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Molecular phylogenetics of shrews (Mammalia: Soricidae) reveal timing of transcontinental colonizations

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Abstract

We sequenced 2167 base pairs (bp) of mitochondrial DNA cytochrome *b* and 16S, and 1390 bp of nuclear genes BRCA1 and ApoB in shrews taxa (Eulipotyphla, family Soricidae). The aim was to study the relationships at higher taxonomic levels within this family, and in particular the position of difficult clades such as *Anourosorex* and *Myosorex*. The data confirmed two monophyletic subfamilies, Soricinae and Crocidurinae. In the former, the tribes Anourosoricini, Blarinini, Nectogalini, Notiosoricini, and Soricini were supported. The latter was formed by the tribes Myosoricini and Crocidurini. The genus *Suncus* appeared to be paraphyletic and included *Sylvisorex*. We further suggest a biogeographical hypothesis, which shows that North America was colonized by three independent lineages of Soricinae during middle Miocene. Our hypothesis is congruent with the first fossil records for these taxa. Using molecular dating, the first exchanges between Africa and Eurasia occurred during the middle Miocene. The last one took place in the Late Miocene, with the dispersion of the genus *Crocidura* through the old world.

Keywords: Soricidae; Colonization; Miocene; Africa, Asia, America; Phylogeny

1. Introduction

Global climate has fluctuated greatly since the Cenozoic, especially between the Late Eocene (40 Myr ago; Ogg, 2004) and the end of the last glacial maximum of the Pleistocene (11,500 yr). The resulting sea level variations and creation of temporary land bridges led to several intercontinental exchanges between the new world and the old world via the Bering Strait (Hunt, 2004). It is commonly acknowledged that Cenozoic intercontinental exchanges between Africa and Eurasia occurred during the Miocene–Pliocene transition (5 Myr) via a land bridge situated at the actual Gibraltar Strait that was caused by the Messinian salinity crisis that partly dried out the Mediterranean sea (Azzaroli and Guazzone, 1979; Thomas et al., 1982; Chevret, 1994). Most of these observations are based on fossil data, especially of large mammals, whose fossils are more easily preserved through time (Alroy, 2003). For small mammals, the situation is different because fewer fossil data are available. Molecular phylogenetic analyses coupled with recent advances in relaxing molecular clock (e.g., Sanderson, 1997, 2002; Thorne and Kishino, 2002) can help put estimated divergence times on nodes without fossils. They are therefore very useful to resolve migration history of such taxa (e.g., Beerli and Edwards, 2002). This is particularly true with shrews (Soricidae, Eulipotyphla), because of their very poor and incomplete fossil record. Any reconstruction of their biogeographic history is therefore largely dependent on the comparison of living species (Butler, 1998).

Here, we analysed a sample of species from the Soricidae, which is one of the largest mammalian clade with more than 300 described species. It has recently been divided into three subfamilies (Hutterer, 2005). The Soricinae

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(red-toothed shrews), which are mainly distributed in the Holartic region, contain 146 species split into 13 genera. The Crocidurinae (white-toothed shrews), which have diversified in Africa and Eurasia contain 210 described species distributed in nine genera. Finally, the Myosoricinae from Africa contain 18 species and three genera (Hutterer, 2005). Despite this diversity, much of the variations observed are not particularly useful for taxonomic purposes, which makes species assignment often problematic and can render discriminant characters at supra-generic levels difficult to find.

The most seminal paper published by Repenning (1967) recognized the importance of the mandibular articulation and allowed the grouping of three genera with white teeth (*Anourosorex*, *Chimarrogale* and *Nectogale*) within the "red-toothed" Soricinae. However, uncertainties remained at three important levels. First, within the African shrews, the basal position of *Myosorex*, which shows a plesiomorphic dental formula, is not clearly demonstrated. This taxon was included by Repenning (1967) and Hutterer (1993) in Crocidurinae (tribe Myosoricini), but it was recently considered, with *Congosorex and* probably *Surdisorex*, as a different subfamily (Myosoricinae; Hutterer, 2005). Second, the relationships between *Suncus* Ehrenberg, 1832 and *Crocidura* Wagler, 1832 remains unclear. The latter shows a reduction of one unicuspid, which is usually considered as derived and it is not clear if the reduction occurred independently several times (Heim de Balsac and Lamotte, 1957; Butler, 1998). Third, uncertainties remain in the taxonomic delimitation of the tribe Neomyini, from which Reumer (1987, 1998) proposed to extract two other tribes, the Anourosoricini and the Notiosoricini. He also proposed to separate the Blarinellini tribe from the Soricini (Reumer, 1998).

Beside classical morphology, techniques such as karyology and biochemical systematics based on enzyme electrophoresis allowed much progress on species level assignment (Catzeflis et al., 1985; Maddalena, 1990; Maddalena and Bronner, 1992; Ivanitskaya, 1994; Maddalena and Ruedi, 1994; Ruedi and Vogel, 1995; Ruedi, 1996; Zima et al., 1998; Schlitter et al., 1999). This resulted in a clarification of the basal position of *Myosorex*, which suggested the creation of the Myosoricinae subfamily (Maddalena and Bronner, 1992; Hutterer, 2005). More recently, mitochondrial DNA (mtDNA) sequences allowed a much finer resolution (George, 1988; George and Sarich, 1994; Ohdachi et al., 1997, 2001; Ruedi et al., 1998; Fumagalli et al., 1999; Motokawa et al., 2000; Brant and Orti, 2002; Han et al., 2002; Vogel et al., 2003; Ohdachi et al., 2004; Dubey et al., 2006; Dubey et al., in press). At supra-generic level, complex relationships emerged with paraphyletic, and or polyphyletic taxa, (e.g., for the basal African Crocidurinae; Quérouil et al., 2001). The latter conclusions based on mtDNA were, however, often not supported. Nevertheless, in our most recent study on Soricinae (Ohdachi et al., 2006), the support was adequate at a tribe level, partially confirming the concept of Reumer (Soricini, Neomyini, Notiosoricini (1987, 1998)), but *Anourosorex* was placed in an unexpected basal position (Anourosoricinae). Such results may be due to misleading information in mitochondrial genes at higher taxonomic level, a situation that has led to erroneous phylogenetic trees for other mammals (Janke et al., 1997) and for fishes (Rasmussen and Arnason, 1999).

In this study, we aim at understanding the higher taxonomic level within the Soricidae using two nuclear and two mitochondrial markers. The lower mutation rate of nuclear genes is expected to help obtaining a good resolution at a higher taxonomic level such as subfamilies, tribes, and genera, while the two mitochondrial markers will allow a good resolution at lower taxonomic level such as intrageneric relationships. Based on the trees obtained, we analysed the potential origin of the different major clades, as well as the number of transcontinental exchanges, using molecular dating and reconstruction of biogeographic area of origins. Our goal was to unravel the diversification history of the family.

2. Materials and methods

2.1. Sampling

We analysed 81 samples of Soricidae, five of Erinaceidae, six of Talpidae, and as outgroup a more distant Laurasiatheria, the Common Pipistrelle (*Pipistrellus pipistrellus*).

Within the Soricinae, all the tribes were represented in our analyses, and we included ten of the thirteen genera recognized by Hutterer (2005). Within the Crocidurinae, the three most widespread genera (*Crocidura*, *Suncus*, and *Sylvisorex*) were represented. The rare and monospecific genera *Scutisorex* and *Ruwenzorisorex* from Africa (formerly considered as *Sylvisorex*) and *Ferroculus* and *Solisorex* from Highlands of Sri Lanka were not treated in the present study. Within the Myosoricinae, only samples of *Myosorex* were analysed. Thus, *Congosorex*, formerly considered as *Myosorex* (two species), and *Surdisorex* (two species) were not treated. For the tribes or the genus *Sorex* containing species on several continents, representative on each ones were analysed.

The samples included material from the following collections: Lausanne (IZEA), Switzerland; Paimpont (Station Biologique), France; and Sapporo (Hokkaido University), Japan (Table 1).

2.2. DNA extraction and amplification

IZEA samples were first frozen in the field in liquid nitrogen and kept for several years at −70 °C before being stored in ethanol until DNA extraction. Samples from 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 the mitochondrial cytochrome *b* gene (*cyt-b*) and 16S ribosomal sequence were performed with the

Table 1
Species and specimens used in the present study, specimen identification code for each species (ID), geographic origin of the samples and area code for the biogeographic reconstruction analysis (1: Eurasia, 2: Africa, 3: North America), collection code, and GenBank accession number of the published *cyt-b* sequences of Ohdachi et al. (2006) used in our study

Genus	Species	Specimen identification code	Origin and biogeographic code	Collection code	GenBank accession of published *cyt-b* sequence
Anourosorex	*squamipes*	1	CN, Wollung Valley (Yunnan), 1	T4738	
Anourosorex	*yamashinai*	1	TW, Nantou Co., 1	ASTW.I	AB175088
Anourosorex	*yamashinai*	2	TW, Chiayi Co., 1	ASTW.2	AB175089
Blarina	*brevicauda*	1	US, Michigan State, 3	02.7.23.1	
Blarina	*brevicauda*	2	US, Michigan State, 3	BLB.I	AB175134
Blarinella	*griselda*	1	VN, Mt. Tay Con Linh II, 3	BLG	AB175144
Chimarrogale	*himalayica*	1	VN, Ha Tinh, Huong Son, 1	VIET.CHV	AB175094
Chimarrogale	*platycephala*	1	JP, Nagasaki Pref., 1	IZEA 7610	
Chimarrogale	*platycephala*	2	TW, Nantou Co., 1	3.3.15.1	
Chodsigoa	*caovansunga*	1	VN, Mt. Tay Con Linh II, 1	COC1	AB175104
Chodsigoa	*caovansunga*	2	VN, Mt. Tay Con Linh II, 1	COC2	AB175103
Chodsigoa	*parca*	1	VN, Mt. Tay Con Linh II, 1	COPI	AB175105
Chodsigoa	*parca*	2	VN, Mt. Tay Con Linh II, 1	COP2	AB175106
Chodsigoa	*sodalis*	1	TW, Kao-Hsiung Co., 1	SIS.2	AB175102
Chodsigoa	*sodalis*	2	TW, Chiayi Co., 1	SIS.I	AB127978
Cryptotis	*goldmani*	1	Mexico, Gurrero State, 3	X2	AB175138
Cryptotis	*magna*	1	Mexico, Oaxaca State, 3	X4	AB175141
Cryptotis	*parva*	1	US, Texas State, 3	CRPI	AB175135
Episoriculus	*fumidus*	1	TW, Chiayi Co., 1	SIF.2	AB175108
Episoriculus	*fumidus*	2	TW, Nantou Co., 1	SIF.I	AB175107
Neomys	*anomalus*	1	CH, Sion, 1	IZEA 5524	
Neomys	*anomalus*	2	YU, Popova Sapka, 1	IZEA 1367	
Neomys	*anomalus*	3	PT, Unhais da Serra Covilha, 1	IZEA 5919	
Neomys	*fodiens*	1	YU, Popova Sapka, 1	IZEA 1368	
Neomys	*fodiens*	2	IT, Laghi di Ceretto, 1	IZEA 5643	
Neomys	*fodiens*	3	CN, Parc Nat. Altaï, 1	IZEA 7453	
Neomys	*fodiens*	4	CH, Bassins, 1	IZEA 5686	
Notiosorex	*crawfordi*	1	US, Texas State, 3	NSC2	AB175146
Notiosorex	*crawfordi*	2	US, Texas state, 3	NSCI	AB175145
Sorex	*alpinus*	1	CH, Pont-de-Nant, 1	IZEA 5444	
Sorex	*araneus*	1	SK, Bratislava, 1	IZEA 5744	
Sorex	*araneus*	2	CN, Parc Nat. Altaï, 1	IZEA 7452	
Sorex	*cinereus*	1	US, 3	99.9.19.1	
Sorex	*cinereus*	2	US, 3	99.9.21.1	
Sorex	*excelsus*	1	CN, Qinghai, 1	MSI 4456	
Sorex	*excelsus*	2	CN, Qinghai, 1	MSI 4470	
Sorex	*fumeus*	1	US, 3	PA110	
Sorex	*fumeus*	2	US, Pennsylvania State, 3	SEF.I	AB175116
Sorex	*granarius*	1	ES, Rascafria, 1	IZEA 639	
Sorex	*isodon*	1	FI, Iisalmi, 1	IZEA 5622	
Sorex	*minutus*	1	CH, Champmartin, 1	IZEA 7622	
Sorex	*raddei*	1	TR, Sumela, 1	IZEA 6080	
Sorex	*raddei*	2	TR, Sumela, 1	IZEA 6081	
Sorex	*saussurei*	1	MX, Guerrero State, 3	SESA2	AB175118
Sorex	*saussurei*	2	MX, Guerrero State, 3	SESA1	AB175117
Sorex	*volnuchini*	1	TR, Sumela Altindere, 1	IZEA 6079	
Crocidura	*brunnea*	1	ID, Java/Cibodas, 1	IZEA 4549	
Crocidura	*buettikoferi*	1	BF, Adiopodoumé, 2	IZEA 2409	
Crocidura	*leucodon*	1	TR, Altindere, 1	IZEA 6040	
Crocidura	*malayana*	1	MY, Ulu Gombak, 1	IZEA 3550	
Crocidura	*nanilla*	1	CI, Lamto, 2	IZEA 2530	
Crocidura	*negligens*	1	MY, Tioman, 1	IZEA 3557	
Crocidura	*nigripes*	1	ID, Bore Katimbo Sulavesi, 1	IZEA 4400	
Crocidura	*olivieri*	1	BF, Bangui, 2	IZEA 2821	
Crocidura	*orientalis*	1	ID, Java/Cibodas, 1	IZEA 4551	
Crocidura	*shantungensis*	1	JP, Tsushima Isl., 1	IZEA 7510	
Crocidura	*suaveolens*	1	HU, Fülophasa, 1	IZEA 6732	
Crocidura	*theresae*	1	BF, Bobo Dioulasso, 2	IZEA 3092	
Crocidura	*viaria*	1	BF, Oursi, 2	IZEA 3108	
Suncus	*dayi*	1	IN, Avallanchi, 1	IZEA V562	

Table 1 (*continued*)

Genus	Species	Specimen identification code	Origin and biogeographic code	Collection code	GenBank accession of published *cyt-b* sequence
Suncus	*dayi*	2	IN, Avallanchi, 1	IZEA V567	
Suncus	*dayi*	3	IN, Kotagiri, 1	IZEA V576	
Suncus	*etruscus*	1	FR, Camargue/Le Pèbre, 1	IZEA 5462	
Suncus	*etruscus*	2	IT, Fivizzano c/o Farina, 1	IZEA 5641	
Suncus	*montanus*	1	IN, Kotagiri, 1	IZEA V573	
Suncus	*murinus*	1	IN, 1	IZEA V546	
Suncus	*murinus*	2	IN, Masinagudi, 1	IZEA V554	
Suncus	*murinus*	3	JP, Okinawa, 1	SUN2	AB175074
Suncus	*remyi*	1	GA, Moueva, 2	SBP GA3650	
Suncus	*varilla*	1	ZA, 2	IZEA "4"	
Suncus	*varilla*	2	ZA, 2	IZEA "3"	
Sylvisorex	*johnstoni*	1	GA, Doudou Mounts, Moueva, 2	SBP GA3691	
Sylvisorex	*johnstoni*	2	GA, Doudou Mounts, Moueva, 2	SBP GA3695	
Sylvisorex	*johnstoni*	3	GA, Doudou Mounts, Moueva, 2	SBP GA3648	
Sylvisorex	*ollula*	1	GA, Doudou Mounts, Moueva, 2	SBP GA3586	
Myosorex	*cafer*	1	ZA, Serala Prov. Nature Res., 2	GB40764	
Myosorex	*sclateri*	1	ZA, Natal, Matubatuba, 2	GB40382	
Myosorex	*sclateri*	2	ZA, Natal, Matubatuba, 2	GB40359	
Myosorex	*varius*	1	ZA, Natal, Ngome Forest, 2	GB39824	
Myosorex	*varius*	2	ZA, Pretoria, 2	GB41102	
Myosorex	*varius*	3	ZA, Pretoria, 2	GB41086	
Erinaceus	*europaeus*	1	CH, Lausanne, 1	IZEA dorigny	
Hylomys	*parvus*	1	ID, Sumatra, 1	IZEA 4494	
Hylomys	*parvus*	2	ID, Sumatra, 1	IZEA 4495	
Hylomys	*parvus*	3	ID, Sumatra, 1	IZEA 4493	
Hylomys	*parvus*	4	ID, Sumatra, 1	IZEA 4484	
Euroscaptor	*mizura*	1	JP, Aomori Prefecture, 1	01.5.26.2	
Talpa	*caeca*	1	CH, Bellinzone, 1	IZEA 5968	
Talpa	*caeca*	2	CH, Bellinzone, 1	IZEA 5976	
Talpa	*europea*	1	CH, Meride, 1	IZEA 5972	
Uropsilus	*sp.*	1	CN, Wollung Valley (Yunnan), 1	IZEA T4739	
Uropsilus	*sp.*	2	CN, Wollung Valley (Yunnan), 1	IZEA T4743	
Pipistrellus	*pipistrellus*	1	CH, Préverenges, 1	IZEA 5408	

Abbreviations of countries: BF, Burkina Faso; CI, Ivory Coast; CH, Switzerland; CN, China; ES, Spain; FI, Finland; FR, France; GA, Gabon; HU, Hungary; ID, Indonesia; IN, India; IT, Italy; JP, Japan; MY, Malaysia; MX, Mexico; PT, Portugal; SK, Slovakia; TR, Turkey; TW, Taiwan; US, United States of America; VT, Vietnam; YU, Yugoslavia; ZA, South Africa.

primer pairs L14724/H15149, C1/C2, C3/H15915, and L14724/H15915 (see Irwin et al., 1991; Dubey et al., 2006), and 16sf (5′cct acc gag cct ggt gat ag 3′)/16Srbis (5′ ata gat aga aac cga cct gg 3′), specifically developed for this study. Amplification of the Breast cancer susceptibility 1 (BRCA1) and Apolipoprotein B (ApoB) nuclear genes (exons) were performed using the primer pairs, B1f/B1r (Dubey et al., 2006) and ApoBf (5′gca atc att tga ctt aag tg 3′)/ApoBr (5′gag caa caa tat ctg att gg 3′), specifically developed for this study. Amplification conditions for the *cyt-b*, 16S, and BRCA1 consisted of 35 thermal cycles (40 for BRCA1) of 60 s denaturation (30 s for the primers pairs L14724/H15149, C1/C2, C3/H15915) at 94 °C, 60 s (45 s for the primers pairs L14724/H15149, C1/C2, C3/H15915) annealing at 50 °C for *cyt-b* (52 °C for BRCA1, and 55 °C for 16S) and 120 s (60 s for the primers pairs L14724/H15149, C1/C2, C3/H15915) extension at 72 °C. Amplification conditions for the ApoB gene consisted of 40 cycles of 45 s denaturation at 94 °C, 45 s annealing at 50 °C and 90 s extension at 72 °C.

PCR products were checked on a 1% agarose electrophoresis gel and visualised with ethidium bromide staining to verify PCR quality. Products were then purified by centrifugal dialysis using the QIAquick PCR Purification Kit (Qiagen), according to the 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 of ABI PRISM™ Dye Terminator 1 (Perkin-Elmer). Sequence reactions were visualised on an ABI 3100 genetic analyser (Applied Biosystems).

2.3. Phylogenetic analyses

The sequences were aligned using the multiple alignment algorithm implemented in ClustalW (Thompson, 1994), and further checked by eye. Due to the problems associated with the ILD test (Yoder et al., 2001; Struck et al., 2006; Wheeler et al., 2006), the congruence between the four markers was tested by performing 100 bootstrap resamples on each marker and comparing the support level thus obtained for each node. The four DNA sequences were combined only if all

supported nodes (defined here by bootstrap values >75%) were present in all four obtained trees (e.g., Barrett et al., 1991; Huelsenbeck et al., 1996; Mason-Gamer and Kellogg, 1996; Cunningham, 1997; Halanych, 1998; Struck et al., 2006). Maximum parsimony analyses on the complete data set were performed using Paup*4.0b10 (Swofford, 2001) with 10,000 random addition sequence followed by TBR branch swapping, and keeping at most 100 trees at each replicate. Support values were estimated using 1000 bootstrap resamples using the same heuristic settings. For maximum likelihood (ML) and Bayesian analyses (BA), the models of DNA substitution were selected for each DNA region using dt_modsel (Minin et al., 2003). The GTR + G (Rodriguez et al., 1990; Yang, 1996) model best fitted the combined data set formed by the concatenation of the four sequences. Fast ML heuristic searches and bootstrap analyses (1000 replicates) were performed on the combined data set only using PHYML (Guindon and Gascuel, 2003). Bayesian analyses were performed with a partition specific models, using MrBayes version 3.0 b4 (Huelsenbeck et al., 2001). The HKY85 + G + I model (Hasegawa et al., 1985; Yang, 1996) was used for the 16S and *cyt-b* partition and the GTR + G for the ApoB and BRCA1 partition. Four independent runs were performed, each consisting of four parallel MCMC chains of five millions generations. Trees were sampled every 1000 generations. The first 3000 trees were discarded as burnin. Both ML and BA analyses were performed on the Vital-IT cluster (Swiss Institute of Bioinformatics).

2.4. Molecular dating

The calibration points used for dating the trees was the oldest known Soricinae–Crocidurinae ancestors (20 Myr, Reumer, 1989, 1994), the oldest known *Cryptotis* (9 Myr BP; Harris, 1998), and *Otisorex* (3.5 Myr BP; Maldonado et al., 2001). Position of fossils in the tree is shown in Fig. 2. The tree with the highest posterior probability found by MrBayes (see above) was selected as the "true" topology for the molecular dating and a Bayesian approach was used to estimate absolute divergence time. The variance–covariance matrix of rates of substitution of each data partition was estimated as implemented in the software estbranch (Thorne and Kishino, 2002). The four matrices were then used to estimate the divergence time with the program multidivtime (Thorne and Kishino, 2002). The fossil calibration points were used as a lower bound constraint, and the root of the tree was constrained to be at most 50 Myr old (required by the software). Two independent runs were done to assess convergence. In each run, a one million generation chain was run, sampling every 100 generations. All the analyses were ran on the Vital-IT cluster (Swiss Institute of Bioinformatics).

2.5. Reconstruction of biogeographic origin

To infer the possible biogeographic origin of the Soricidae and different genera, we reconstructed ancestral geographical origins of each clades with a maximum likelihood approach using Mesquite 1.05 (Maddison and Maddison, 2004). The current geographic distribution of extant species was coded as, (1) for Eurasian taxa, (2) for African taxa, and (3) for North American taxa (Table 1). The model of character evolution was a simple stochastic model (Mk1; Lewis, 2001), which assumes a symmetric and equal rate of change between any two states. The probability that a character changes along a branch of the tree is then a function of the branch length, a change being more likely on longer branches than on shorter ones. Here, we used the calibrated tree obtained by molecular dating (see section above) in order to have branch lengths representing absolute time of divergence.

3. Results

3.1. Phylogenetic relationships

The 93 sequences of 3577 bp (on which 338 bp were excluded from the analyses) used in this study showed 1892 variable sites, of which 1638 were parsimony-informative. GenBank accession numbers are as follows: for *cyt-b*, DQ630379-DQ630437, DQ42541, DQ521043-DQ521045, DQ065609, DQ065611, for 16S, DQ630291-DQ630378, for BRCA1, DQ630209-DQ630290, and for ApoB, DQ630122-DQ630208. Other *cyt-b* sequences were taken from our previous study (Ohdachi et al., 2006; see Table 1). The alignment file is deposited on TreeBASE under the submission ID number SN3114. The congruence test showed that no nodes with bootstrap higher than 75% were contradicted by another partition of the data (data not shown). We thus considered that the four markers could be combined for further analyses. Using the combined data matrix, trees obtained by ML, MP, and BA showed identical supported clades. Consequently, only the tree obtained by ML on the complete dataset is shown in Fig. 1. All families, subfamilies, and tribes (Fig. 1) were monophyletic and were supported by 100% bootstrap, for MP, ML, and a posterior probability of 1.0 for BA (branches support is always cited in the same order in the text, i.e., bootstrap for MP, ML, and posterior probability for BA). All genera, except *Suncus*, were also supported by all analyses (support of >78%, >83% and 1.0; Fig. 1). Using a vespertilionid bat as outgroup, the Talpidae was the most basal clade, followed by the Erinaceidae, which is the sister clade of the Soricidae (support of 95%, 80%, and 1.0 for the Talpidae, and 100%, 100% and 1.0 for the Erinaceidae). Within the Soricidae, the Crocidurinae and Myosoricinae (*sensu* Hutterer, 2005) were both monophyletic (support of each subfamily of 100%, 100% and 1.0), and clustered together (support of 100%, 100% and 1.0), formed a sister clade to the Soricinae (support of 100%, 100% and 1.0). Within the Crocidurinae, the genus *Suncus* was

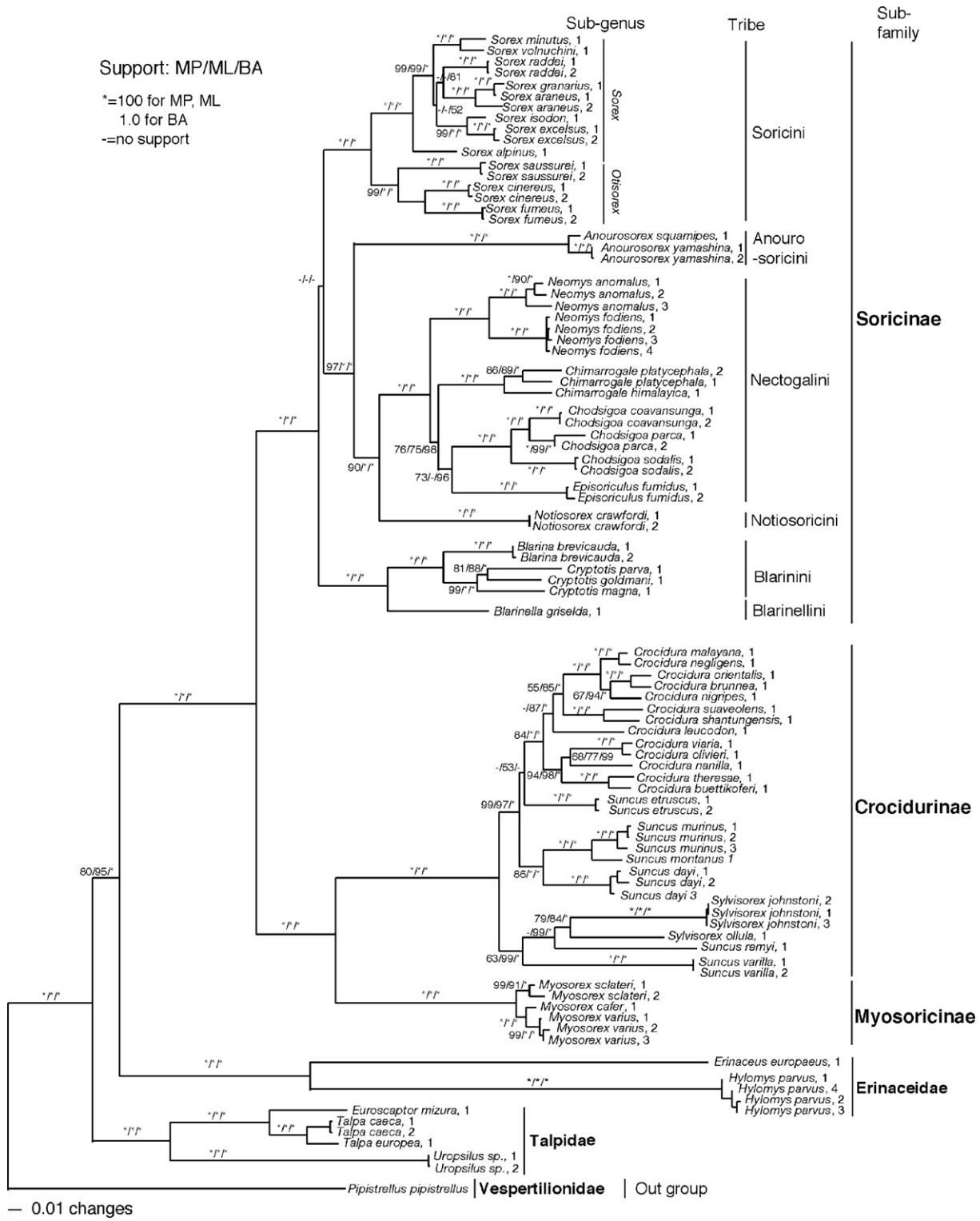

Fig. 1. Phylogeny of the 3314 bp analysed with maximum likelihood, using the TVM + G model of substitution and TBR branch swapping. Values in branches are supports, for the major branches for maximum parsimony (MP) and maximum likelihood (ML) analyses, and Bayesian posterior probabilities (BA). Specimens codes are as in Table 1.

paraphyletic. The African *Suncus* (*S. varilla* and *S. remyi*) and the representatives of the genus *Sylvisorex* (*S. ollula, S. johnstoni*) formed a basal clade (support of 63%, 99% and 1.0; Fig. 1) to the Asiatic *Suncus* (*S. dayi*, *S. montanus, S. murinus*; support of 86%, 100% and 1.0). The pygmy white-toothed shrew *Suncus etruscus* was found to be the sister group of the monophyletic genus *Crocidura* (support of <50%, 53% and <0.5).

Three major clades are included in the Soricinae subfamily. The first contained the Soricini tribe, where

the subgenera *Sorex*, and *Otisorex* were monophyletic (support of 100%, 100% and 1.0, and 100%, 99% and 1.0, respectively). The second corresponded to the Blarinini and Blarinellini tribes (support of 100%, 100% and 1.0). Finally, the third included the Anourosoricini tribe that is basal to the Notiosoricini, and the Nectogalini (Hutterer, 2005; former Neomyini, Hutterer, 1993) tribes. Within this latter tribe, the genus *Neomys* is the sister clade to all other genera (support of 90%, 100% and 1.0).

3.2. Molecular dating

The two independent runs of MCMC (Markov chain Monte Carlo) gave very similar results (Pearson correlation = 0.98) and the major dates obtained during the first analysis are shown below. The separation between Soricidae tribes occurred during the middle Miocene between 16.5 Myr (95% CI: 12.5–20.5) and 13.8 Myr (95% CI: 10.2–17.4) ago (Fig. 2). The separation of the Palaearctic and Nearctic Soricinae happened during the same period, i.e., between *Otisorex* and *Sorex* 13.9 Myr ago (95% CI: 10.2–17.5), Notiosoricini and Nectogalini 13.8 Myr ago (95% CI: 10.2–17.4), and *Blarinella*, *Blarina* and *Cryptotis* 12.1 Myr ago (95% CI: 5.1–17.7).

The split between the Myosoricinae and Crocidurinae occurred 16.5 Myr ago (95% CI: 12.5–20.5). The split between the basal African Crocidurinae clade (*Sylvisorex*, and *Suncus*) and the Eurasian *Suncus* occurred 10.8 Myr ago (95% CI: 7.6–14.0) whereas the split between this latter and the genus *Crocidura* happened more recently around 9.3 Myr ago (95% CI: 6.4–12.3). Finally, the split between Eurasian and African *Crocidura* occurred in the Late Miocene around 8.0 Myr (95% CI: 5.4–10.7).

3.3. Biogeographic reconstruction

The common pipistrelle that was chosen as outgroup to reconstruct the phylogenetic trees was removed because of the difficulty to assign a biogeographic area for its distribution. It is furthermore a very distant taxa of the studied families.

Therefore, the Erinaceidae and Talpidae were the most basal groups for the biogeographic reconstruction. Fig. 3a shows the results of the ancestral biogeographic

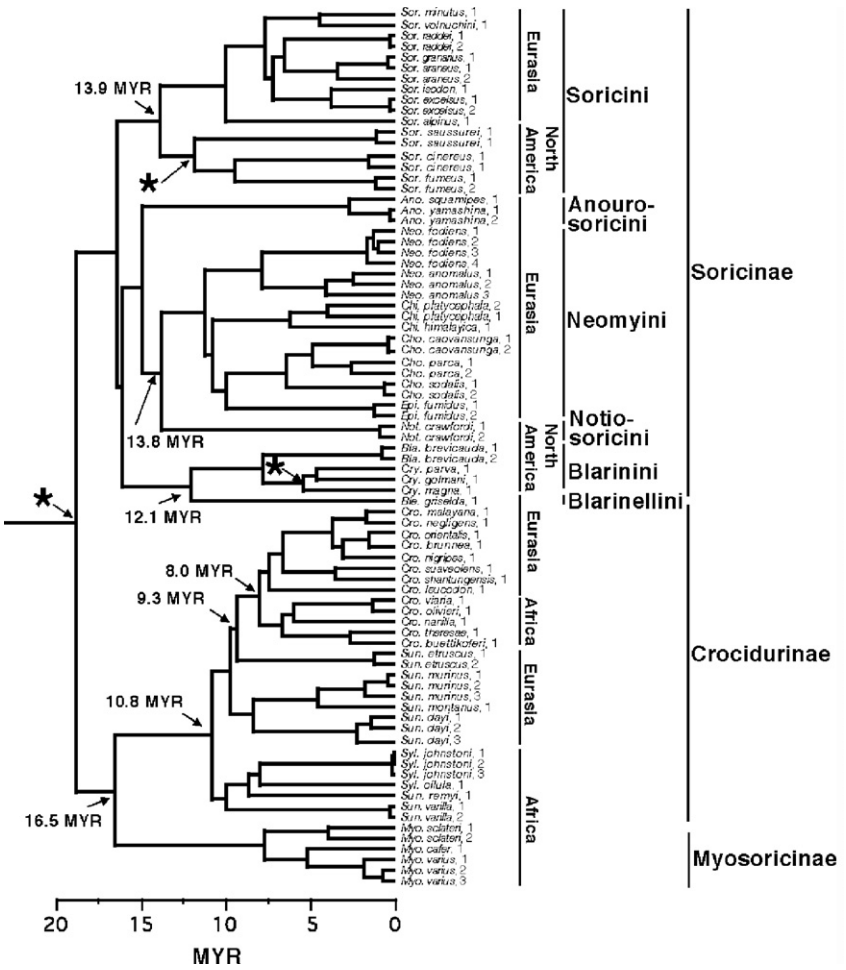

Fig. 2. Molecular dating of splits. The stars indicate the calibration points that were taken as a lower bound on the indicated node. Specimens codes are as in Table 1, and genus abbreviations are Sor. for *Sorex*, Ano. for *Anourosorex*, Neo. for *Neomys*, Chi. for *Chimarrogale*, Cho. for *Chodsigoa*, Epi. for *Episoriculus*, Not. for *Notiosorex*, Bla. for *Blarina*, Cry. for *Cryptotis*, Ble for *Blarinella*; Cro. for *Crocidura*, Sun. for *Suncus*, Syl. for *Sylvisorex*, Myo. for *Myosorex*.

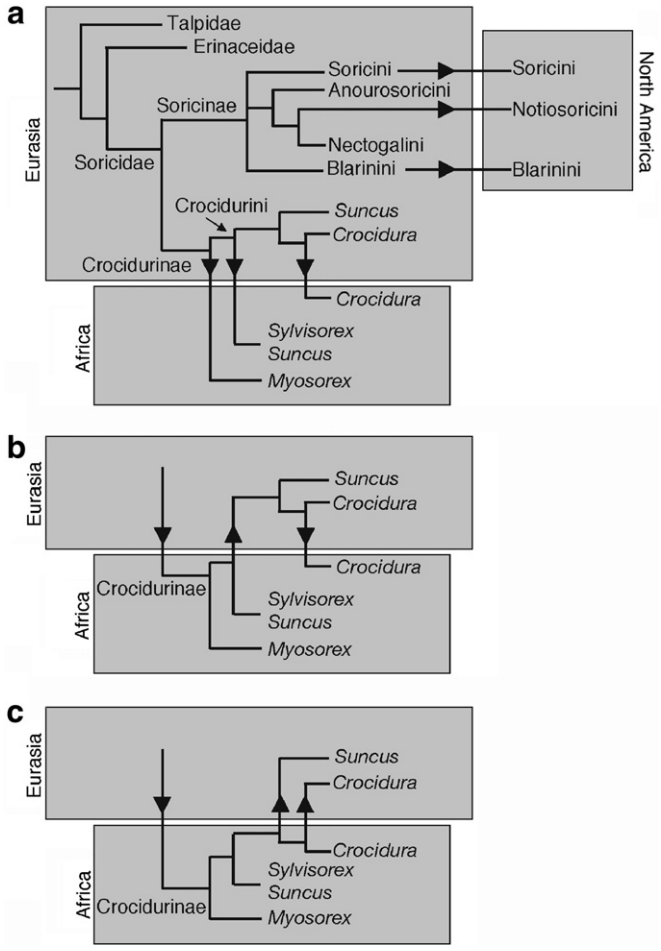

Fig. 3. Hypothesized history of shrews based on the present phylogeny (no time scale), taking into account for the biogeographic analysis (a) and alternative scenarios for the Crocidurinae, only based on fossils records, see Section 4.2 (b,c).

origins based on maximum likelihood reconstruction of ancestral areas. The Crocidurinae–Myosoricinae–Soricinae ancestor presents a probability of 99.5% of being Eurasian, and the basal nodes of Soricinae, and Crocidurinae a probability of, respectively, 99.9% and 76.4% of being of Eurasian origin. The basal nodes between American and Eurasian Soricinae, i.e., Notiosoricini vs Nectogalini, *Otisorex* vs *Sorex*, and *Cryptotis* and *Blarina* vs *Blarinella* have a probability of, respectively, 99.8%, 98.1%, and 95.6% of being Eurasian. The basal nodes within *Otisorex*, and between *Blarina* and *Cryptotis* have a probability of, respectively, 94.9% and 98.6% of being of American origin. Concerning the Crocidurinae, the basal nodes of Crocidurini have a ML prob. of 74.9% of being Eurasian. The basal node of the Eurasiatic *Suncus* and *Crocidura*, and the basal node to the previous genus have a ML prob. of 99.4%, and 99.3% of being Eurasian. The basal nodes of the African taxa, i.e., *Myosorex*, *Sylvisorex*, African *Suncus*, and African *Crocidura* have probabilities up to 96% of being of an African origin.

4. Discussion

4.1. Molecules vs morphology

The Talpidae, which were considered by most morphologists as sister group to the Soricidae and part of the Eulipotyphla (Simpson, 1945; Macphee and Novacek, 1993; Symonds, 2005), are replaced in our results by the Erinaceidae (Fig. 1), which are represented in this study by two subfamilies (Erinaceinae, and Galericinae). This position was also supported by recent studies based on nuclear genes (Murphy et al., 2001; Douady and Douzery, 2003).

With the combined DNA regions used in this study, the Soricinae were found as sister group to the Crocidurinae (*sensu* Repenning, 1967; Fig. 1). This position confirmed the classical hypothesis of Repenning (1967).

Although *Myosorex* formed a monophyletic group, its definition as a distinct subfamily (Maddalena and Bronner, 1992; Quérouil et al., 2001; Hutterer, 2005) cannot be advocated from our results. The genetic distance, expressed by the branch lengths, is comparable to those of different tribes of Soricinae. This close relationship with Crocidurinae (sensu Hutterer, 2005) contrasts with Querouil's results (2001) that tend to cluster the *Myosorex* with Soricinae on the basis of 16SrRNA data. However, their results were not well supported.

Similarly, the classification of the *Anourosorex* in a distinct subfamily (Ohdachi et al., 2006) was not supported by our analysis (Fig. 1), the *Anourosorex* being in a central position within the Soricinae. Thus, we propose to attribute a tribe level for these two clades, which should be named, respectively, Myosoricini (included within the Crocidurinae; Repenning, 1967), and Anourosoricini (included within the Soricinae). The term Myosoricini (Crocidurinae), and Anourosoricini (Soricinae) will be used in the rest of the discussion and in Fig. 3.

Within the Soricinae, five tribes received bootstrap support of 100%, 100% and posterior probabilities of 1.0 (Fig. 1). As in Repenning (1967), the Soricini remained a basal tribe, but *Blarinella* does no longer belong to it (Fig. 1). The split between the Eurasian subgenus *Sorex* and the American subgenus *Otisorex* confirmed earlier hypotheses (Fumagalli et al., 1999; Ohdachi et al., 2006). Furthermore, the Asian genus *Blarinella*, should not be placed in a separate tribe Blarinellini (Reumer, 1998; Hutterer, 2005), as it was found to be the sister taxon of the American genera *Blarina* and *Cryptotis* (support of 100%, 100% and of 1.0, Fig. 1). This position confirmed the hypothesis of Thomas (1911) and some of our previous results (Ohdachi et al., 2006). Consequently, *Blarinella* will be considered in the rest of the discussion and in Fig. 3 as a member of the Blarinini tribe. Moreover, the Neomyini (Repenning, 1967) should be split into three tribes, as proposed by Reumer (1998): (i) the Nectogalini (Hutterer, 2005) with *Neomys* basal to *Chodsigoa*, *Episoriculus, and Chimarrogale*; this last genus being basal to *Chodsigoa* and *Episoriculus*, (ii) the Notiosoricini, and (iii) the Anourosoricini (support of 100%, 100%, and of 1.0

for each tribe, Fig. 1). The relationships between the Soricini, the Blarinini and the other tribes remained unresolved (Fig. 1).

Within the Crocidurini, the genus *Suncus* appeared paraphyletic, the African species forming a basal clade with the genus *Sylvisorex* Thomas, 1904, which is strictly confined to Africa. In contrast, the three Asian species (*S. dayi*, *S. montanus*, and *S. murinus*) formed a monophyletic clade (support of 100%, 86%, and 1.0, Fig. 1), clustered with the pygmy white-toothed shrew, *Suncus etruscus*, which is basal to the monophyletic genus *Crocidura* (support of <50%, 53% and of <0.5, Fig. 1). These results are in agreement with two molecular studies using the mitochondrial *cyt-b* gene or 16S (Quérouil et al., 2001; Ohdachi et al., 2006), which suggested that none of the *Suncus* species are included within *Crocidura*.

Consequently, as the type species of the genus *Suncus* is the Asian *Suncus murinus* (Linnaeus, 1766), the Eurasian *Suncus* should then conserve their taxonomic status. However, the position of the African clade represented by the genera *Suncus* and *Sylvisorex* is not yet clearly understood, and no morphological synapomorphies are yet known to define these clades. The analyses of additional species of these genera are needed to unravel their phylogenetic relationships and draw systematic conclusions. This also applies to the questionable position of the monospecific genera *Scutisorex* Thomas, 1913, and *Ruwenzorisorex* Hutterer, 1986, whose type specimens were first described as *Sylvisorex somereni* Thomas, 1910, and *Sylvisorex suncoides* Osgood, 1936, respectively.

4.2. Biogeography

Our study is the first molecular biogeographic reconstruction of the Soricidae, based on molecular dating and reconstruction of biogeographic area of origin. We should note that the biogeographic analyses are based on a simple model. The exchanges between continents are considered as equiprobable in both directions, and they can occur at any time. Under these assumptions, the family originated in Eurasia as suggested by fossils records (Repenning, 1967; Rzebik-Kowalska, 1998; Storch et al., 1998). North America was colonized by three independent lineages of Eurasian Soricinae (Fig. 3) during the middle Miocene (from 13.9 to 12.1 Myr, Fig. 2). These events are congruent with the first fossil records of the genera *Cryptotis* and *Notiosorex* in North America between 9 and 12 Myr (Harris, 1998), and with the molecular study of Fumagalli et al. (1999) for the subgenera *Otisorex*. They occurred after the climatic transition from 14.8 to 16.0 Myr, which was marked by major short-term variations in global climate and a global low sea level between 14.8 and 12.9 Myr (Flower and Kennett, 1994). This led to the formation of a land connection between Siberia and Alaska through the Bering Strait. These intercontinental colonizations are contemporary to those of other taxa such as felids (Tedford et al., 1987; Hunt, 2004; Wang et al., 2004), but older than major colonizations of both continents by small and big mammals (Late Miocene, e.g., 7 and 4.5 Myr, and since 9 Myr; Tedford et al., 1987; Hunt, 1998; Webb and Opdyke, 1995; Tedford and Martin, 2001; and Van der Made et al., 2002).

According to the biogeographic reconstruction and the molecular dating (Fig. 3a), the first diversification of the monophyletic Crocidurinae occurred in Eurasia, where they differentiated in Crocidurini and Myosoricini (16.5 Myr, 95% CI: 12.5–20.5; middle Miocene; Figs. 2 and 3). The Myosoricini were the first Soricidae to colonize Africa in the Upper Miocene. Our result coincides with the first fossil record of "*Myosorex sp.*" in Africa (12 Myr; Robinson and Black, 1974), and could be associated with the presence of forested corridors during the Neogene connecting Africa and Asia, 19 Myr ago (Thomas, 1985; Cox and Moore, 1993), as previously suggested by Quérouil et al. (2001). This was also hypothesized for bats (Juste et al., 1999), and is in accordance with the colonization of Africa by the Muridae (Butler, 1984; Jacobs, 1985).

In the late middle Miocene, two other Eurasian lineages emerged (10.8 Myr, 95% CI: 7.6–14.0). One colonized Africa and differentiated in the actual *Sylvisorex* and in the African "*Suncus*" as hypothesized by Butler (1998). Another one differentiated in Eurasia leading to the actual Eurasian *Suncus*. From this last lineage emerged *Crocidura* (9.3 Myr, 95% CI: 6.4–12.3) in Eurasia. Thus, the origin of *Crocidura* is anterior to the oldest European and Asian fossils known, respectively, of the early Pliocene (5 Myr) and the middle Pleistocene (Rzebik-Kowalska, 1998; Storch et al., 1998). Nevertheless, the discovery in Africa of two very different *Crocidura* fossils, dating of the middle Pliocene (3 Myr; Butler, 1998), suggests that the diversification of the genus took place much earlier on this continent. This discrepancy illustrated the very poor and incomplete fossil record of the family, which could lead to erroneous interpretations. The reconstruction of its biogeographic history is therefore largely dependent on the comparison of living species (Butler, 1998). Finally, in the Late Miocene, the genus differentiated in an African and an Eurasian lineage (8.0 Myr, 95% CI: 5.4–10.7), which is older than the Messinian regression. This differentiation corresponds with the beginning of a more humid phase (Late Tortonian) when savannah and subtropical grasslands replaced the Sahara desert (Griffin, 1999, 2002; Micheels, 2003). This created a potential route of colonization between Eurasia and Africa through the middle east, rather than Gibraltar, as suspected by several authors (Azzaroli and Guazzone, 1979; Thomas et al., 1982; Chevret, 1994). Nevertheless, additional *Crocidura* species should be analysed to test for the presence of two distinct biogeographic lineages, and not one undifferentiated, as suggest by Quérouil et al. (2001). Under this scenario, three independent colonizations from Eurasia to Africa occurred during the Miocene (Fig. 3a).

Without taking into account the biogeographic analysis, two alternative equally parsimonious scenario can be proposed (Fig. 3b and c). (i) First colonization of Africa

16.5 Myr ago (95% CI: 12.5–20.5) led to the actual *Myosorex*, *Sylvisorex*, and African *Suncus*. Then, a back colonization happened from Africa to Eurasia, leading to the actual Asian *Suncus* and *Crocidura*. Some *Crocidura* then migrated back to Africa to diversify into the actual African *Crocidura* (Fig. 3b). (ii) First colonization of Africa 16.5 Myr ago (95% CI: 12.5–20.5) led to the actual *Myosorex*, *Sylvisorex*, African *Suncus*, and African *Crocidura*. Then, two successive back colonizations occurred from Africa to Eurasia, first by the African *Suncus*, and second by the African *Crocidura*, leading to the actual diversity of Eurasian *Suncus* and *Crocidura* (Fig. 3c).

These scenarios imply that the ancestral Crocidurinae disappeared from Eurasia, and were replaced by the lineage emerging from Africa. This hypothesis is supported by the presence during the lower and middle Miocene of a very rich Soricinae fauna in Eurasia and in North Africa, from where they have since disappeared. In contrast, only one doubtful Crocidurinae fossil is known from Eurasia (Turkey; Engesser, 1980; Storch et al., 1998; Rzebik-Kowalska, 1998). The first biogeographic scenario is in agreement with a former hypothesis of Butler (1998), who proposed that three different lineages emerged from Asia and colonized Africa (actual Myosoricini, *Suncus* and the other Crocidurini). However, this is in contrast with several authors (Meester, 1953; McLellan, 1994; Quérouil et al., 2001) who gave an African origin to the genus *Suncus*. The first two biogeographic scenarios proposed (Fig. 3a and b) also contradict all the former assumptions concerning *Crocidura* evolution, who pled for an African origin of this genus (Butler, 1998; Meester, 1953; McLellan, 1994; Quérouil et al., 2001).

Nevertheless, the three scenarios have two points in common. They all hypothesize: (i) a colonization of Africa during the middle Miocene, and (ii) two independent origins of the *Crocidura* lineages.

Thus, at least three exchanges occurred between Africa and Eurasia, first in the middle Miocene, and second in the Late Miocene with the dispersion of the genus *Crocidura* through the old world. Nevertheless, more investigations are needed. Additional material from, for example, *Crocidura*, *Suncus* and endemic African genera such as *Scutisorex*, as well as the enigmatic Asian genera *Ferroculus* and *Solisorex*, should be included in further analyses. These taxa are of major interest to select between these three biogeograhic hypotheses for the Crocidurinae.

5. Conclusions

We highlight for the first time a clear relationship between the major groups of taxa within the Soricidae. *Anourosorex* should be definitively classified inside the Soricinae and not in a different subfamily. Accordingly, *Myosorex* should be included in the Crocidurinae. Our results also suggest a complex relationship between *Suncus* and *Sylvisorex*. *Suncus* is a paraphyletic unit including at least two Eurasian clades and an African one, the latter comprising *Sylvisorex*. It therefore needs a taxonomic revision. The biogeographic analyses showed a clear pattern inside the Soricinae, which originated in Eurasia and colonized subsequently North America with three different lineages. However, part of the biogeographical history of Crocidurinae remains uncertain.

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