DNA structure across its Eurasiatic range. The seven identified phylogeographical clades are ancient, reflecting isolation during Pliocene climatic fluctuations and Pleistocene glaciations, and likely to have survived in southern refugia during recurrent Ice Ages, as shown in the root vole (Brunhoff et al., 2003; Galbreath and Cook, 2004) and the woodmouse (Michaux et al., 2003). The Western European barriers isolating the populations are those commonly accepted; in contrast, eastern barriers, such as the 'Anatolian Diagonal' and the Caucasus Mountains, are not as well known.

Our results are in agreement with some previous *cyt-b* gene studies using fewer specimens and restricted to smaller areas in Europe (Vogel et al., 2003) and East Asia

(Han et al., 2002; Motokawa et al., 2000; Ohdachi et al., 2004). Our sampling, being more thorough, allow for the first time a fairly complete description of the phylogeographic structure of *C. suaveolens* in Eurasia, and suggest that the validity of many taxa in the *C. suaveolens* group has to be re-evaluated. On a more general level, this investigation produced a comprehensive portrait of Pliocene and Pleistocene refugia within the region.

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References

Arntzen, J.W., Wallis, G.P., 1991. Restricted gene flow in a moving hybrid zone of the newts *Triturus cristatus* and *Triturus marmoratus* in Western France. Evolution 45, 805–826.

Atallah, S.I., 1977. Mammals of the Eastern Mediterranean Region: their ecology, systematics and zoogeographical relationships. Säugetierkd. Mitt. 25, 241–320.

Bannikova, A.A., Lavrenchenko, L.A., Kramerov, D.A., 1993. Molecular phylogeny of the Paleartic white-toothed shrew (pp. 35–39) In: Averianov A., Abramson N. (Eds.), Systematics, Phylogeny and Paleontology of Small Mammals. Russian Academy of Sciences, St. Petersburg, 246 p.

Bannikova, A.A., Lavrenchenko, L.A., Kramerov, D.A., 2005. Phylogenetic relationships between Afrotropical and Palaearctic *Crocidura* species inferred from Inter-SINE-PCR. Biochem. Syst. Ecol. 33, 45–59.

Bilton, D.T., Mirol, P.M., Mascheretti, S., et al., 1998. Mediterranean Europe as an area of endemism for small mammals rather than a source for northwards postglacial colonization. Proc. R. Soc. Lond. Ser. B Biol. Sci. 265, 1219–1226.

Bradley, R.D., Baker, R.J., 2001. A test of the genetic species concept: cytochrome-*b* sequences and mammals. J. Mammal. 82, 960–973.

Brändli, L., Handley, L.-J.L., Vogel, P., Perrin, N., in press. Evolutionary history of the greater white-toothed shrew (*Crocidura russula*) inferred from analysis of mtDNA, Y, and X chromosome markers. Mol. Phylogenet. Evol., doi:10.1016/j.ympev.2005.06.019.

Brunhoff, C., Galbreath, K.E., Fedorov, V.B., Cook, J.A., Jaarola, M., 2003. Holarctic phylogeography of the root vole (*Microtus oeconomus*): implications for late Quaternary biogeography of high latitudes. Mol. Ecol. 12, 957–968.

Brünner, H., Lugon-Moulin, N., Hausser, J., 2002. Alps, genes, and chromosomes: their role in the formation of species in the *Sorex araneus* group (Mammalia, Insectivora), as inferred from two hybrid zones. Cytogenet. Genome Res. 96, 85–96.

Catzeflis, F., Maddalena, T., Hellwing, S., Vogel, P., 1985. Unexpected findings on the taxonomic status of East Mediterranean *Crocidura russula* Auct (Mammalia, Insectivora). Z. Saugetierkd.-Int. J. Mamm. Biol. 50, 185–201.

Chandler, M., Rind, D., Thompson, R., 1994. Joint investigations of the Middle Pliocene Climate. 2. Giss Gcm Northern-Hemisphere Results. Glob. Planet. Change 9, 197–219.

Combourieu-Nebout, N., Fauquette, S., Quezel, P., 2000. What was the late Pliocene Mediterranean climate like: a preliminary quantification from vegetation. Bull. Soc. Geol. Fr. 171, 271–277.

Cooper, S.J.B., Ibrahim, K.M., Hewitt, G.M.., 1995. Postglacial expansion and genome subdivision in the European Grasshopper *Chorthippus parallelus*. Mol. Ecol. 4, 49–60.

Corbet, G.B., 1978. The Mammals of the Palaearctic Region: A Taxonomic Review. Cornwell University Press, British Museum (Natural History). 314 p.

Cosson, J.F., Hutterer, R., Libois, R., Sara, M., Taberlet, P., Vogel, P., 2005. 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). Mol. Ecol. 14, 1151–1162.

Dansgaard, W., Johnsen, S.J., Clausen, H.B., et al., 1993. Evidence for general instability of past climate from a 250-Kyr ice-core record. Nature 364, 218–220.

Davis, P.H., 1971. Distribution patterns in Anatolia with particular reference to endemism. In: Davis, P.H., Harper, P.C., Hedge, I.C. (Eds.), Plant Life of South-West Asia. Royal Botanic Garden, Edinburgh, pp. 15–27.

Dumolin-Lapegue, S., Demesure, B., Fineschi, S., LeCorre, V., Petit, R.J., 1997. Phylogeographic structure of white oaks throughout the European continent. Genetics 146, 1475–1487.

Edwards, S.V., 1997. Relevance of microevolutionary processes to higher level molecular systematics. In: Mindell, D.P. (Ed.), Avian Molecular Evolution and Systematics. Academic Press, New York, pp. 251–278.

Fauquette, S., Bertini, A., 2003. Quantification of the northern Italy Pliocene climate for pollen data: evidence for a very peculiar climate pattern. Boreas 32, 361–369.

Ferris, C., Oliver, R.P., Davy, A.J., Hewitt, G.M., 1993. Native oak chloroplasts reveal an ancient divide across Europe. Mol. Ecol. 2, 337–344.

Ferris, C., King, R.A., Vainola, R., Hewitt, G.M., 1998. Chloroplast DNA recognizes three refugial sources of European oaks and suggests independent eastern and western immigrations to Finland. Heredity 80, 584–593.

Fu, Y.X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147, 915–925.

Fumagalli, L., Taberlet, P., Stewart, D.T., et al., 1999. Molecular phylogeny and evolution of *Sorex* shrews (Soricidae: Insectivora) inferred from mitochondrial DNA sequence data. Mol. Phylogenet. Evol. 11, 222–235.

Galbreath, K.E., Cook, J.A., 2004. Genetic consequences of Pleistocene glaciations for the tundra vole (*Microtus oeconomus*) in Beringia. Mol. Ecol. 13, 135–148.

Grafodatsky, A.S., Radzhabli, S.I., Sharshov, A.V., Zaitsev, M.V., 1988. Karyotypes of five *Crocidura* species of the USSR fauna. Tsytologya 30, 1247–1250.

Han, S.H., Iwasa, M.A., Ohdachi, S.D., et al., 2002. Molecular phylogeny of *Crocidura* shrews in northeastern Asia: a special reference to specimens on Cheju Island, South Korea. Acta Theriol. 47, 369–379.

Haywood, A.M., Valdes, P.J., Francis, J.E., Sellwood, B.W., 2002. Global middle Pliocene biome reconstruction: A data/model synthesis. Geochem. Geophys. Geosyst. 3, 1072.

Hellborg, L., Gunduz, I., Jaarola, M., 2005. Analysis of sex-linked sequences supports a new mammal species in Europe. Mol. Ecol. 14, 2025–2031.

Hewitt, G.M., 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biol. J. Linn. Soc. 58, 247–276.

Hewitt, G.M., 1999. Post-glacial re-colonization of European biota. Biol. J. Linn. Soc. 68, 87–112.

Hewitt, G.M., 2000. The genetic legacy of the quaternary ice ages. Nature 405, 907–913.

Hewitt, G.M., 2003. Ice ages: their impact on species distributions and evolution. In: Rothschild, L.J., Lister, A.M. (Eds.), Evolution on Planet Earth. Academic Press, New York, pp. 339–361.

Huelsenbeck, J.P., Ronquist, F., Nielsen, R., Bollback, J.P., 2001. Evolution: Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294, 2310–2314.

Hutterer, R., 1981. Der Status von *Crocidura ariadne* Pieper, 1979 (Mammalia: Soricidae). Bonn. Zool. Beitr. 32, 3–12.

Irwin, D.M., Kocher, T.D., Wilson, A.C., 1991. Evolution of the cytochrome *b* gene of mammals. J. Mol. Evol. 32, 128–144.

Jaarola, M., Searle, J.B., 2002. Phylogeography of field voles (*Microtus agrestis*) in Eurasia inferred from mitochondrial DNA sequences. Mol. Ecol. 11, 2613–2621.

Jiang, X.L., Hoffmann, R.S., 2001. A revision of the white-toothed shrews (*Crocidura*) of Southern China. J. Mammal. 82, 1059–1079.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Manceau, V., Despres, L., Bouvet, J., Taberlet, P., 1999. Systematics of the genus *Capra* inferred from mitochondrial DNA sequence data. Mol. Phylogenet. Evol. 13, 504–510.

Michaux, J.R., Magnanou, E., Paradis, E., Nieberding, C., Libois, R., 2003. Mitochondrial phylogeography of the Woodmouse (*Apodemus sylvaticus*) in the Western Palearctic region. Mol. Ecol. 12, 685–697.

Michaux, J.R., Libois, R., Paradis, E., Filippucci, M.G., 2004. Phylogeographic history of the yellow-necked fieldmouse (*Apodemus flavicollis*) in Europe and in the Near and Middle East. Mol. Phylogenet. Evol. 32, 788–798.

Michaux, J.R., Libois, R., Filippucci, M.G., 2005. So close and so different: comparative phylogeography of two small mammal species, the Yellow-necked fieldmouse (*Apodemus flavicollis*) and the Woodmouse (*Apodemus sylvaticus*), in the Western Palearctic region. Heredity 94, 52–63.

Motokawa, M., Suzuki, H., Harada, M., et al., 2000. Phylogenetic relationships among East Asian species of *Crocidura* (Mammalia, Insectivora) inferred from mitochondrial cytochrome *b* gene sequences. Zool. Sci. 17, 497–504.

Nesbo, C.L., Fossheim, T., Vollestad, L.A., Jakobsen, K.S., 1999. Genetic divergence and phylogeographic relationships among European perch (*Perca fluviatilis*) populations reflect glacial refugia and postglacial colonization. Mol. Ecol. 8, 1387–1404.

Ogg, J.G., 2004. Status of divisions of the International Geologic Time Scale. Lethaia 37, 183–199.

Ohdachi, S., Dokuchaev, N.E., Hasegawa, M., Masuda, R., 2001. Intraspecific phylogeny and geographical variation of six species of northeastern Asiatic *Sorex* shrews based on the mitochondrial cytochrome *b* sequences. Mol. Ecol. 10, 2199–2213.

Ohdachi, S.D., Iwasa, M.A., Nesterenko, V.A., Abe, H., Masuda, R., Haberl, W., 2004. Molecular phylogenetics of *Crocidura* shrews (Insectivora) in east and central Asia. J. Mammal. 85, 396–403.

Palme, A.E., Vendramin, G.G., 2002. Chloroplast DNA variation, postglacial recolonization and hybridization in hazel, *Corylus avellana*. Mol. Ecol. 11, 1769–1780.

Parker, S.R., 1997. Sequence Navigator. Multiple sequence alignment software. Methods Mol. Biol. 70, 145–154.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Ramos-Onsins, S.E., Rozas, J., 2002. Statistical properties of new neutrality tests against population growth. Mol. Biol. Evol. 19, 2092–2100.

Robinson, M., Gouy, M., Gautier, C., Mouchiroud, D., 1998. Sensitivity of the relative-rate test to taxonomic sampling. Mol. Biol. Evol. 15, 1091–1098.

Rogers, A.R., 1995. Genetic-evidence for a Pleistocene population explosion. Evolution 49, 608–615.

Rozas, J., Sanchez-DelBarrio, J.C., Messeguer X., Rozas R., 2005. DNA Sequence Polymorphism version 4.10.2. Departament de Genetica, Universitat de Barcelona, Spain, (www document) URL <http://www.ub.es/dnasp/>.

Ruedi, M., Smith, M.F., Patton, J.L., 1997. Phylogenetic evidence of mitochondrial DNA introgression among pocket gophers in New Mexico (family Geomyidae). Mol. Ecol. 6, 453–462.

Santucci, F., Emerson, B.C., Hewitt, G.M., 1998. Mitochondrial DNA phylogeography of European hedgehogs. Mol. Ecol. 7, 1163–1172.

Schneider, I., 2000. Software programs for DNA sequence analysis. Genet. Eng. News 20, 8.

Schneider, S., Excoffier, L., 1999. Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. Genetics 152, 1079–1089.

Seddon, J.M., Santucci, F., Reeve, N., Hewitt, G.M., 2002. Caucasus Mountains divide postulated postglacial colonization routes in the white-breasted hedgehog, *Erinaceus concolor*. J. Evol. Biol. 15, 463–467.

Swofford, D.L., 1998. PAUP*. Phylogenetic Analysis Using Parsimony (* and other Methods). Version 4.0b1. Sinauer Associates, Sunderland, MA.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.G., Cosson, J.F., 1998. Comparative phylogeography and postglacial colonization routes in Europe. Mol. Ecol. 7, 453–464.

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.

Tegelstrom, H., 1987. Transfer of mitochondrial-DNA from the Northern Red-Backed Vole (*Clethrionomys rutilus*) to the Bank Vole (*Clethrionomys glareolus*). J. Mol. Evol. 24, 218–227.

Tembotova, F.A., 1999. The regularities of variability and the evolution of insectivora (Mammalia) of the Caucasus. PhD thesis (English translation), Nalchic.

Thompson, R.S., Fleming, R.F., 1996. Middle Pliocene vegetation: Reconstructions, paleoclimatic inferences, and boundary conditions for climate modeling. Mar. Micropaleontol. 27, 27–49.

Thorpe, R.S., 1984. Primary and secondary transition zones in speciation and population differentiation—a phylogenetic analysis of range expansion. Evolution 38, 233–243.

Vogel, P., Cosson, J.F., Jurado, L.F.L., 2003. Taxonomic status and origin of the shrews (Soricidae) from the Canary Islands inferred from a mtDNA comparison with the European *Crocidura* species. Mol. Phylogenet. Evol. 27, 271–282.

Vogel, P., Madallena, T., Sara, M., 2004. *Crocidura cossyrensis* Contoli, 1989 (Mammalia, Soricidae): karyotype, biochemical genetics and hybridization experiments. Rev. Suisse Zool. 111, 925–934.

Webb, T., Bartlein, P.J., 1992. Global changes during the last 3 million years: climatic controls and biotic responses. Annu. Rev. Ecol. Syst. 23, 141–173.

Willis, K.J., Kleczkowski, A., Crowhurst, S.J., 1999. 124,000-year periodicity in terrestrial vegetation change during the late Pliocene epoch. Nature 397, 685–688.

Wu, C.-I., Li, W.-H., 1985. Evidence for higher rates of nucleotide substitutions in rodents than in man. Proc. Natl. Acad. Sci. USA 82, 1741–1745.

Yasuda, S.P., Vogel, P., Tsuchiya, K., Han, S.H., Lin, L.K., Suzuki, H., in press. Phylogenetic patterning of mtDNA in a widely distributed Eurasian temperate rodent, the harvest mouse *Micromys minutus* (Muridae, Rodentia), suggests dramatic cycles of range contraction and expansion during mid- to late Pleistocene. Can. J. Zool.

Yudin, B.S., 1989. Insectivorous Mammals of Siberia. Nauka, Novosibirsk. 360 p.

Zaitsev, M.V., 1993. Species composition and questions of systematics of white-toothed shrews (Mammalia, Insectivora) of the fauna of USSR. In: Zaitsev, M.V. (Ed.), Questions of Systematics, Faunistics and Paleontology of Small Mammals. Proc. Zool. Inst. USSR Acad. Sci. vol. 243, pp. 3–46.