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Asynchronous Colonization of Madagascar by the Four Endemic Clades of Primates, Tenrecs, Carnivores, and Rodents as Inferred from Nuclear Genes

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Abstract.— Madagascar harbors four large adaptive radiations of endemic terrestrial mammals: lemurs, tenrecs, carnivorans, and rodents. These rank among the most spectacular examples of evolutionary diversification, but their monophyly and origins are debated. The lack of Tertiary fossils from Madagascar leaves molecular studies as most promising to solve these controversies. We provide a simultaneous reconstruction of phylogeny and age of the four radiations based on a 3.5-kb data set from three nuclear genes (ADRA2B, vWF, and AR). The analysis supports each as a monophyletic clade, sister to African taxa, and thereby identifies four events of colonization out of Africa. To infer the time windows for colonization, we take into account both the divergence from the closest noninsular sister group and the initial intrainsular radiation, which is a novel but conservative approach in studies of the colonization history of Madagascar. We estimate that lemurs colonized Madagascar between 60 million years ago (Mya) (split from lorises) and 50 Mya (lemur radiation) (70–41 Mya taking 95% credibility intervals into account), tenrecs between 42 and 25 Mya (50–20 Mya), carnivorans between 26 and 19 Mya (33–14 Mya), and rodents between 24 and 20 Mya (30–15 Mya). These datings suggest at least two asynchronous colonization events: by lemurs in the Late Cretaceous–Middle Eocene, and by carnivorans and rodents in the Early Oligocene–Early Miocene. The colonization by tenrecs may have taken place simultaneously with either of these two events, or in a third event in the Late Eocene–Oligocene. Colonization by at least lemurs, rodents, and carnivorans appears to have occurred by overseas rafting rather than via a land bridge hypothesized to have existed between 45 and 26 Mya, but the second scenario cannot be ruled out if credibility intervals are taken into account. [Bayesian analyses; endemic mammals; island colonization; Madagascar; maximum likelihood; molecular dating; molecular phylogeny.]

The study of adaptive radiations on islands has been essential for understanding processes of evolutionary diversification (Grant, 1998; Losos et al., 1998). Reconstructing the origin and phylogeny of endemic island taxa provides crucial insight into transoceanic dispersal mechanisms and in the factors triggering radiation processes. Among major islands, Madagascar has long been renowned for the uniqueness of its fauna and flora (Myers et al., 2000), with a species-level endemism in non-flying vertebrates of over 95% that is mainly due to a few speciose endemic radiations (e.g., Bossuyt and Milinkovitch, 2001; Nagy et al., 2003; Vences et al., 2003). Madagascar became isolated from India 96 to 84 million years ago (Mya), and overland connections with Africa were severed approximately 160 to 158 Mya (Briggs, 2003).

Terrestrial mammals are represented in Madagascar by about 100 endemic species (Goodman et al., 2003) belonging to four taxonomic groups: lemurs, tenrecs, nesomyine rodents, and carnivorans (Fig. 1). These represent four of the 16 orders of land-dwelling placental mammals. Recent molecular studies have provided compelling evidence that Malagasy lemurs and carnivorans, despite their striking morphological diversity, are two monophyletic groups that presumably originated from single African ancestors (Yoder et al., 2003; Roos et al., 2004). However, morphological and molecular data are inconsistent with regard to the monophyly and intercontinental relationships of Malagasy tenrecs (Eisenberg, 1981; Asher, 1999; Douady and Douzery, 2003; Olson and Goodman, 2003) and nesomyine rodents (Lavocat, 1978; Dubois et al., 1998; Jansa et al., 1999; Michaux et al., 2001; Jansa and Weksler, 2004; Steppan et al., 2004), possibly because of extraordinary similarities to non-Malagasy forms. The Malagasy tenrec lineage has spawned hedgehog-like tenrecines, mole- and shrew-like oryzoryctines, and a semiaquatic form (*Limnogale*), whereas nesomyine rodents comprise vole- and gerbil-like species (*Brachyuromys* and *Macrotarsomys*) as well as arboreal and giant jumping rats (*Brachytarsomys* and *Hypogeomys*).

Fossil evidence to help resolve the origin of Madagascar's mammals is scarce. Relevant fossils are absent from Madagascar for the whole of the Tertiary period, and the rich findings from the Late Cretaceous include gondwanatheres, multituberculates, and marsupials, but no fossils related to extant taxa (Krause et al., 1997a, 1997b; Krause, 2001). The extant mammal groups probably arrived during the Cenozoic after the complete isolation of Madagascar (Krause et al., 1997a). However, most terrestrial mammals are poor over-water dispersers as indicated by their rareness on isolated oceanic islands (Lawlor, 1986). To reconcile these facts, a land bridge has been proposed that might have connected Africa and Madagascar from ~45 to ~26 Mya (McCall, 1997). Alternatively, mammals may have reached Madagascar by "rafting" or island-hopping (e.g., Krause et al., 1997a).

We here apply a DNA sequence data set of almost 3.5 kb from three independent nuclear genes to the reconstruction of phylogeny and age of the four Malagasy mammalian radiations and find compelling support for their respective monophyly. We argue in favor of a more

conservative approach to date ages of island colonization by taking into account both the divergence from the closest noninsular sister group and the deepest intrainsular divergence, and apply this method to test alternative hypotheses for the origin of endemic Malagasy mammals. Because all four Malagasy clades are included in the same analyses, the obtained molecular datings are directly comparable and strongly support independent colonizations by overseas rafting.

Materials and Methods

Sampling, DNA Amplification, and Sequencing

Fragments of the intronless gene of alpha 2B adrenergic receptor (ADRA2B), of exon 28 of the von Willebrand factor (vWF) gene, and of exon 1 of the androgen receptor (AR) gene were amplified and sequenced. These genes were selected because (i) they are located in the nuclear genome, as single-copy genes in at least human and mouse, and such genes are generally superior to mitochondrial genes for reconstruction of ancient relationships (Springer et al., 2001) and for time estimations (Glazko and Nei, 2003); (ii) a considerable number of sequences are already available for ADRA2B and vWF and have been useful in deeper mammalian phylogeny; and (iii) they are functionally and genetically unrelated. We selected 60 mammal species to represent for each of the three genes (i) the major lineages of all four Malagasy mammal radiations; (ii) their potential sister groups; (iii) groups needed for multiple calibration of the molecular clock; (iv) other basal mammal clades; and (v) appropriate outgroups. A total of 103 new sequences were obtained and complemented with 72 sequences from GenBank (Appendix 1). The full data matrix is available from Treebase (accession number: M2279).

Genomic DNA was isolated from ethanol-preserved tissue, following the protocols of either the DNeasy Tissue Kit (Qiagen) or the Wizard SV Genomic DNA Purification System (Promega). Fragments of the intronless ADRA2B gene and of exon 28 of the vWF gene were amplified using previously published primers (Porter et al., 1996; Springer et al., 1997). Two new vWF primers were designed for some species (vWF-for and vWF-rev), and exon 1 of the AR gene was amplified with the primers F-AR1 and R-AR1 (Appendix 2, available at www.systematicbiology.org). Polymerase chain reactions (PCRs) were performed on 50 to 200 ng DNA with Expand DNA polymerase (Expand High Fidelity PCR system, Roche) using the following program: 2 min at 94°C; 30 to 35 cycles of 15 s at 94°C, 1 min at 52 to 58°C, 56–61°C, or 55–59°C (for vWF, A2AB, or AR, respectively), and 1 min 30 s at 72°C; and a final step of 2–10 min at 72°C. DMSO (1.3% to 2.5%) and/or betaine (1 M) was added for some samples. PCR products were purified from a 1% agarose gel, using GFX PCR DNA & Gel Band Purification Kit (Amersham Biosciences) and reamplified if necessary. Gel-extracted PCR products were sequenced directly on ABI 3700 or 3730 96-capillary sequencers (Applied Biosystems).

Some specimens were polymorphic for glutamine tracks in AR or a glutamic acid track in ADRA2B. These PCR-products were cloned into a pGEM-T Vector (Promega), transformed into competent *E. coli* TOP 10 cells, and positive clones sequenced. Internal primers were used to get complete sequences of both strands. For one tenrec, *Microgale*, no vWF sequence could be obtained; this species was excluded from the molecular dating.

Phylogenetic Analyses

Sequences were assembled with PreGAP and GAP4 (Staden package, http://www.mrc-lmb.cam.ac.uk/pubseq). Alignments were obtained using GCG PILEUP (Wisconsin Package Version 10.3, Accelrys Inc.) and manually adjusted considering amino acid properties. Amino acids repeats and sites not sequenced or gapped in more than 25% of the taxa were excluded from analysis. This resulted in a data set of 1134 bp for ADRA2B, 1141 bp for vWF, and 1212 bp for AR.

Phylogenetic reconstructions on the concatenated data set were performed by maximum likelihood (ML) with PAUP*, version 4b10 (Swofford, 2003), and Bayesian analyses with MRBAYES, version 3.0b4 (Huelsenbeck and Ronquist, 2001). The best fitting model under the ML criterion was selected by the hLRT output of ModelTest, version 3.5 (Posada and Crandall, 1998). ML analyses included heuristic searches with a neighbor-joining starting tree and tree bisection-reconnection branch swapping. Node stability was estimated by 100 nonparametric bootstrap replicates (Felsenstein, 1985).

A major advantage of Bayesian phylogenetic inference is the possibility of partitioning the data, giving each partition its own best-fitting model of sequence evolution. However, overpartitioning may introduce unnecessary sampling variances, which could influence the phylogenetic estimates. For the nine possible codon partitions (each codon position of each gene), ModelTest was used to calculate the best fitting model of sequence evolution. As further explained in Table 1, codon partitions with similar models and model parameters were merged, resulting in six partitions for the Bayesian analyses: the first codon positions of ADRA2B and vWF, and the second positions of AR; the second positions of ADRA2B and vWF; the first position of AR; and the third codon positions of each gene separately. Four Markov chains were run simultaneously for 1,000,000 and 500,000 generations, with initial equal probabilities for all trees and starting with a random tree. Tree sampling frequency was each 20 generations and the consensus tree with posterior probabilities was calculated after removal of the first 2500 trees ("burn in" as determined from the likelihood values).

Molecular Dating

We used the Bayesian approach (Thorne et al., 1998) as implemented in the MULTIDIVTIME program package (Thorne and Kishino, 2002), which relaxes the molecular clock by allowing continuous autocorrelation of

TABLE 1. Best-fitting evolutionary model for each codon position. Best models and parameters were found with the hierarchical likelihood ratio test as implemented in ModelTest 3.5 for each codon position of the three gene fragments. Codon positions with similar model and model parameters were regrouped into the same partition and resulted in six partitions: (1) first codon positions of ADRA2B and vWF, and second positions of AR; (2) second positions of ADRA2B and vWF; (3) first positions of AR; and (4–6) third codon positions of each gene separately. Codon positions were merged into the same partition when none of their model parameters (e.g., TRatio of position 1 compared to TRatio of position 2, PInvar 1 to PInvar 2, etc.) differed by more that 100%. The maximum difference between model parameters within one partition was 58%. TRatio, transition/transversion ratio; rmat, rate matrix; π, frequency of base; PInvar, proportion of invariable sites; alpha, shape of gamma distribution.

Gene	Codon position	Length	π_A	π_C	π_G	Best model	TRatio or Rmat	alpha	PInvar	Partition number
ADRA2B	1	378	0.25	0.25	0.25	K80+Γ	1.47	0.42	0	1
	2	378	0.19	0.32	0.23	TrN+Γ+I	(1.0 4.4 1.0 1.0 2.9)	1.08	0.45	2
	3	378	0.13	0.38	0.32	TVM+Γ	(1.0 4.5 1.7 0.4 4.5)	2.46	0	4
vWF	1	381	0.27	0.30	0.31	HKY+Γ	1.30	0.59	0	1
	2	380	0.30	0.28	0.16	TrN+Γ+I	(1.0 5.8 1.0 1.0 4.6)	0.81	0.31	2
	3	380	0.1	0.38	0.38	TVM+Γ	(1.6 8.1 3.5 0.6 8.1)	2.9	0	5
AR	1	404	0.23	0.26	0.30	TrN+Γ	(1.0 5.3 1.0 1.0 3.7)	0.62	0	3
	2	404	0.25	0.34	0.21	HKY+Γ	1.1	0.55	0	1
	3	404	0.24	0.28	0.24	HKY+Γ	2.5	1.65	0	6

substitution rates among the branches of the phylogenetic tree. This approach estimates rates accurately (Ho et al., 2005), and was here chosen instead of penalized likelihood (Sanderson, 2002) because the MULTIDIVTIME software does not require the root of the tree to be fixed at a particular date but estimates its age starting from a prior value.

The concatenated sequence data set was partitioned into the same six categories as for the Bayesian phylogenetic analyses and branch lengths calculated under the F84+gamma model of sequence evolution, which is the most complex model available in MULTIDIVTIME. The prior for the root was set at 100 Mya. Markov chain Monte Carlo (MCMC) analyses were run for 3,000,000 and 1,000,000 generations with a "burn in" of 100,000 generations. The chains were sampled every 100 generations. To assess the influence of our particular partitioning on the dating results, we performed additional analyses using five other partitioning schemes, and without partitioning, running MCMC analyses for 1,000,000 generations. The results of these six supplementary analyses were close to each other. Notably, all datings for the nodes that we were interested in remained within the 95% credibility intervals of the datings obtained in the original analysis using six partitions (cf. Table 2). Our conclusions are therefore not affected by the choice of our partitioning.

Six well-established fossil constraints on divergence times were used: (i) a minimum of 54 and a maximum of 65 Mya for the base of Paenungulata (Gheerbrant et al., 2001); (ii) a minimum of 37 Mya for the split between ochotonids and leporids (McKenna and Bell, 1997); (iii) a minimum of 63 and a maximum of 90 Mya for the radiation of primates (Martin, 1993; Gingerich and Uhen, 1994; Tavare et al., 2002); (iv) a minimum of 50 and a maximum of 63 Mya for the split between feliform and caniform carnivorans (Benton, 1993; McKenna and Bell, 1997); (v) a minimum of 54 and a maximum of 58 Mya for the split beween hippomorph and ceratomorph Perissodactyla (Garland, 1993); (vi) a minimum of 55 and a maximum of 65 Mya for the base of Cetartiodactyla (Gatesy and O'Leary, 2001). To assess the reciprocal compatibility of these calibrations, calculations were repeated after their removal one by one, the Markov chains being sampled 1,000,000 times.

RESULTS AND DISCUSSION

Assessing Relationships of Malagasy Mammals

To determine the phylogenetic relations of Malagasy mammals we analyzed sequences from the nuclear genes for ADRA2B, vWF, and AR. Our sampling included 13 of the 18 orders of placental mammals, and two marsupial outgroup orders (Appendix 1). Phylogenetic analysis of the concatenated 3487-bp data set, by maximum likelihood (ML) and Bayesian methods, recovered inter- and intraordinal relationships (Fig. 2) in perfect agreement with more comprehensive recent phylogenies (reviewed by Springer et al., 2004). These include the superordinal clades Afrotheria, Boreoeutheria, Euarchontoglires, and Laurasiatheria, as well as Glires (rodents and lagomorphs) and Paenungulata (elephants, sea cows and hyraxes). This concordance with previous results increases the confidence in the phylogenetic relationships newly deduced here. Our analysis found each of the four endemic Malagasy mammal radiations to be monophyletic, with maximal bootstrap percentages and posterior probabilities (BP = 100, PP = 1.00) for Malagasy tenrecs, rodents, and carnivorans. Only the monophyly of the lemurs was poorly supported (BP = 47, PP = 0.86), but corroborated by a unique 15-bp deletion in the vWF sequence of all Lemuriformes, including the most basal aye-aye (*Daubentonia*) (Appendix 3A, available at www.systematicbiology.org).

The monophyly of Malagasy carnivorans and their relationship to herpestids, here represented by *Suricata*, confirmed previous molecular data (Yoder et al., 2003). The same applied to the monophyletic lemurs that are sister to the Lorisiformes, here represented by *Nycticebus* (Yoder et al., 1996, 2003). Our data further confirmed the phylogenetic relationships among Malagasy carnivoran and lemuriform taxa (Yoder et al.,1996, 2003; Pastorini

TABLE 2. Posterior estimates of divergence ages (± standard deviation) inferred from the concatenated data set using the Bayesian relaxed molecular clock method. The 95% credibility intervals are given between brackets.

			Removal of the following calibration point during the analysis[c]:					
Radiation or branching (/)	Calibration time frame (Mya)[a]	All calibration points[b]	1. Paenungulata	2. Ochotona/ Leporidae split	3. Primates	4. Feliformia/ Caniformia split	5. Perissodactyla	6. Cetartiodactyla
Malagasy tenrecs		25.3 ± 3.1 (31.8–19.7)	26.7 ± 3.6	25.3 ± 3.0	25.4 ± 3.1	25.4 ± 3.0	25.3 ±3.0	25.2 ± 3.0
Malagasy tenrecs/ Potamogalines		41.8 ± 4.1 (50.3–34.1)	43.9 ± 5.1	41.7 ± 4.1	41.9 ± 4.2	41.8 ± 4.1	41.7 ± 4.1	41.5 ± 4.1
Nesomyine rodents		20.1 ± 2.6 (25.7–15.4)	20.6 ± 2.8	20.2 ± 2.7	20.3 ± 2.6	20.1 ± 2.6	20.0 ± 2.7	19.9 ± 2.7
Nesomyine rodents/ (*Cricetomys* + *Steatomys*)		23.5 ± 2.9 (29.6–18.2)	24.1 ± 3.0	23.6 ± 2.9	23.7 ± 2.9	23.5 ± 2.9	23.4 ± 3.0	23.2 ± 2.9
Malagasy lemurs		49.6 ± 4.4 (58.5–41.1)	50.6 ± 4.6	49.6 ± 4.4	49.8 ± 4.5	49.4 ± 4.4	49.3 ± 4.6	49.0 ± 4.6
Malagasy lemurs/ Lorisiformes		60.4 ± 4.6 (69.6–51.6)	61.5 ± 4.8	60.4 ± 4.6	60.6 ± 4.8	60.2 ± 4.7	60.0 ± 4.8	59.6 ± 4.9
Malagasy carnivorans		19.0 ± 2.7 (24.8–14.1)	19.1 ± 2.8	19.0 ± 2.7	19.0 ± 2.7	18.9 ± 2.9	18.9 ± 2.7	18.9 ± 2.7
Malagasy carnivorans/ *Suricata*		25.9 ± 3.2 (32.5–20.1)	26.1 ± 3.2	25.9 ± 3.2	25.9 ± 3.2	25.7 ± 3.4	25.7 ± 3.2	25.7 ± 3.1
1. Paenungulata	54–65	60.9 ± 2.8	64.8 ± 5.7					
2. Ochotona/ Leporidae	>37	52.8 ± 4.7		52.8 ± 4.7				
3. Primates	63–90	78.9 ± 4.5			79.1 ± 4.7			
4. Feliformia/ Caniformia	50–63	55.6 ± 3.1				55.2 ± 4.0		
5. Perissodactyla	54–58	55.9 ± 1.1					54.8 ± 2.3	
6. Cetartiodactyla	55–65	58.6 ± 2.5						56.2 ± 4.3

[a] Paleontological time constraints used as calibrations, numbered as indicated in Figure 4.
[b] Estimates of the age of the Malagasy lineages as used in Figure 4 and in the text.
[c] The influence of each calibration point was tested by computing divergence ages after removing that calibration point. The lower panel shows that all six calibrations points are correctly recovered when the point itself is excluded from the constraints. The recovered ages for the excluded calibration points are in very good agreement with the original calibrations, all of them falling within the calibration time frame set for that point, and close to the times obtained with all calibration points.

et al., 2003; Roos et al., 2004), and provide the first compelling evidence for a close relationship of the specialized worm-eating civet *Eupleres* to *Fossa* (BP = 100, PP = 1.00).

Monophyly of Malagasy tenrecs was strongly supported in our analyses and relations among included taxa were resolved completely and with high support (Fig. 2), whereas morphological data have been ambiguous in this respect (Asher, 1999). The African otter shrews, here represented by *Micropotamogale*, were found as sister group of all Malagasy tenrecs. The semiaquatic web-footed tenrec *Limnogale* which morphologically resembles the otter shrew (Asher, 1999) actually appeared closely related to the shrew tenrec *Microgale* (BP = 100, PP = 1.00). This relationship was corroborated by a molecular synapomorphy, a shared 3-bp deletion in the ADRA2B gene (Appendix 3B, available at www.systematicbiology.org).

Nesomyine rodents belong to the Muridae, the most speciose family of mammals. Previous studies identified various major clades within nesomyines but have been unable to resolve relationships between these and several non-Malagasy murid taxa (Jansa et al., 1999; Jansa and Weksler, 2004). Our analysis included representatives of each of these clades (cf. Fig. 1) and the monophyly of Malagasy rodents was firmly established (Fig. 2). Their sister group was a clade comprising the African murids *Steatomys* (Dendromurinae) and *Cricetomys* (Cricetomyinae) (BP = 100, PP = 1.00).

Hence, monophyly and relations to African taxa were unambiguously suggested for Malagasy tenrecs and nesomyine rodents, where the evidence was so far controversial, and confirmed for the Malagasy carnivorans. The sister group of the Lemuriformes contains African an Asian taxa, but an African origin of the strepsirrhine clade is now supported (Seiffert et al., 2003; Roos et al., 2004). This strongly suggests that each of the four clades originated by a single colonization event out of Africa.

Timing the Origins and Radiations of Malagasy Mammals

DNA sequences can be used in various statistical approaches to estimate times of divergence (Hedges and Kumar, 2003). Such molecular datings face two main problems. First, the assumption of evolutionary rate constancy is in general not valid (Bromham and Penny, 2003), as obvious in our data set from branch lengths in Figure 2. Second, the fossil ages used in the analyses may not be accurate (Graur and Martin, 2004). Biases can in both cases result in erroneous time estimates. We

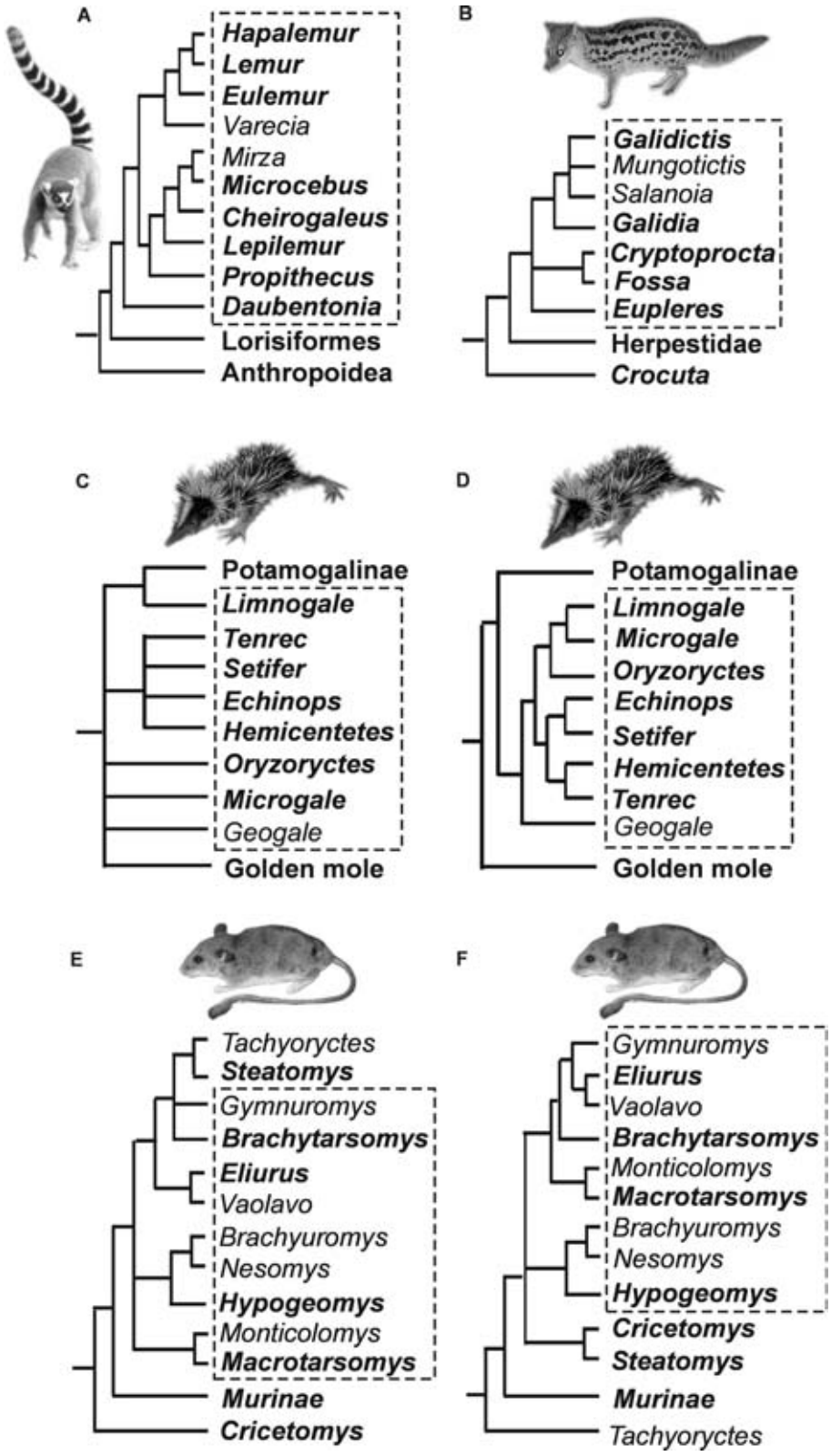

FIGURE 1. Phylogenetic hypotheses for lemurs (A), carnivores (B), tenrecs (C, D) and rodents (E, F) of Madagascar. Dashed boxes enclose endemic Malagasy taxa. Taxa in bold were included in the present study. Molecular data unequivocally suggested a monophyletic origin for lemurs (A) and Malagasy carnivorans (B) (Yoder et al., 1996, 2003). Morphological data indicated paraphyly of Malagasy tenrecs (C; consensus of alternative morphological trees; Asher, 1999), but molecular data support their monophyly (D; Olson and Goodman, 2003). Analysis of cytochrome *b* sequences suggested paraphyly of Malagasy rodents (E; Jansa et al., 1999), whereas IRBP sequences could not resolve their relationships (F; Jansa and Weksler, 2004). In the case of tenrecs and rodents some taxa are excluded to make trees comparable. Photos show *Lemur catta* (A), *Fossa fossana* (B), *Hemicentetes semispinosus* (C, D), and *Eliurus* sp. (E, F).

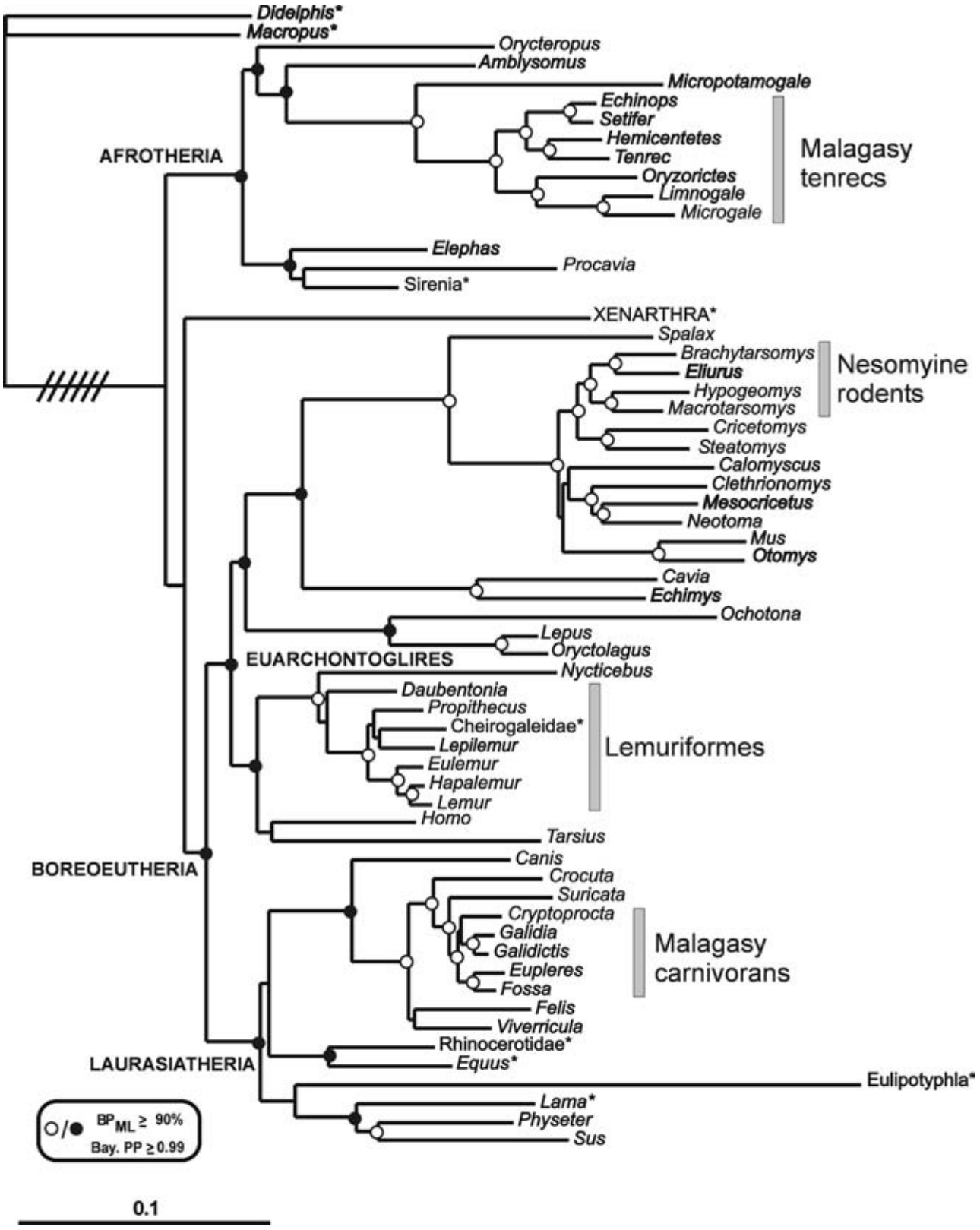

FIGURE 2. Phylogenetic relationships of Malagasy mammals as inferred by maximum likelihood analysis of the concatenated 3487-bp data set of ADRA2B, vWF, and AR sequences. Bayesian analyses result in an identical topology. Nodes receiving high support (BP $\geq$ 90% and PP $\geq$ 0.99) are marked with circles; filled circles correspond with generally accepted ordinal and superordinal relationships. The length of the branch connecting eutherians to the marsupial outgroup was reduced six times. Asterisks mark taxa represented by different species in the concatenated sequences (Appendix 1).

here simultaneously used six independent fossil calibrations, specified in Materials and Methods, in a relaxed clock approach that takes into account the variations of the molecular substitution rate. By constraining the time estimates on the fossil calibrations as ranges rather than fixed values, the method takes also the paleontological uncertainties into account.

The age of colonization of Madagascar has usually been seen as equivalent either to the initial diversification of the Malagasy lineages (Yoder et al., 1996, 2003; Roos et al., 2004) or to the split from their non-Malagasy sister group (Nagy et al., 2003; Vences et al., 2003). However, a radiation may take place long after the initial colonization, or early radiations may go extinct. Moreover, the extant mainland sister group of an insular clade is not necessarily its closest mainland relative which may have gone extinct (Fig. 3A). The same rationale has been applied for the colonization of South America by rodents and primates (Poux et al., in press). Hence, in order to obtain a conservative and more reliable estimate of the time period during which colonization has occurred, we here suggest that the two divergence times for the latest outgroup split and the earliest ingroup split, and their 95% credibility intervals, need to be taken into account (Fig. 3B).

Applying these extended intervals, our results (Table 2 and Fig. 4) indicated that the colonization events can be reliably dated into the Late Cretaceous–Middle Eocene for lemurs (70 to 41 Mya), Early Eocene–Early Miocene for tenrecs (50 to 20 Mya), and Early Oligocene–Middle Miocene for carnivorans and rodents (33 to 14 and 30 to 15 Mya, respectively). The time windows were synchronous for carnivorans and rodents, but there was no overlap between any of these two clades and the lemurs. The timing of the tenrec colonization overlapped in the Eocene with the lemurs and in the Oligocene-Miocene with the rodents and carnivorans. Because *Geogale*, possibly the most basal tenrec (Olson and Goodman, 2003), was absent from our data set, the Malagasy tenrec radiation may actually be somewhat older and consequently their colonization window a bit narrower. In conclusion, Madagascar was colonized at a later period by carnivorans and rodents than by lemurs. Colonization by tenrecs may have occurred in the Late Eocene–Oligocene in a third, separate event, but we cannot exclude that it occurred simultaneously either with lemurs or with carnivorans and rodents.

Our dating of the lemur radiation at 50 Mya (59–41 Mya) is more recent than the previous estimate by Yoder et al. (2003) at 66 Mya (75–55 Mya) using the same method, but agrees with a previous estimate of 48 to 41 Mya based on the epsilon-globin gene and its 5′ flanking region (Porter et al., 1997; analyses performed with a local molecular clock approach). These differences could be due to the use of different phylogenetic markers (nuclear and mitochondrial genes) and to the fact that the IRBP gene (exon 1), used by Yoder et al. (2003), evolves significantly slower in lemurs than in other mammals, except perissodactyles (Poux et al., 2004). Our estimates for Malagasy carnivorans displayed a radiation time at

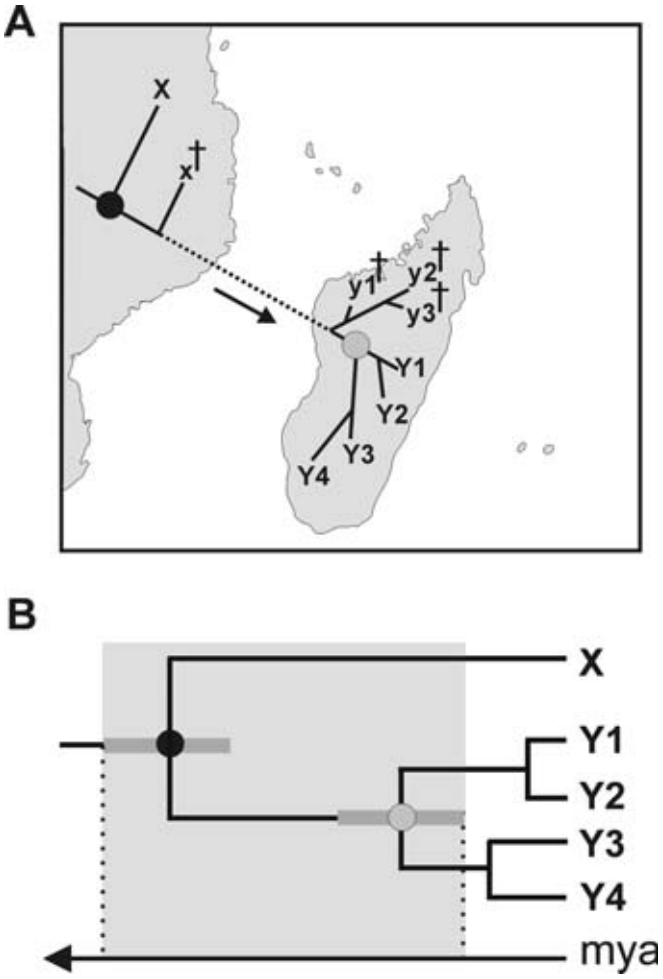

FIGURE 3. Estimating the colonization time of an island (here Madagascar) using molecular clock data from extant taxa. (A) Molecular datings provide estimates for the time of divergence of the extant insular taxa Y from their nearest extant noninsular sister taxon X (black circle) and for the earliest divergence among the extant insular taxa Y1–Y4 (grey circle). These two estimates provide the maximum time window for possible colonization. Any of the estimates may be close to the actual time of colonization, but extinct species x from the mainland may have been closer to the colonizing ancestor, and early radiations in Madagascar (y1–y3) may have gone extinct. Fossil data might therefore shorten the time window for colonization (dashed line). (B) The most conservative window of possible colonization times (shaded area) is given by the upper 95% confidence interval of the first estimate (black circle) and the lower 95% confidence interval of the second estimate (grey circle).

19 Mya (25–14 Mya), which is in perfect accordance with previous estimates (Yoder et al., 2003) of 20 Mya (26–15 Mya). Similarly, our datings for the split of nesomyine rodents and Malagasy tenrecs from their sister groups at 24 Mya (30–18 Mya) and 42 Mya (50–34 Mya), respectively, are not far from previously published results, $16 \pm 0.5/19 \pm 1$ Mya (Michaux et al., 2001; with global clock approach) and 43 Mya (52–34 Mya) (Douady and Douzery, 2003; with Bayesian dating method), respectively.

To exclude the possibility that individual calibration constraints may bias our dating analyses, we repeated them after removing each calibration point in turn. All

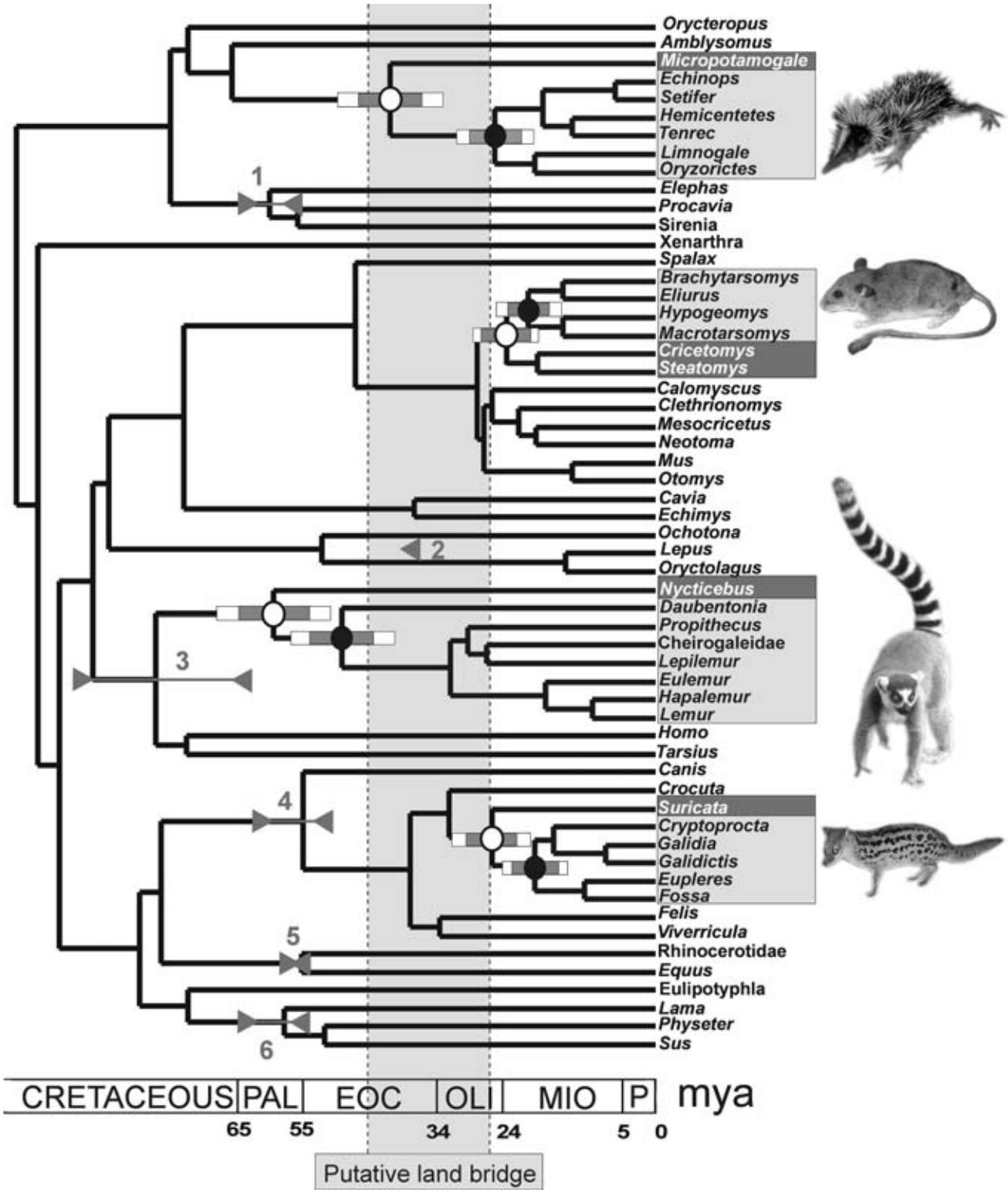

FIGURE 4. Asynchronous colonizations of the Malagasy mammal clades. Eutherian tree topology as in Figure 2. Divergence times were estimated from the concatenated data set by a Bayesian relaxed molecular clock method with six time constraints from fossil calibrations (nodes numbered as in Table 2). Malagasy clades are displayed in light grey boxes and their sister groups in dark grey boxes. Black circles indicate the initial divergence within each Malagasy radiation and open circles indicate divergences from non-Malagasy sister groups, with standard deviations (grey bars) and 95% credibility intervals (open bars) (see Table 2). Time estimates for all other nodes are given in Appendices 4 and 5 (available at www.systematicbiology.org). The period of a putative land bridge between Madagascar and Africa at 45 to 26 Mya (McCall, 1997) is shaded.

TABLE 3. Comparison of estimated divergence times (in Mya) with standard deviations (SD) and 95% credibility intervals (CI) from the present study, Springer et al. (2003), and Hasegawa et al. (2003). Divergence times in Springer et al. (2003) are based on a large data set (~16,000 bp) of mainly nuclear genes, and in Hasegawa et al. (2003) on a mitochondrial protein data set (3392 bp). Node numbers as in Appendices 4 and 5. In Hasegawa et al. (2003) the age of Laurasiatheria and 95% credibility intervals were not tabulated and could therefore not be included here (−). ND, not determined.

	This study		Springer et al. (2003)		Hasegawa et al. (2003)	
Clade and node number	age ± SD	95% CI	Age ± SD	95% CI	Age ± SD	95% CI
Afrosoricida, 53	66.9 ± 4.5	58.1–75.7	66.4 ± 3.3	59.5–72.4	ND	ND
Afrotheria, 55	76.5 ± 3.9	68.9–83.9	79.9 ± 3.0	73.0–85.8	79.9 ± 2.9	—
Glires, 41	86.3 ± 4.6	77.3–95.4	82.6 ± 3.2	76.6–89.0	74.6 ± 1.6	—
Euarchontoglires, 42	89.0 ± 4.4	80.4–97.8	87.3 ± 3.2	81.5–93.9	89.0 ± 1.9	—
Laurasiatheria, 15	81.6 ± 3.3	75.3–88.4	85.1 ± 2.5	80.3–90.3	—	—
Eutheria, 56 (root)	101.0 ± 4.7	92.1–110.5	106.7 ± 4.9	97.8–117.1	101.6 ± 1.3	—

relevant datings remained highly congruent when any of the six calibrations was removed (Table 2). Moreover, the reciprocal compatibility of the calibrations was evident: after excluding any of them, the remaining five calibrations always recovered a posterior estimate for the excluded node within the time window independently obtained from the corresponding fossil evidence (Table 2). In addition, the observed congruence of our interordinal divergence times with previously published data, based on much larger data sets, gives further confidence in our results (Table 3).

Biogeographic Scenarios

In contrast to all other molecular studies of the mammalian colonization of Madagascar (Yoder et al., 1996, 2003; Michaux et al., 2001; Douady and Douzery, 2003; Roos et al., 2004), we included all four Malagasy clades simultaneously in one analysis. Therefore, our estimates of divergence ages are directly comparable because they were affected by the same, if any, calibration biases. Our study is moreover based on the comparatively greatest length of concatenated nucleotides and includes representatives of 13 mammalian orders, which reduces sampling bias and long-branch attractions. The robustness of our results strengthens the evaluation of the different scenarios that have been proposed to explain the origin of extant Malagasy mammals: (i) ancient vicariance; (ii) terrestrial migration or island hopping along a land bridge or island arc; (iii) overseas rafting across the 400 km of open sea that make up the Mozambique channel.

The first scenario, vicariance, has been invoked for lemurs (Arnason et al., 2000) and would assume an age of colonization older than 84 Mya, the time when Madagascar became isolated (Briggs, 2003). According to our data, lemurs were the first to diverge from their African sister group, not earlier than 70 Mya (including the 95% credibility interval). Vicariance can thus be excluded as an explanation for the origin of lemurs and any other Malagasy mammal lineage.

The second scenario involves a more or less continuous land bridge between Africa and Madagascar during the period 45 to 26 Mya (McCall, 1997). Our results do not match the colonization pattern expected under this hypothesis. Instead of showing large overlapping periods between the four clades during the Middle Eocene–Late Oligocene, our results display colonization ages spread over the Tertiary (Fig. 4). The radiation of lemurs dated at 75 to 55 Mya (Yoder et al., 2003) invalidated the land bridge hypothesis for this clade. However, in our study, the estimated age of the lemur colonization (70 to 41 Mya) is younger and therefore overlaps slightly with the postulated land bridge period. The windows of colonization (using the 95% credibility intervals) of tenrecs, carnivorans, and rodents likewise overlap to different extents the period of the putative land bridge, and migration via the land bridge route therefore cannot be excluded based on our data. However, the hypothesis remains unlikely because in three out of the four clades (all except tenrecs), both our ingroup and outgroup age estimates are outside of the landbridge period, the overlap only concerning the credibility intervals. Moreover, the existence of an emerged land bridge during the Eocene/Oligocene period has been seriously challenged (e.g., Rogers et al., 2000), and if this land bridge had been uninterrupted, a much greater variety of mammalian lineages could be expected to have colonized Madagascar.

The third scenario, transoceanic dispersal on rafting flotsam, predicts colonizations to occur probably randomly over time (Krause et al., 1997). The clearly asynchronous timing of at least two colonization events supports this scenario. Also considering that the estimated colonization times for lemurs, carnivorans, and rodents are largely outside the assumed time frame for the land bridge (with only the credibility intervals overlapping), we favor the transoceanic dispersal scenario. This agrees with the pattern observed in the majority of nonflying Malagasy vertebrate groups (Vences, 2004) and in at least some plants (Yuan et al., 2005), and supports recent claims that the importance of oceanic dispersal has been strongly underestimated in historical biogeography (de Queiroz, 2004).

In conclusion, the extant diversity of endemic Malagasy mammals reflects four adaptive radiations that probably colonized the island in at least two asynchronous waves of overseas dispersal. Studying ancient DNA from subfossil remains of two extinct lineages of Malagasy mammals, hippos and the enigmatic *Plesiorycteropus* (Goodman et al., 2003), bears the potential to add additional colonization ages and thereby test the hypothesis of random timing. Relating the age, pattern, and diversity of radiations to the emergence of eastern Malagasy rainforests in the Eocene or Oligocene (Wells,

2003) is a further exciting perspective for studies on the Malagasy biota.

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APPENDIX 1. Taxonomic sampling and accession numbers for the three nuclear genes. Upperscore numbers[(1–10)] refer to data sets in which different taxa were available for each gene, and were concatenated, or to taxa that were not included in all analyses. *Sequences taken from the database.

	Species	ADRA2B	vWF	AR
		EUTHERIA		
RODENTIA				
Sciurognathi				
Muridae				
Murinae	*Mus musculus*	L00979*	AJ238390*	NM_013476*
Spalacinae	*Spalax ehrenbergii*	AJ891078	U31621*	AJ893519
Nesomyinae	*Eliurus* sp.	AJ891058	AJ891086	AJ893520
	Hypogeomys antimena	AJ891066	AJ891094	AJ893521
	Macrotarsomys ingens	AJ891070	AJ402705*	AJ893522
	Brachytarsomys albicauda	AJ891049	AJ891083	AJ893523
Arvicolinae	*Clethrionomys glareolus*	AJ891053	AJ402709*	AJ893524
Calomyscinae	*Calomyscus mystax*	AJ891050	AJ402702*	AJ893525
Cricetinae	*Mesocricetus auratus*	AJ891071	AJ402706*	AJ893526
Cricetomyinae	*Cricetomys gambianus*	AJ891054	AJ402694*	AJ893527
Dendromurinae	*Steatomys* cf.*gautuni*	AJ891079	AJ402704*	AJ893528
Otomyinae	*Otomys angoniensis*	AJ891075	AJ402711*	AJ893529
Sigmodontinae	*Neotoma fuscipes*	AJ891073	AJ402703*	AJ893530
Hystricognathi				
Echimyidae	*Echimys chrysurus*	AJ427269*	AJ251141*	AJ893532
Caviidae	*Cavia porcellus*	AJ271336*	AJ224663*	AJ893531
LAGOMORPHA				
Leporidae	*Oryctolagus cuniculus*	Y15946*	U31618*	AJ893533
	Lepus crawshayi	AJ427254*	AJ224669*	AJ893534
Ochotonidae	*Ochotona princeps*	AJ427253*	AJ224672*	AJ893535
PRIMATES				
Lemuridae	*Lemur catta*	AJ891067	AJ410292*	AJ893536
	Eulemur fulvus fulvus	AJ891059	AJ891087	AJ893537
	Hapalemur simus	AJ891064	AJ891092	AJ893538
Megaladapidae	*Lepilemur edwardsi*	AJ891068	AJ891095	AJ893539
Cheirogaleidae	*Cheirogaleus/Microcebus*[1]	AJ891052	AJ410295*	AJ893540
Daubentoniidae	*Daubentonia madagascariensis*	AJ891057	AJ410293*	AJ893541
Indridae	*Propithecus verreauxi coronatus*	AJ891076	AJ410294*	AJ893542
Loridae	*Nycticebus coucang*	AJ251186*	AJ410291*	AJ893543
Tarsiidae	*Tarsius bancanus*	AJ891081	AJ410296*	AJ893544
Hominidae	*Homo sapiens*	M34041*	X06828*	M27423*

(Continued on next page)

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	Species	ADRA2B	vWF	AR
CARNIVORA				
Canidae	*Canis familiaris*	AJ891051	L16903*	AF197950*
Felidae	*Felis catus*	AJ251174*	U31613*	AJ893545
Hyaenidae	*Crocuta crocuta*	AJ891055	AJ891084	AY128705*
Herpestidae/Viverridae				
Galidiinae	*Galidictis fasciata*	AJ891063	AJ891091	AJ893547
	Galidia elegans	AJ891062	AJ891090	AJ893546
Herpestinae	*Suricata suricata*	AJ891080	AJ891099	AJ893548
Cryptoproctinae	*Cryptoprocta ferox*	AJ891056	AJ891085	AJ893549
Euplerinae	*Eupleres goudoti*	AJ891060	AJ891088	AJ893550
	Fossa fossana	AJ891061	AJ891089	AJ893551
Viverrinae	*Viverricula indica*	AJ891082	AJ891100	AJ893552
PERISSODACTYLA				
Rhinocerotidae	*Ceratotherium/Diceros*[2]	AJ251184*	U31604*	AJ893553
Equidae	*Equus* sp.[3]	Y15945*	U31610*	AJ893554
CETARTIODACTYLA				
Camelidae	*Lama*[4]	AJ315941*	AF108835*	AJ893555
Suidea	*Sus scrofa*	AJ251177*	S78431*	AF161717*
Physeteridae	*Physeter catodon*	AJ427417*	AF108834*	AJ893556
EULIPOTYPHLA	*Erinaceus/Crocidura*[5]	Y12521*	AY057834*	AJ893557
XENARTHRA	*Bradypus/Cyclopes*[6]	AJ251179*	U31603*	AJ893558
SIRENIA	*Trichechus/Dugong*[7]	AJ251109*	U31608*	AJ893559
PROBOSCIDEA	*Elephas maximus*	Y12525*	U31611*	AJ893560
HYRACOIDEA	*Procavia capensis*	Y12523*	U31619*	AJ893561
TUBULIDENTATA	*Orycteropus afer*	Y12522*	U31617*	AJ893563
AFROSORICIDA				
Chrysochloridea	*Amblysomus hottentotus*	Y12526*	U97534*	AJ893562
Tenrecidea				
Tenrecinae	*Setifer setosus*	AJ891077	AJ891098	AJ893566
	Echinops telfairi	Y17692*	AF076478*	AJ893565
	Tenrec ecaudatus	AJ251108*	AF390536*	AJ893564
	Hemicentetes semispinosus	AJ891065	AJ891093	AJ893567
Oryzoryctinae	*Oryzorictes hova*	AJ891074	AJ891097	AJ893568
	Microgale brevicaudata[8]	AJ891072	—	AJ893569
	Limnogale mergulus	AJ891069	AJ891096	AJ893570
Potamogalinae	*Micropotamogale lamottei*	AJ251107*	AF390538*	AJ893571
	MARSUPIALIA			
DIDELPHIMORPHIA	*Didelphis*[9]	Y15943*	AF226848*	AJ893572
DIPROTODONTIA	*Macropus*[10]	AJ251183*	AJ224670*	AJ893573

[1] *Cheirogaleus medius* (ADRA2B, AR) combined with *Microcebus murinus* (vWF).
[2] *Diceros bicornis* (ADRA2B, AR) combined with *Ceratotherium simum* (vWF).
[3] *Equus caballus* (ADRA2B, AR) combined with *E. asinus* (vWF).
[4] *Lama pacos* (ADRA2B, AR) combined with *L. glama* (vWF).
[5] *Erinaceus europaeus* (ADRA2B, AR) combined with *Crocidura russula* (vWF).
[6] *Bradypus tridactylus* (ADRA2B, vWF) combined with *Cyclopes didactylus* (AR).
[7] *Trichechus manatus* (ADRA2B, AR) combined with *Dugong dugon* (vWF).
[8] *Microgale brevicaudata* was removed from the dating analyses.
[9] *Didelphis marsupialis* (ADRA2B, AR) combined with *D. virginiana* (vWF).
[10] *Macropus rufus* (ADRA2B, AR) combined with *M. giganteus* (vWF).