Biogeographic origin and radiation of the Old World crocidurine shrews (Mammalia: Soricidae) inferred from mitochondrial and nuclear genes

Sylvain Dubey [a,b,*], Nicolas Salamin [b], Manuel Ruedi [c], Patrick Barrière [d], Marc Colyn [d], Peter Vogel [b]

[a] School of Biological Sciences, University of Sydney, Sydney, NSW 2006, Australia
[b] Department of Ecology and Evolution, University of Lausanne, CH-1015 Lausanne, Switzerland
[c] Natural History Museum, P.O. Box 6434, 1211 Geneva 6, Switzerland
[d] Laboratoire Ecobio UMR 6553—CNRS, Université de Rennes 1, Station Biologique, F-35380 Paimpont, France

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ABSTRACT

The crocidurine shrews include the most speciose genus of mammals, *Crocidura*. The origin and evolution of their radiation is, however, poorly understood because of very scant fossil records and a rather conservative external morphology between species. Here, we use an alignment of 3560 base pairs of mitochondrial and nuclear DNA to generate a phylogenetic hypothesis for the evolution of Old World shrews of the subfamily Crocidurinae. These molecular data confirm the monophyly of the speciose African and Eurasian *Crocidura*, which also includes the fossorial, monotypic genus *Diplomesodon*. The phylogenetic reconstructions give further credit to a paraphyletic position of *Suncus* shrews, which are placed into at least two independent clades (one in Africa and sister to *Sylvisorex* and one in Eurasia), at the base of the *Crocidura* radiation. Therefore, we recommend restricting the genus *Suncus* to the Palaearctic and Oriental taxa, and to consider all the African *Suncus* as *Sylvisorex*. Using molecular dating and biogeographic reconstruction analyses, we suggest a Palaearctic–Oriental origin for *Crocidura* dating back to the Upper Miocene (6.8 million years ago) and several subsequent colonisations of the Afrotropical region by independent lineages of *Crocidura*.

1. Introduction

During most of the Tertiary Period, the African and Eurasian continental plates were isolated from each other by the Tethys Sea (Rögl, 1998). It was only during the Lower Miocene, about 18 million years (Myr) ago, that both continents were first connected by the closure of the Eastern Mediterranean seaway, i.e. the Gomphotherium land bridge (Rögl, 1998; Harzhauser et al., 2002). A land connection existed in the northwest (i.e. at the location of the actual Gibraltar Strait) 16–14 Myr ago during the Bethic crisis, and again during the Miocene–Pliocene transition via extensive land bridges during the Messinian salinity crisis, 5.6–5.3 Myr ago (Krijgsman et al., 1999).

Paleontological data revealed that major terrestrial faunal exchanges between the African and Eurasian continents took place during these periods of connections (Azzaroli and Guazzone, 1979; Thomas et al., 1982; Chevret, 1994). In addition to these geological events, climatic variations also played a crucial role in faunal diversification of Old World biotas. From the Late Eocene (40 Myr ago) up to now, warm and wet periods have intersected

cold and dry ones, leading to deep faunal and floral turnovers. These climatic fluctuations led to major changes in habitat, e.g. the emergence of grassland savannah and open woodland in Africa during cold periods. As a consequence, the diversification of arid-adapted fauna was facilitated (De Menocal, 2004), as well as the colonization of Africa by Eurasian-born species adapted to these habitats (Fu, 1998; Caujapé-Castells and Jansen, 2003; De Menocal, 2004; Amer and Kumazawa, 2005).

Usually, time estimates of faunal turnovers and diversifications based on paleontological data are available only for large mammals, due to a better preservation of large fossils compared to small ones over time (Alroy, 2003). This is indeed the case for small mammals such as shrews that left a scant fossil record. In such cases, molecular phylogenetic analyses coupled with recent advances in relaxed molecular clocks (Sanderson, 1997, 2002; Thorne and Kishino, 2002; Drummond et al., 2006) can help put estimated divergence times on nodes without fossils.

The Soricidae is one of the most diversified mammalian families, as it comprises 26 genera and 376 species (Hutterer, 2005). Traditional systematic accounts and recent molecular data suggest that this family is subdivided into two subfamilies, the Soricinae and the Crocidurinae (Dubey et al., 2007d), although the Myosoricinae is sometimes also considered as a distinct subfamily (Hutterer, 2005; Maddalena and Bronner, 1992). Soricine shrews have essen-

tially a Holarctic distribution with 142 species and 13 genera living in the northern Hemisphere. Crocidurine shrews are restricted to the Old World and represent 209 species from 10 genera, pertaining to the Crocidurini. In addition, 18 species from two genera belong to Myosoricini (Hutterer, 2005). The family Soricidae is believed to have originated in Eurasia, based on the oldest known fossil (20 Myr; Reumer, 1989, 1994), on the high diversity of genera present today on this continent, and according to recent biogeographic reconstructions inferred from extensive molecular data (Dubey et al., 2007d).

Molecular studies based on nuclear and mitochondrial sequences confirmed the monophyly of the tribes and genera within the Soricidae (Dubey et al., 2007d), except for the genus *Suncus*, which appeared to be paraphyletic. *Crocidura* shrews were also monophyletic, but only 13 of the 209 current species were analyzed in that previous study. The molecular data suggested several transcontinental exchanges at a higher taxonomic level and complex faunal exchanges of Crocidurinae between Eurasia and Africa (Dubey et al., 2007d). Because few crocidurine shrews were analyzed, and because the focus was on higher taxonomic levels, their biogeographic evolution is still unclear. Indeed, of three proposed scenarios, two argued for a Eurasian origin of *Crocidura* and one for an African origin (Fig. 3. in Dubey et al., 2007d). Other studies focusing on karyological data of crocidurine shrews (Maddalena and Ruedi, 1994) hypothesized that the genus *Crocidura* could have evolved in two independent lineages displaying opposite patterns of chromosomal evolution: the first one, endemic to the Afrotropical region, is characterized by species with high chromosome numbers, while the second one, with a Eurasian distribution, is composed of species bearing a presumed ancestral or reduced chromosome number. The only exceptions among Afrotropical species were *C. luna* and *C. bottegi*, both characterized by a plesiomorphic chromosome formula ($2n = 36$ and FN = 56; see Maddalena and Ruedi, 1994).

Here, we expand the taxon sampling to most of the Old World genera and to approximately 90 species of crocidurine shrews, with particular emphasis on the widespread genus *Crocidura*. We performed a biogeographic analysis based on a tree resulting from analyses of an alignment of mitochondrial and nuclear genes. The main questions addressed are: (i) Which previous scenarios of biogeographic and karyological evolution are supported by the new molecular reconstructions? (ii) Where is the geographic origin of the crocidurine shrew radiation? (iii) How many faunal exchanges occurred in this group between Africa and Eurasia? (iv) Is there a correlation between this radiation and major geological and/or climatic changes?

2. Materials and methods

2.1. Sampling

We analyzed a total of 189 samples of Soricidae, that were mostly collected during the course of our previous studies (e.g. Quérouil et al., 2001; Maddalena, 1990a; Ruedi, 1996). In addition, few other samples were obtained from private collections (see Supplementary material 1). Specimens were identified by informed specialists, but some African specimens in the genus *Crocidura* were only provisionally identified, because several species complexes are still in need of thorough taxonomic revisions. Additional taxa with sequences already deposited in GenBank (Dubey et al., 2007d; Ohdachi et al., 2004, 2006; and Quérouil et al., 2001; Ruedi et al., 1998) were also incorporated into the final dataset (see Supplementary material 1).

New sequences were deposited in GenBank with the following Accession No. for *cyt-b*, EF524580–EF524796, for *16S*, EF524797–EF524905, for *BRCA1*, EF525050–EF525167, and for *ApoB*,

EF524906–EF525049. The alignment file is deposited in TreeBASE under the submission ID number SN3669.

2.2. DNA extraction and amplification

Prior to extraction, most frozen tissue samples were preserved for several years at −70 °C, then transferred to 80% ethanol at room temperature. 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*) were performed with the primer pairs L14724/H15149 and C3/H15915 (Irwin et al., 1991; Dubey et al., 2006), and that of the *16S* ribosomal sequence (*16S*) with the primer pair 16sf/16Srbis (Dubey et al., 2007d). Amplification of the breast cancer susceptibility 1 (*BRCA1*) nuclear gene was performed using the primer pair B1f/B1r (Dubey et al., 2006, 2007d), while the Apolipoprotein B (*ApoB*) nuclear gene was amplified with the primer pairs ApoBf/ApoBr and ApoBf2 (5′ctg gga aat att aca tat gac 3′)/ApoBr2 (5′tca ctg gca ata gtt cc 3′), specifically developed for this study. Amplification conditions for *16S* and *BRCA1* consisted of 35 thermal cycles (40 for *BRCA1*) of 60 s denaturation at 94 °C, 60 s annealing at 55 °C for *16S* (52 °C for *BRCA1*) and 120 s extension at 72 °C. Amplification conditions for *cyt-b* consisted of 35 thermal cycles of 30 s denaturation at 94 °C, 45 s annealing at 50 °C, and 60 s 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 visualized on 1% agarose gels, and purified by centrifugal dialysis using the QIAquick PCR Purification Kit (QIAgen).

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

2.3. Phylogenetic analyses

The coding sequences of *cyt-b* were aligned by eye, while sequences of *16S*, *BRCA1*, and *ApoB* were aligned with help of ClustalW (Thompson et al., 1994); the latter alignments were further checked by eye to minimize gaps. Phylogenetic analyses were first performed on the dataset A consisting of 139 taxa with complete sequence data (i.e. all four concatenated genes). Analyses were then performed on a larger dataset B that contained more species (189 taxa), but not all of them were represented by the four genes. The congruence between the four markers was tested on dataset A by performing 100 bootstrap re-samples on each marker with a maximum parsimony (MP) criterion, and comparing the support level thus obtained for each node. Heuristic searches in maximum parsimony (MP; dataset A) analyses were performed using PAUP*4.0b10 (Swofford, 2001) with 100 random additions of sequences followed by tree bisection-reconnection (TBR) branch swapping, and keeping a maximum of 100 trees during each search. Branch support was estimated using 1000 bootstrap pseudoreplicates using the same heuristic settings. For maximum likelihood analyses (ML), best-fitting models of DNA substitution were selected using hierarchical likelihood ratio tests (hLRTs) implemented in Modeltest 3.06 (Posada and Crandall, 1998). The GTR + I + G model (Rodriguez et al., 1990; Yang, 1996) best fitted both datasets, with an unequal distribution of substitution rates at variable sites (dataset A: $a = 0.343$; dataset B: $a = 0.219$) and 6 different substitution types (dataset A: rate [A–C] = 1.5476, rate [A–G] = 6.6468, [C–T] = 17.0424, rate [A–T] = 1.6272, rate [C–G] = 0.7895, rate [G–T] = 1.0000; dataset B: rate [A–C] = 1.4908, rate [A–G] = 6.7349, [C–T] = 16.8883, rate [A–T] = 1.5270, rate [C–G] = 0.7512, rate [G–T] = 1.0000). ML heuristic searches (dataset
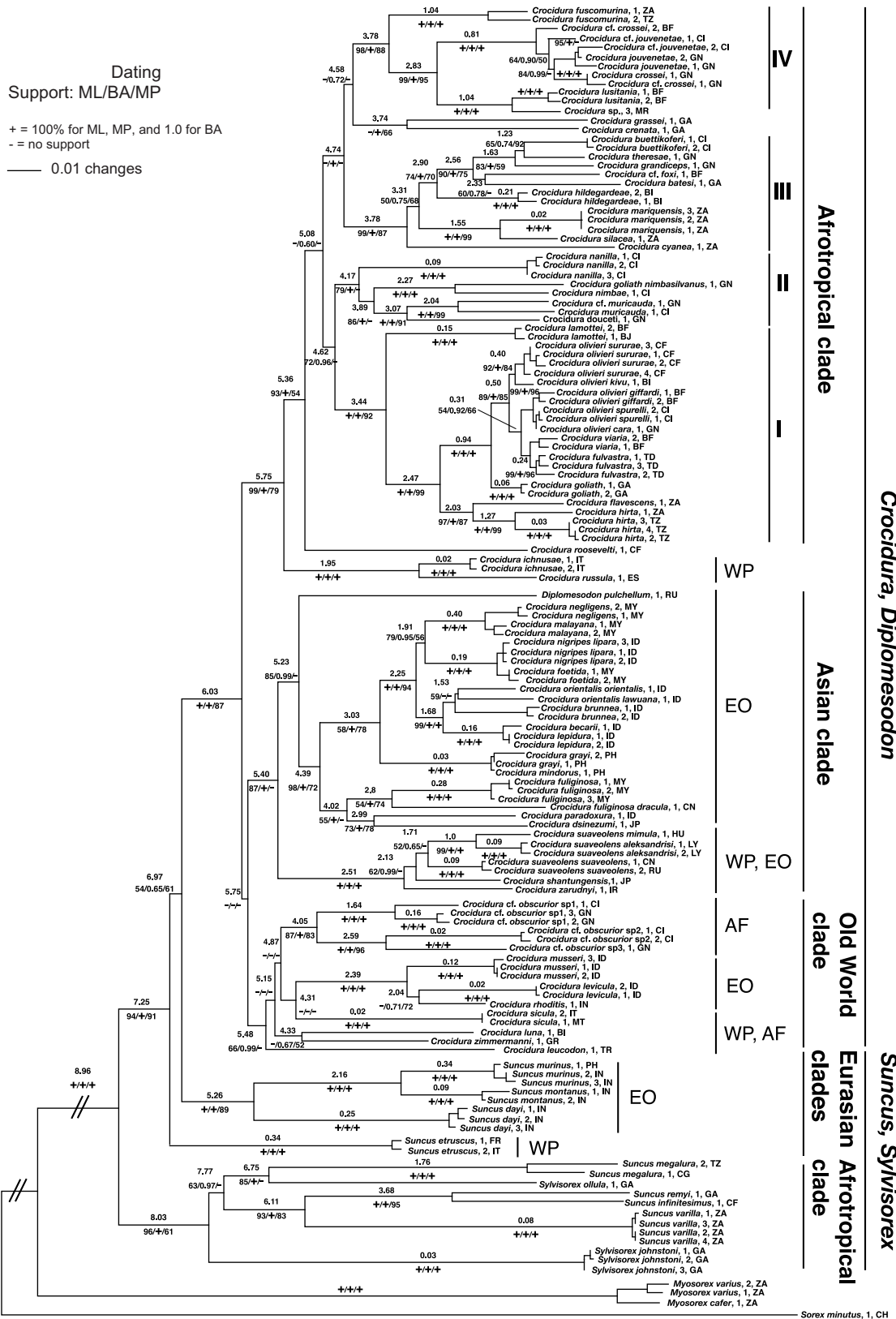
Fig. 1. Phylogeny of 139 taxa of shrews sequenced for 3306 bp of mitochondrial and nuclear genes (dataset A) analyzed with maximum likelihood, using the GTR + I + G model of substitution. Values above and below the branches are, respectively, molecular dating of nodes and supports for the major branches, for maximum likelihood (ML), bayesian posterior probabilities (BA), and maximum parsimony (MP) analyses (+: support of 100% for ML and MP, and of 1.0 for BA, -: support <50% for ML and MP, and <0.5 for BA). The symbol // indicates that the branch length has been shortened. Abbreviations for the distribution of samples are AF for Afrotropical region, WP for West Palaearctic region, and EO for East Palaearctic–Oriental regions. Specimen codes are as in Supplementary material 1.
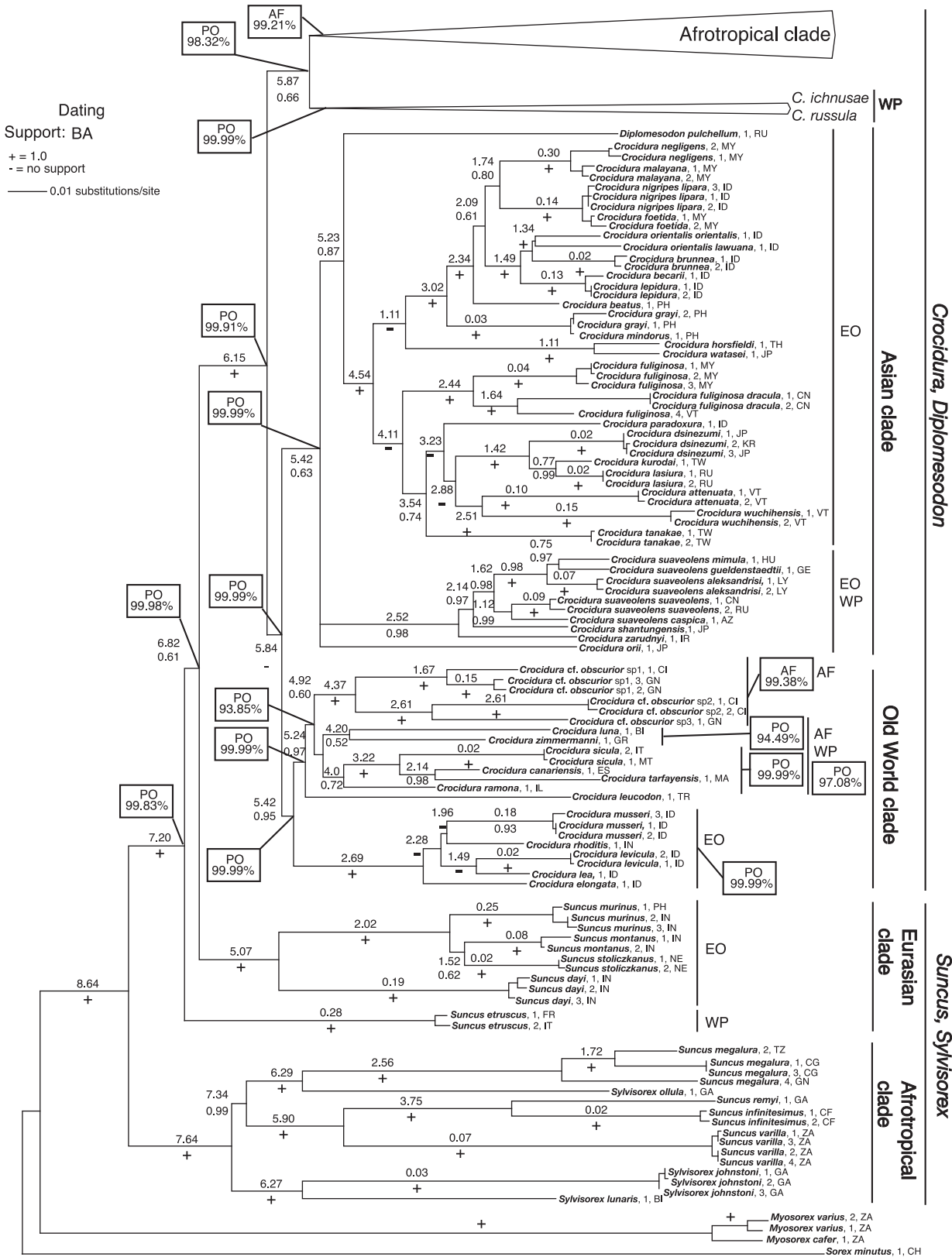
Fig. 2. Phylogeny of 189 taxa of shrews with partial or complete alignment of 3306 bp (dataset B) analyzed with maximum likelihood, using the GTR + I + G model of substitution. Values above and below the branches are, respectively, molecular dating of nodes and supports for the major branches, for bayesian posterior probabilities (BA; +: support of 1.0, -: support <0.5). Values within boxes are probabilities of nodes being of a Palaearctic–Oriental origin (PO) or Afrotropical origin (AF) according to the biogeographic reconstruction analyses. Abbreviations for the distribution of samples are AF for Afrotropical region, WP for West Palaearctic region, and EO for East Palaearctic–Oriental regions. Specimen codes are as in Supplementary material 1.
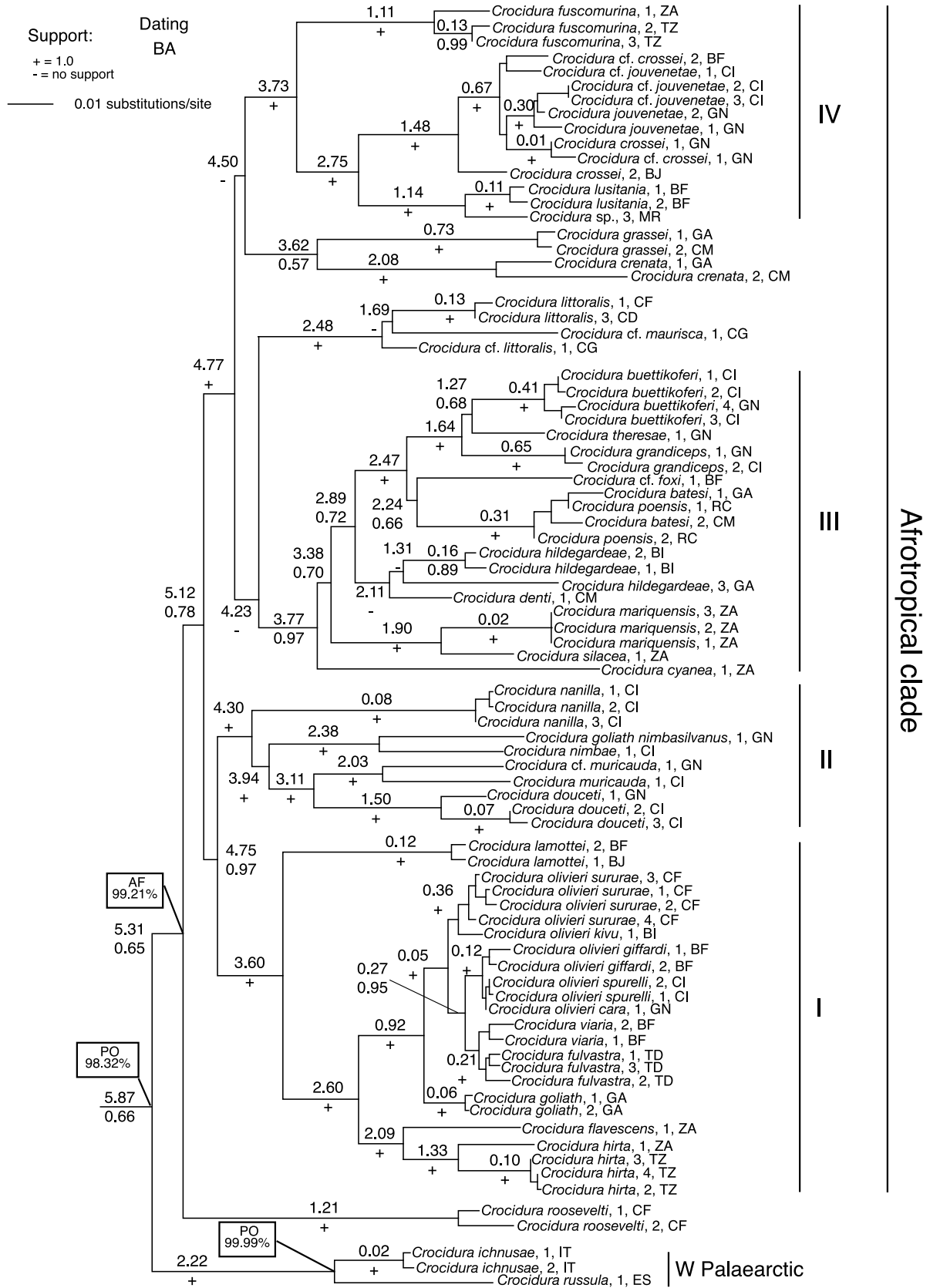
Fig. 3. Phylogeny of dataset B, with focus on the African clade. See Fig. 2 for a global phylogenetic position of this clade. Values above and below the branches are the molecular dating of nodes and supports for the major branches, for bayesian posterior probabilities (BA; +: support of 1.0, -: support <0.5). The value in the rectangle is the percentage of the node being of an Afrotropical origin (AF) from the biogeographic reconstruction analyses. Specimen codes are as in Supplementary material 1.

A and B) and bootstrap analyses (1000 replicates; dataset A) were performed with PHYML (Guindon and Gascuel, 2003). Bayesian analyses (BA) were run with MrBayes version 3.1.2.1 (Huelsenbeck et al., 2001) using a GTR + I + G model, and considering a partitioning of the data, corresponding to the four genes, the values of parameters varying between the partitions. Two independent runs were performed, each consisting of four parallel MCMC chains of five million generations (dataset A) and 10 million generations (dataset B), allowing a good convergence of the independent runs. Tree parameters reached stationarity after a burn-in period of two hundred thousand and one million generations (datasets A and B, respectively). Optimal trees were then sampled every 100 generations to obtain the final consensus BA tree and associated posterior probabilities.

2.4. Molecular dating

The calibration points used for dating the trees were the oldest known Soricinae–Crocidurinae ancestors (20 Myr, Reumer, 1989, 1994) and the oldest fossil attributed to *Crocidura* (5.3 Myr, Rzebik-Kowalska, 1998), both originating from Eurasia.

A Bayesian approach was used to estimate absolute divergence time. The ML tree was selected as the best topology for the molecular dating. Each data partition was used separately to estimate the variance-covariance matrix of rates of substitution 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 (minimum age constraint), and the root of the tree was constrained to be at most 50 Myr old. Two independent runs were done to assess convergence. In each run, a one million generation chain was run, with sampling every 100 generations.

2.5. Reconstruction of biogeographic origin

To infer the possible biogeographic origin of the Crocidurinae subfamily and of the different genera, we reconstructed ancestral areas of each clade 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 Palaearctic + Oriental (PO) taxa or as 2 for Afrotropical taxa (AF; Supplementary material 1), with the limits between the Palaearctic and Afrotropical regions taken from Corbet (1978). The model of character evolution was a simple stochastic model (Mk1; Lewis, 2001), which assumes a symmetrical and equal rate of change between any two states. The character state frequencies were estimated from the transition probabilities. In this likelihood approach, the probability that a character changes along a branch of the tree is therefore a function of the branch length, with changes being more likely along longer branches than along shorter ones; it also depends on the taxon sampling. We thus based these biogeographic inferences on the ML tree issued from the taxon-rich dataset B. We used the calibrated ML 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 most complete dataset (dataset A), i.e. composed of sequences of all four genes for each sample, included 139 taxa with 3560 bp each, of which 254 bp were excluded from the analyses, due to uncertainties in the alignment within the non-coding *16S* gene. Thus, the final dataset A contained 3306 bp with 1552 variable nucleotide positions (*ApoB*: 851 bp; *BRCA1*: 874 bp; *16S*: 834 bp; *cyt-b*: 747 bp), of which 1173 were parsimony-informative.

The MP bootstrap analyses, performed on each marker, revealed congruent results between the four markers (results not shown). Nevertheless, due to the lower mutation rate of nuclear genes compared to the mitochondrial ones, the topology of terminal branches of *ApoB* and *BRCA1* trees was poorly supported. Inversely, the topology of the basal branches of *16S* and *cyt-b* trees was poorly supported. These differences of resolution resulted in some conflicting topologies between the four markers. However, they were not supported by MP bootstrap values higher than 75%.

All three methods of phylogenetic construction (MP, ML, and BA), based on the whole dataset A, were congruent in recovering the same main clades with high support (Fig. 1). Consequently, only the tree obtained by ML is shown here (Fig. 1).

In particular, the two tribes Crocidurini and Myosoricini were each monophyletic with high support (100% bootstrap, 1.0 posterior probability and 100% bootstrap for ML, BA, and MP analyses, respectively; the same order of nodal support for the different analyses is reported hereafter). Within the Crocidurini, the genera *Suncus* and *Sylvisorex* were polyphyletic, with the African *Suncus* (*S. infinitesimus*, *S. megalura*, *S. remyi*, and *S. varilla*) intermingled with species of the genus *Sylvisorex* (*Sy. ollula*, *Sy. johnstoni*). Together, these Afrotropical shrews formed a well-defined clade (96, 1.0, 61; Fig. 1), where *Sy. johnstoni* was placed in a basal position (63, 0.97, <50). This Afrotropical clade was itself an early offshoot of the remaining Crocidurini, with the Eurasian *Suncus etruscus* being basal to the Asian *Suncus* (clade *S. dayi*, *S. montanus*, *S. murinus*; 100, 1.0, 89) and the remaining Crocidurini (94, 1.0, 91). In addition, the Asian *Suncus* were placed further at the base of all representatives of the genus *Crocidura* (support of *Crocidura* clade, including *Diplomesodon*; 100, 1.0, 87).

Within *Crocidura*, three major clades were strongly supported:

(i) The first clade included all Afrotropical *Crocidura* (Afrotropical clade, Fig. 1) plus the West Palaearctic pair *C. russula/ichnusae* (99, 1.0, 79). The two sister species *C. ichnusae* and *C. russula* were found to be in a basal position (93, 1.0, 54), followed by the central African *C. roosevelti*, and a succession of four well-supported sub-clades (I–IV) and a few other single species.

Sub-clade I (100, 1.0, 92) included the samples of *C. lamottei*, which were basal to the samples of *C. olivieri* from western and central Africa, of *C. viaria* and *C. fulvastra* from northern Africa, *C. hirta* and *C. flavescens* from southern Africa, and the Gabonese samples of *C. goliath*. Sub-clade II (79, 1.0, <50) included west African samples of *C. nanilla*, which were basal to two sister units from west Africa including (i) *C. nimbae* and *C. goliath nimbasilvanus* and (ii) *C. muricauda*, *C. cf. muricauda* and *C. douceti*. Sub-clade III (99, 1.0, 87) included samples of the south African species *C. cyanea*, *C. silacea*, and *C. mariquensis*, and the central African species *C. hildegardeae*, *C. batesi*, and the western African *C. buettikoferi*, *C. cf. foxi*, *C. grandiceps*, and *C. theresae*. Sub-clade IV (98, 1.0, 88) included samples of *C. fuscomurina*, which were situated in a basal position to two well-differentiated units, including first the west African taxa of *C. cf. crossei*, *C. cf. jouvenetae*, *C. crossei*, and *C. jouvenetae* and, second, the samples of *C. lusitania* from Burkina Faso, and one undetermined taxon from Mauritania. The remaining taxa, *C. grassei* and *C. crenata* from central Africa, formed a partially supported unit (<50, 1.0, 66), which clustered with sub-clade IV (<50, 0.72, <50%).

(ii) The second major clade was essentially formed by Asian representatives of *Crocidura* and by the monotypic genus *Diplomesodon* (Asian clade; 87, 0.99, <50; Fig. 1). The Palaearctic samples of *C. suaveolens*, *C. shantungensis*, and *C. zarudnyi* formed a monophyletic unit (100, 1.0, 100), which was basal to *Diplomesodon pulchellum* and all other species of the clade (85, 0.97, <50). The latter species was the sister taxon to Oriental species (98, 1.0, 72). The

continental *C. fuliginosa* samples clustered with the insular *C. paradoxura* from Sumatra and *C. dsinezumi* from Japan (55, 1.0, <50). The latter clade was basal to insular SE Asian samples of *C. grayi* and *C. mindorus* from the Philippines, *C. lepidura* and *C. becarii* from Sumatra, *C. brunnea* and *C. orientalis* from Java, *C. nigripes* from Sulawesi, *C. foetida* from Borneo, *C. negligens* from Tioman, and to *C. malayana* from Peninsular Malaysia.

(iii) The third major clade (Old World clade, 66, 0.99, <50, Fig. 1) was composed of species from all three continents. In particular, it included species from the "Old Sulawesian clade" described in a previous study (Ruedi et al., 1998), a sub-clade consisting of the West African samples *C. cf. obscurior* sp1, sp2, and sp3 (87, 1.0, 83), the west Palaearctic *C. leucodon*, *C. sicula*, and *C. zimmermanni*, and the Afrotropical *C. luna*, which was sister to *C. zimmermanni* (50, 0.67, 52). The precise order of taxa within this clade was not ascertained as none of the basal nodes received strong support in the different phylogenetic reconstructions (Fig. 1). Clade III appears to be the sister clade of the Asian clade, although the support for this relationship is again limited (all <50).

The dataset B is the same alignment of 3360 bp from four genes, but includes 50 additional taxa (see Supplementary material 1), for which only some of those genes were available. The missing positions were replaced by stretches of Ns and treated as missing data. The taxon sampling of this extended dataset thus consisted of 189 taxa and resulted in 1580 variable positions, of which 1208 were parsimony-informative. The phylogenetic reconstruction based on this expanded taxon sampling (Figs. 2 and 3) did not differ significantly from those obtained from the dataset A, as all additional species were nested within the clades already defined in the previous analyses.

Within the Crocidurinae, the Afrotropical *Suncus* and *Sylvisorex* remain in a basal position to *Crocidura* (BA support of 1.0; in the following, BA support will be given between brackets), and the additional *Suncus megalura*, *S. infinitesimus*, and *Sylvisorex lunaris* were included within the latter. Within the Eurasian *Suncus*, *S. etruscus* remained in a basal position (0.61), and *S. stoliczkanus* clustered with *S. montanus* (0.62).

Within the clade including Afrotropical *Crocidura* (0.66), the west Palaearctic *C. ichnusae* and *C. russula* samples remained in a basal position (0.65), followed by *C. roosevelti* (0.78). In addition, the different sub-clades (I–IV) were highly supported (⩾ 0.97). The new samples of *C. cf. jouvenetae* and *C. crossei* from West Africa and of the widespread *C. fuscomurina* clustered with their close relative within sub-clade IV. The additional samples of *C. buettikoferi*, *C. batesi*, *C. poensis*, *C. hildegardeae*, *C. denti*, and *C. grandiceps* from West or Central Africa were all included within sub-clade III. The samples of *C. douceti* were included within sub-clade II. Finally, the additional samples of *C. crenata* and *C. grassei* clustered with their conspecifics, and the samples of *C. littoralis*, *C. cf. littoralis*, and *C. cf. maurisca* from Central Africa formed a well-supported unit (1.0), distinct to the other sub-clades.

Within the Asian clade, the additional Oriental and East Palaearctic species, *C. beatus* from the Philippines, *C. horsfieldii* from Thailand, *C. watasei* from Japan, *C. lasiura* from Russia, *C. kurodai* and *C. tanakae* from Taiwan, and *C. attenuata* and *C. wuchihensis* from Vietnam clustered with species from the same region (support for this Asian sub-clade: 0.74). The additional samples of *C. suaveolens caspica* and *C. suaveolens gueldenstaedtii* clustered with their conspecifics and formed a well-supported clade with *C. shantungensis* and *C. zarudnyi* (0.98). The phylogenetic position of the Japanese *C. orii* remained uncertain.

Within the Old World clade, *C. canariensis* from the Canary Islands clustered with *C. tarfayaensis* from Morocco and with *C. sicula* from Sicily (1.0), and *C. ramona* from Israel was basal to these taxa. The Sulawesian samples *C. elongata* and *C. lea* clustered with the other samples from the same island (1.0). As for dataset A, the basal nodes within this clade were not well-supported.

3.2. Molecular dating

Molecular dating based on the two datasets were generally congruent, as there was less than 5% difference between both estimates. For the sake of simplicity, we report divergence times based on dataset B only, to cover the largest sample of taxa. The split between the clade including the African *Sylvisorex* and *Suncus*, and the remaining Crocidurini (Eurasian *Suncus*, *Crocidura*, and *Diplomesodon*) occurred approximately 8.6 Myr ago (95% CI: 7.6–9.7; Fig. 2). The split between *S. etruscus* and the other Eurasian *Suncus* (*dayi*, *montanus*, *murinus*) and the remaining taxa happened 7.2 Myr ago (95% CI: 6.3–8.1). The split between the Eurasian *Suncus* and *Crocidura* (including *Diplomesodon*) occurred 6.8 Myr ago (95% CI: 6.0–7.7). Within *Crocidura*, the split between the Afrotropical clade (including the West Palaearctic *C. russula* and *C. ichnusae*) and the other clades occurred 6.2 Myr ago (95% CI: 5.4–6.9), and the split between the strictly Afrotropical clade and the latter West Palaearctic species occurred 5.9 Myr ago (95% CI: 5.2–6.6). The split between the Asian and the Old World clades occurred 5.8 Myr ago (95% CI: 5.1–6.5), whereas the basal nodes of the Afrotropical, Asian, and Old World clades dated, respectively, from 5.3 Myr (95% CI: 4.6–6.0), 5.4 Myr (95% CI: 4.7–6.1) and 5.4 Myr (95% CI: 4.7–6.1).

Within the Afrotropical clade, the splits between the sub-clades I to IV occurred between 5.1 and 4.8 Myr ago (Fig. 3), and the basal nodes of these sub-clades were situated between 4.3 and 3.6 Myr ago. Within the Asian clade, the split between the *C. suaveolens* group and the other Asian taxa occurred 5.4 Myr ago (95% CI: 4.7–6.1), and the basal node of the *C. suaveolens* group was dated at 2.5 Myr ago (95% CI: 2.1–3.0). Within this latter group, the split between the northeastern African and European *C. s. mimula* and *C. s. gueldenstaedtii* occurred 1.0 Myr ago (95% CI: 0.7–1.2). Within the Old World clade, the split between Palaearctic and Afrotropical taxa occurred between 5.4 (95% CI: 4.7–6.1) and 4.2 Myr ago (95% CI: 3.6–4.8).

3.3. Biogeographic reconstruction

As the divergence times estimated from dataset A (Fig. 1) and dataset B (Figs. 2 and 3) were similar, only the results based on dataset B were used in the biogeographic reconstructions.

Because the choice of basal taxa in such analyses can strongly influence the biogeographic reconstruction of the focus taxa, we performed successive analyses by deleting each time the most basal taxon (first *Myosorex*, then *Sylvisorex* and the Afrotropical *Suncus*, and finally the Asian *Suncus*) and compared the results. None of the reconstructions resulting from these successive changes in outgroup resulted in a change in the inferred biogeographic scenarios, nor in the level of ML support of each node.

The common ancestor to all *Crocidura* and *Diplomesodon* was most likely of Palaearctic–Oriental origin (ML support of 100%), as well as the basal node of the Asian and Old World clades (ML support of 100%). The common ancestors of these two latter clades were also clearly of Palaearctic–Oriental origin (ML support of 100%), while the basal node of the Afrotropical clade and the pair of West Palaearctic taxa *C. russula* and *C. ichnusae* had a probability of 98% of being Palaearctic–Oriental (for more details, see Figs. 2 and 3).

4. Discussion

4.1. Current taxonomic and molecular discrepancies

The African *Suncus* analyzed here are clearly intermingled within a clade containing all tested *Sylvisorex* (endemic to Africa), but excluding the Eurasian *Suncus* (Figs 1 and 2). Two Afrotropical species, *Sy. ollula* and *S. megalura*, are clearly sister species and closely

related on the molecular tree. Indeed, the latter was usually classified into the genus *Sylvisorex* until Hutterer (2005) proposed to assign it to the genus *Suncus* based on a closer 16s rRNA genetic distance with the Asian *Suncus dayi* (Quérouil et al., 2001). This genus assignment is not supported by more comprehensive character and taxon sampling analyses (Figs 1–3), which all show that Eurasian *Suncus* (i.e. including *S. dayi*) are clearly distinct from any Afrotropical species assigned either to the genera *Suncus* (*S. megalura, S. remyi, S. infinitesimus, S. varilla*) or *Sylvisorex* (*Sy. ollula, Sy. johnstoni, Sy. lunaris*). In turn, all these Afrotropical species formed a monophyletic unit in all reconstructions and with very high support (Figs. 1 and 2). Owing to their very close morphological (Butler and Greenwood, 1979; Butler et al., 1989), biochemical (Jenkins et al., 1998) and molecular similarities (Dubey et al., 2007d, and Figs. 1 and 2), all Afrotropical representatives of *Suncus* should be transferred to the genus *Sylvisorex*, which would become a monophyletic genus endemic to this biogeographic region.

In turn, all analyzed Eurasian taxa of *Suncus*, including the type-species of the genus (*Suncus murinus*, see Hutterer, 2005), are monophyletic, except for *S. etruscus*, which is apparently more basal to all crocidurine shrews (Figs. 1 and 2). However, the nodal support for the latter placement is weak, and the alternative placement of *S. etruscus* within the other Eurasian *Suncus* clade in a basal position remains probable. We thus recommend restricting the genus *Suncus* to these Palaearctic and Oriental taxa, distinguished morphologically from *Crocidura* shrews by four pairs of upper unicuspids (total 30 teeth), a plesiomorphic character shared with *Sylvisorex* (Jenkins et al., 1998).

The genus *Crocidura* is clearly monophyletic (support for dataset A: 100%, 1.0 and 87%, and for dataset B: 1.0) and genetically well distinct from *Suncus* and *Sylvisorex*. It differs morpho-anatomically from the latter two genera at least by its dental formula, characterized by the loss of one pair of upper unicuspids (28 teeth), a character considered as derived by Jenkins et al. (1998). The sand-adapted, monospecific genus *Diplomesodon*, is further distinguished from *Crocidura* shrews by the reduction of a second pair of upper unicuspids (26 teeth). Molecular reconstructions (Fig. 1) clearly suggest that it is imbedded within *Crocidura* radiation, with a basal position to the Oriental and Japanese species (support from dataset A: 98%, 1.0, and 72%, for dataset B: 1.0). The inclusion of *Diplomesodon* within the genus *Crocidura* is also consistent with previous reconstructions based on allozyme data (Maddalena, 1990a,b). Consequently, if taxa have to be strictly monophyletic, then *Diplomesodon* should be considered as a junior synonym of *Crocidura* and the binomen *Diplomesodon pulchellum* should therefore be replaced by *Crocidura pulchella*. We propose however to follow a less strict definition of the genera (e.g. Brummiti, 2002) and keep this morphologically highly distinctive taxon in its own genus, although this would render *Crocidura* a paraphyletic taxon.

4.2. Evolution of karyological versus molecular characters

According to the present results, *Crocidura* shrews are split into three major clades. Two of them are represented, respectively, by unique Afrotropical and Palaearctic taxa, and are therefore defined by the common geographic origin of their component taxa. A previous review of karyological data of crocidurine shrews (Maddalena and Ruedi, 1994) also indicated this dichotomy of lineages as most Afrotropical species (including those of the current Afrotropical clade depicted in Figs. 1–3) shared a derived karyotype characterized by increased chromosome number (diploid number $2n = 42–60$), while the karyotype of most Palaearctic and Oriental species (including those of the current Asian clade depicted in Figs. 1–3) where characterized by less chromosomes ($2n = 22–40$; see references in Maddalena and Ruedi, 1994). The presumed ancestral karyotype ($2n = 36$ and a fundamental number FN = 54–58) was

present in the African taxa *C. bottegi* and *C. luna* or in the West Palaearctic *C. canariensis* and *C. tarfayaensis* (Vogel et al., 2006). The latter three taxa were also sequenced and analyzed here, and all fell together within the mixed Old World clade, the third well-supported, major clade apparent in molecular reconstructions (Figs. 1–3).

Within the Asian and Afrotropical clades of *Crocidura*, the karyotype also appears to be a good indicator of phylogenetic relationships. For instance, the widespread Palaearctic taxa of the *C. suaveolens* group (*C. s. suaveolens, C. s. aleksandrisi, C. s. mimula, C. s. gueldenstaedtii, C. shantungensis*, and *C. zarudnyi*, see Dubey et al., 2006, 2007b,c) are characterized by a karyotype of $2n = 40$ and FN = 50, and are indeed monophyletic in the molecular reconstructions. Likewise, the monophyly of the African *C. olivieri* group (*C. olivieri, C. flavescens, C. fulvastra, C. goliath, C. hirta*, and *C. viaria*; see Dubey et al., 2007a) is well established both by molecular data (Figs. 1–3) and by their unique, shared chromosomal formula of $2n = 50$ and FN = 66 or 74 (Maddalena et al., 1987).

The good correspondence between molecular reconstructions and the proposed general framework of karyological evolution in *Crocidura* shrews bear important phylogenetic information allowing a better understanding of the evolution in this diversified group.

4.3. Biogeography

If we assume that the genus *Crocidura* is composed of four major clades (Afrotropical, West Palaearctic, Asian and Old World), as suggested by the molecular reconstructions (Fig. 1–3), then the most likely origin for the latter three is Palaearctic or Oriental, while the first is Afrotropical. These biogeographic inferences are robust to the choice of the outgroup (*Myosorex, Suncus*, or *Sylvisorex*) and are supported by very high likelihoods (>98%). We thus envision four possible scenarios of diversification compatible with these biogeographic analyses: the first scenario (Fig. 4A) would be an origin of the genus *Crocidura* in the Palaearctic–Oriental region and a first basal split before the common ancestor to the Afrotropical clade colonized the African continent. A second transcontinental migration in the same direction would then have occurred, when representatives of the Old World clade colonized secondarily Africa.

Another, less parsimonious scenario (Fig. 4B) would imply an early colonization of the Afrotropical region by the common ancestor to the West Palaearctic and Afrotropical clades, with a subsequent return of a descendant of the common ancestor to the former clade into the West Palaearctic region; then a third transcontinental migration would have occurred from a single lineage of the Old World clade entering Africa secondarily.

A third scenario of the *Crocidura* radiation originating in the PO region (Fig. 4C) would involve four transcontinental migrations: the same initial two as in scenario 4B, but then an early colonization of Africa by the descendant of the common ancestor to all the Old World clade, followed by a secondary return of one lineage back into the PO region.

The last envisioned scenario would be compatible with an Afrotropical origin of the *Crocidura* radiation (Fig. 4D), as proposed by palaeontologists (Butler, 1998; Meester, 1953) or by the higher species diversity found today in this region (McLellan, 1994; Quérouil et al., 2001). This scenario, however, contradicts the current MESQUITE analyses (Figs. 2 and 3), which clearly support a Palaearctic–Oriental origin. Except for the origin of the radiation, this scenario 4D is identical to 4B, and would also imply three transcontinental faunal exchanges.

We favor the scenario A to represent the evolution of the *Crocidura* shrews, because it minimizes the number of transcontinental exchanges and is compatible with the scant fossil record. Accord-
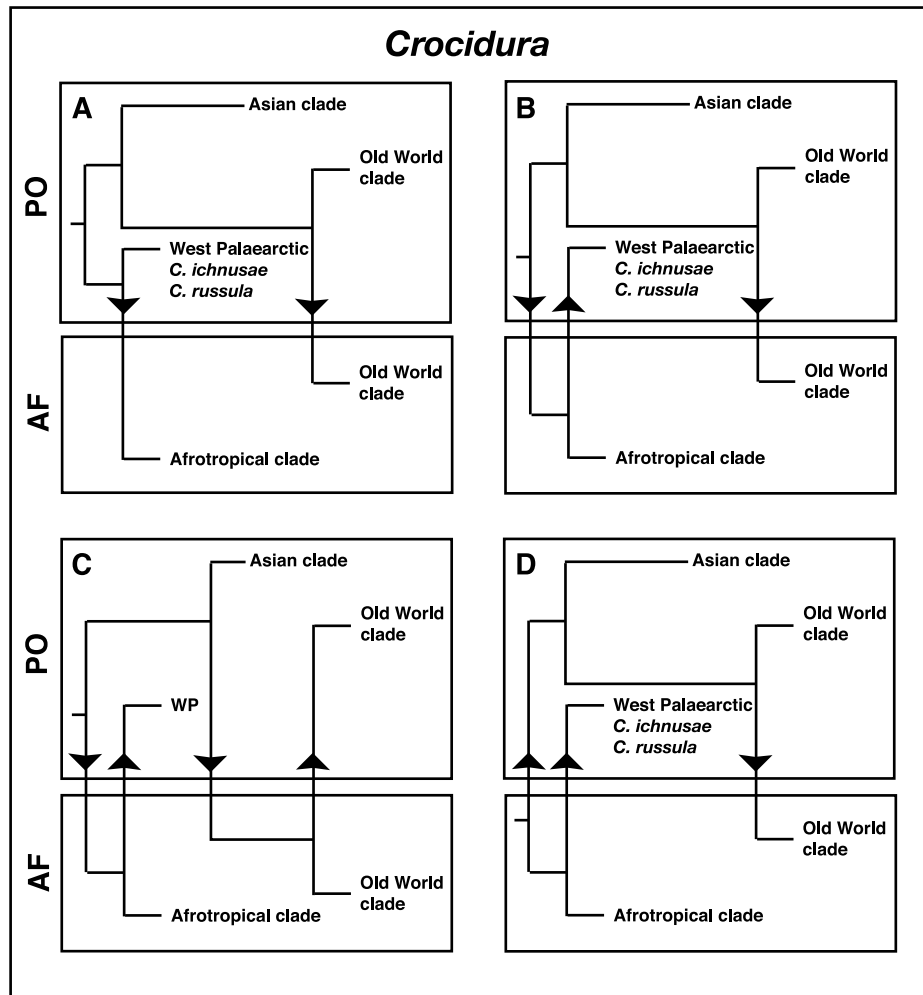
Fig. 4. Distribution (PO: Palaearctic–Oriental region; AF: Afrotropical region) and scenarios of diversification for the major clades within *Crocidura,* based on the present phylogeny (no time scale), considering a Palaearctic–Oriental (A, B and C) or Afrotropical (D) origin of *Crocidura.*

ing to this scenario A and to the molecular datings, the first event of colonization apparently occurred around 5.9 Myr ago (95% CI: 5.2–6.6; Figs. 2 and 4) during the Messinian (Upper Miocene). This period is characterized by a salinity crisis (5.3–5.6 Myr ago; Krijgsman et al., 1999) that partially dried out the Mediterranean Sea, which permitted faunal exchanges between Europe and Africa. Thus, it is likely that the first colonizing shrews entered the Afrotropical region via this route at that time, as suggested for several other small mammals (Thomas et al., 1982; Chevret, 1994). This proposed Messinian colonization is reinforced by the clearly basal position of the West Palaearctic *C. russula-C. ichnusae* compared to the Afrotropical clade. Both species are presently distributed across the western Mediterranean Sea (Brändli et al., 2005; Cosson et al., 2005), which indeed suggests a reminiscence of an ancient colonization through the Gibraltar Strait.

The second event of colonization into Africa from a member of the Old World clade, may have occurred 1 Myr later, at the beginning of the Lower Pliocene (4.9 Myr ago; Fig. 2). This second transcontinental event is more recent, but also coincident with the Messinian salinity crisis, as the confidence interval of the molecular dating fully includes this event (95% CI: 4.3–5.6).

Within the Afrotropical clade, the splits between the sub-clades (I–IV) occurred apparently within a short time period and shortly after the colonization of Africa by their common ancestor. In fact, the splits are all dated to the Messinian period of the Upper Miocene and the beginning of the Pliocene (5.1–4.8 Myr ago). Similar

events occurred within the Asian and Old World clades, where the *C. suaveolens* group and *C. orii* diverged from the other Asian taxa 5.4 Myr ago. A noteworthy fact is the early (about 5.4 Myr ago) differentiation of the endemic species of Sulawesi (*C. elongata, C. lea, C. levicula, C. musseri,* and *C. rhoditis,* i.e. the Old Sulawesian clade in Ruedi et al., 1998) from the other Asian, continental taxa, and a subsequent *in situ* diversification in the Upper Pliocene (2.3 Myr ago).

Consequently, the present species diversity of *Crocidura* in the Old World results from a relatively long evolution of distinct lineages, which started in the Upper Miocene, as hypothesized by Maddalena (1990a), Butler (1998) and Quérouil et al. (2001), rather than a rapid radiation during the Pleistocene.

The presumed origin of *Crocidura* shrews, dated to the Upper Miocene (6.8 Myr; 95% CI: 6.0–7.7) according to our molecular datasets, is slightly older than the age of the first European and Asian fossils found, respectively, in early Pliocene (5 Myr) and Middle Pleistocene deposits (Rzebik-Kowalska, 1998; Storch et al., 1998). Molecular dating of the diversification of *Crocidura* shrews in Africa (1–5 Myr, Figs. 2 and 3) are also compatible with the age of two different fossils of *Crocidura* discovered in the Afrotropical region and dating back to the Middle Pliocene. However, the marked morphological differences observed between these two African fossils (Butler, 1998) suggested an older presence of *Crocidura* on this continent, which is again consistent with the molecular dating.

Therefore, we highlight a long independent evolution of taxa of different biogeographic affinities, which started in the Upper Miocene for *Crocidura*. In addition, it reveals a strong influence of the Messinian salinity crisis on transcontinental dispersions and radiations. Our results also show that a large taxon and character sampling is necessary in order to fully understand the phylogenetic relationships in this highly speciose family.

5. Conclusions

The phylogenetic relationships between *Crocidura* shrews appear to be largely consistent with the geographic origin of the species, as well as with their karyological characteristics. Indeed extensive molecular reconstructions clearly highlight that *Crocidura* shrews are made up of three major clades, two reflecting distinct biogeographic origins (Afrotropical and Asian clades), as suggested by Ruedi et al. (1998), and one including Afrotropical and Asian species (Old World clade) sharing the presumed ancestral karyotype of *Crocidura* (Vogel et al., 2006).

Previous taxonomic conclusions based on parallel, convergent or plesiomorphic morphological traits, were inappropriate and resulted in the grouping of unrelated taxa, e.g. within the genera *Suncus* and *Sylvisorex* (Quérouil et al., 2001). The combination of our new genetic data with previous karyological, paleontological and molecular data was shown to be helpful in resolving the phylogenetic relationships within the subfamily Crocidurinae, as well as its biogeographic origin and radiation.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.07.002.

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