

Molecular Evidence for the Monophyly of Tenrecidae (Mammalia) and the Timing of the Colonization of Madagascar by Malagasy Tenrecs

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Tenrecs are a diverse family of insectivores, with an Afro-Malagasian biogeographic distribution. Three subfamilies (Geogalinae, Oryzorictinae, Tenrecinae) are restricted to Madagascar and one subfamily, the otter shrews (Potamogalinae), occurs on the mainland. Morphological studies have generated conflicting hypotheses according to which both tenrecids and Malagasy tenrecs are either monophyletic or paraphyletic. Competing hypotheses have different implications for the biogeographic history of Tenrecidae. At present, there are no molecular studies that address these hypotheses. The present study provides sequences of a nuclear protein-coding gene (vWF) and the mitochondrial 12S rRNA, tRNA valine, and 16S rRNA genes from a potamogaline (*Micropotamogale*). New sequences of these genes are also reported for the tenrecine, *Tenrec ecaudatus*. The 12S sequences from these taxa were combined with data already available for this locus from two other tenrecids (*Echinops telfairi*, subfamily Tenrecinae and *Oryzorictes talpoides*, subfamily Oryzorictinae). Phylogenetic analyses provided strong bootstrap support for the monophyly of Tenrecidae and Malagasy tenrecs. The majority of statistical tests rejected morphological claims for both a Tenrecinae–Chrysochloridae clade and an Oryzorictinae–Potamogalinae clade. Molecular clock estimates suggest a split of otter shrews and Malagasy tenrecs at approximately 53 MYA. We estimate that the ancestor of Malagasy tenrecs dispersed to Madagascar subsequent to this split but prior to about 37 MYA. © 2002 Elsevier Science (USA)

INTRODUCTION

The taxonomic wastebasket “Insectivora” remains one of the most misunderstood mammalian groups. Throughout the history of mammalian systematic study, the taxonomic composition of the group has been debated and modified (see, e.g., Van Valen, 1967; McKenna, 1975; Yates, 1984; McKenna and Bell, 1997). While the overall primitive condition of insectivores has undoubtedly contributed to this enigmatic status, presumed similarities between the insectivores and the ancestral placental stock (Simpson, 1945) have also made insectivores a group of special interest in the study of mammalian evolution. Among the six lipotyphlan insectivore families [Chrysochloridae (= golden moles), Erinaceidae (= hedgehogs and gymnures), Solenodontidae (= solenodons), Soricidae (= shrews), Talpidae (= moles), and Tenrecidae (= tenrecs)], the Afro-Malagasy tenrecs have traditionally been placed in the suborder Soricomorpha with shrews, moles, solenodons, and, more variably, golden moles (Butler, 1988; MacPhee and Novacek, 1993; but see Van Valen, 1967 and McKenna, 1975). In contrast, phylogenetic analyses with DNA sequences demonstrate that tenrecs are closely related to golden moles but not to other soricomorphs (Stanhope *et al.*, 1998a,b). Further, tenrecs and golden moles share a common ancestry with five African-origin orders to the exclusion of other lipotyphlans (Springer *et al.*, 1997; Stanhope *et al.*, 1998a,b; Liu and Miyamoto, 1999; Madsen *et al.*, 2001). Stanhope *et al.* (1998a) suggested the name “Afrosoricida” for a new order of mammals that includes tenrecids and chrysochlorids. Afrotheria (Stanhope *et al.*, 1998a) is the superordinal group that includes “Afrosoricida,” Sirenia, Proboscidea, Hyracoidea, Macroscelidea, and Tubulidentata. Whereas some authors have argued for a tenrecid–chrysochlorid alliance based on morphology (e.g., Butler, 1988), the inclusion of “Afrosoricida” in Afrotheria is unprece-

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dented based on morphological data and contradicts more than one century of morphological investigation (e.g., Haeckel, 1866; Gregory, 1910; Butler, 1988; MacPhee and Novacek, 1993). Even the recent morphological study of Asher (1999), which fails to support lipotyphlan monophyly, finds no support for Afrotheria.

The family Tenrecidae has conventionally been viewed as monophyletic (Butler, 1972). In contrast, four of eight analyses presented by Asher (1999) challenge the monophyly of Tenrecidae and instead suggest that a subfamily of tenrecs, Tenrecinae, are more closely related to chrysochlorids (golden moles) than to other tenrecs, creating a paraphyletic Tenrecidae. Asher's analyses never recovered the monophyly of the Malagasy tenrecs and instead placed *Limnogale* (subfamily Oryzorictinae) as the sister taxon to the African otter shrews (subfamily Potamogalinae) in eight of eight analyses. Finally, Asher's (1999) study supports the hypothesis that there have been multiple colonization events of Madagascar by tenrecs.

At present, there are no published molecular studies that address questions pertaining to tenrecid monophyly, the monophyly of Malagasy tenrecs, and the biogeographic history of Tenrecidae. The present paper provides DNA sequences for a potamogaline and examines the evolutionary history of the Tenrecidae from a molecular perspective.

MATERIALS AND METHODS

Phylogenetic Loci and Taxa

Given the suggestion that basal tenrecid divergences may trace to the Paleocene (Eisenberg, 1981), we selected moderately conserved loci that have phylogenetic signal at this temporal level (Springer *et al.*, 2001). Specifically, we collected and analyzed DNA sequences for two independent data sets: exon 28 of the gene encoding von Willebrand Factor (vWF) and the colinear mitochondrial 12S rRNA, tRNA valine, and 16S rRNA genes. The data sets that we analyzed encompassed 36 taxa that represent all orders of placental mammals for both vWF and 12S–16S rRNA (Table 1; sequences indicated with an asterisk are new to this study). The Tenrecidae included in these analyses are *Micropotamogale lamottei* (Nimba Otter Shrew), *Tenrec ecaudatus* (Tailless Tenrec), and *Echinops telfairi* (Lesser Hedgehog Tenrec). The *Micropotamogale* and *Tenrec* vWF genes were amplified and sequenced as described previously (Porter *et al.*, 1996; Springer *et al.*, 1997). The 12S rRNA–tRNA valine–16S rRNA amplification primers for *Micropotamogale* were modified to correspond to “afrotherian versions” of primers 12C (Springer *et al.*, 1995) and 16R (Springer *et al.*, 1997). The sequences of these two primers are 5' AAA GCA AAR CAC TGA AAA TGC YTA GAT G 3' and 5' TGT

TAA GGA GAG GAT TTG AAC CTC TG 3', respectively. Mitochondrial RNA gene sequences of other mammals (Table 1) were obtained as previously described (Springer *et al.*, 1997).

The resulting data sets were aligned using Clustal X (Thompson *et al.*, 1994) and subsequently refined by eye. Positions that could not be unambiguously aligned by eye were excluded from analysis. The secondary structures of the 12S and 16S rRNA molecules (Springer and Douzery, 1996; Burk, 1999) allowed some further refinement of the final alignment. The resulting vWF alignment was 1254 bp in length and the 12S rRNA–tRNA valine–16S rRNA alignment was 2053 bp in length. Both alignments are available upon request from M.J.S. (Michael_J_Stanhope@gsk.com).

Finally, we analyzed a matrix of 12S rRNA gene sequences that also included the *Oryzorictes talpoides* sequence from Emerson *et al.* (1999). *Oryzorictes* was added manually to the previously described rRNA alignment.

Analysis

Phylogenetic reconstructions. Maximum-likelihood (ML), minimum-evolution (ME), and maximum-parsimony (MP) were used to infer phylogenetic relationships. The majority of these analyses were carried out using PAUP*4.0b2 (Swofford, 1998); Puzzle 4.0.2 (Strimmer and von Haeseler, 1996) was used to perform quartet puzzling (QP) with vWF amino acid sequences. ML and QP analyses employed the Tamura and Nei model (Tamura and Nei, 1993). For each data set, ML and QP searches were performed with and without a gamma distribution (G) of rates across sites and an allowance for a proportion of invariant sites (I). Substitution rates, the shape parameter of the gamma distribution, and the proportion of invariant sites were estimated from the most parsimonious trees for the corresponding data or, in the case of QP, directly from the data set. ME trees were computed using LogDet (Lockhart *et al.*, 1994) and GTR (General Time Reversible; Lanave *et al.*, 1984) distances. In all analyses, gaps were treated as missing data, branches were swapped using the tree bisection and reconnection branch swapping option, and the taxon addition sequence was random. The statistical significance of *a priori* hypotheses was evaluated with the Winning Sites (WS; Prager and Wilson, 1988), Templeton (T; 1983), and Kishino-Hasegawa (KH; 1989) tests. These calculations were also done using PAUP*4.0b2. Clade support was assessed using the bootstrap, employing 200 replicates for ML, 500 replicates for ME and MP, and 10,000 puzzling steps for QP. Trees were rooted using marsupial outgroups.

Molecular clock estimates of splitting events. The relative evolutionary rate homogeneity between taxa was tested using the method proposed by Wu and Li

TABLE 1

Accession Numbers and Taxa Common Names

Species	Common name	vWF Accession No.	12S-16S Accession No.
<i>Didelphis virginiana</i>	Opossum	AF226848	Z29573
<i>Macropus giganteus</i>	Kangaroo	AJ224670	AF027985
<i>Bradypus tridactylus</i>	Sloth	U31603	AF069535, AF038022
<i>Chaetophractus villosus</i>	Armadillo	AF076480	U61080, AF069534
<i>Erinaceus europaeus</i>	Hedgehog	U97536	X88898
<i>Scalopus aquaticus</i>	Mole	AF076479	AF069539
<i>Tupaia (glis/tana)</i>	Tree shrew	U31623, AF061063	AF038021, AF203727
<i>Cynocephalus variegatus</i>	Flying lemur	U31606	AF038018, AF038018
<i>Megaderma lyra</i>	Microbat	U31616	AF069538
<i>Tadarida brasiliensis</i>	Microbat	U31623, AF061061	AF179288
<i>Cynopterus sphinx</i>	Megabat	U31605	U93068, AF203740
<i>Dobsonia moluccensis</i>	Megabat	U31609	U93065, AF179290
<i>Otolemur crassicaudatus</i>	Galago	U31614, AFO61064	AF019080
<i>Homo sapiens</i>	Human	M25851	J01415
<i>Felis catus</i>	Cat	U31613, AFO61062	U20753
<i>Canis familiaris</i>	Dog	L76227	U96639
<i>Phocoena phocoena</i>	Porpoise	AF061060	—
<i>Balaenoptera physalus</i>	Fin whale	—	X61145
<i>Sus scrofa</i>	Pig	S78431	AJ002189
<i>Bos taurus</i>	Cow	X63820, AF004285	J01393
<i>Equus (asinus/caballus)</i>	Horse	U31610	X79547
<i>Ceratotherium simum</i>	Rhino	U31604	Y07726
<i>Manis</i> sp.	Pangolin	U97535	U97340, U61079
<i>Mus domesticus</i>	Mouse	U27810	J01420
<i>Cavia porcellus</i>	Guinea pig	—	L35585
<i>Dasyprocta agouti</i>	Agouti	U31607	—
<i>Oryctolagus cuniculus</i>	Rabbit	U31618	AJ001588
<i>Ochotona princeps</i>	Pika	AJ224672	AF390540*
<u><i>Dugong dugong</i></u>	Dugong	U31608	U60185, AF179291
<u><i>Loxodonta africana</i></u>	African elephant	U31615	U60182, AF039436
<u><i>Elephas maximus</i></u>	Asian elephant	U31611	AF390541*
<u><i>Procavia capensis</i></u>	Hyrax	U31619	U97335, U60184
<u><i>Orycteropus afer</i></u>	Aardvark	U31617	U97338
<u><i>Elephantulus rufescens</i></u>	Elephant shrew	U31612	U97339
<u><i>Amblysomus hottentotus</i></u>	Golden mole	U97534	U97336
<u><i>Tenrec ecaudatus</i></u>	Tailless tenrec	AF390536*	AF390537*
<u><i>Echinops telfairi</i></u>	Lesser hedgehog tenrec	AF076478	AF069540
<u><i>Micropotamogale lamottei</i></u>	Nimba otter shrew	AF390538	AF390539*

Note. Afrotheria members appear underlined.

(1985), as developed in the program K2WuLi-1.0 (Jer-miin, 1996), and by the Unambiguous Parsimony Sites test (e.g., Mindell and Honeycut, 1990; Waddell *et al.*, 1999). Taxa showing rate heterogeneity were removed from the analysis (taxa retained for each of the loci were as follows: vWF: *Macropus*, *Bos*, *Loxodonta*, *Elephas*, *Procavia*, *Orycteropus*, *Elephantulus*, *Micropotamogale*, *Echinops*; 12S: *Macropus*, *Manis*, *Loxodonta*, *Elephas*, *Procavia*, *Orycteropus*, *Elephantulus*, *Micropotamogale*, *Echinops*, *Dugong*; 16S: *Cyclopes*, *Loxodonta*, *Elephas*, *Procavia*, *Orycteropus*, *Elephantulus*, *Micropotamogale*, *Echinops*, *Dugong*; 12S–16S: *Balaenoptera*, *Loxodonta*, *Elephas*, *Procavia*, *Orycteropus*, *Elephantulus*, *Micropotamogale*, *Echinops*, *Dugong*). The resulting pruned taxon set was then analyzed by ML (TN model with parameters estimated as above) under a clock constraint.

We used basal divergences among paenungulate orders (i.e., a hyrax–elephant split, chosen because vWF dugong was not clocklike) at 60 million years ago (MYA) (Amrine and Springer, 1999) as a calibration point for estimating cladogenic events within Tenrecidae. An estimate of variation on these dates was obtained by calculating the split using four different data sets: vWF, 12S rRNA, 16S rRNA, and a concatenation of 12S and 16S rRNA.

RESULTS AND DISCUSSION

Tenrecidae Monophyly

Both the 12S–16S rRNA and the vWF data sets resulted in high support for the monophyly of Tenrecidae (Fig. 1). For the vWF locus, support values (boot-

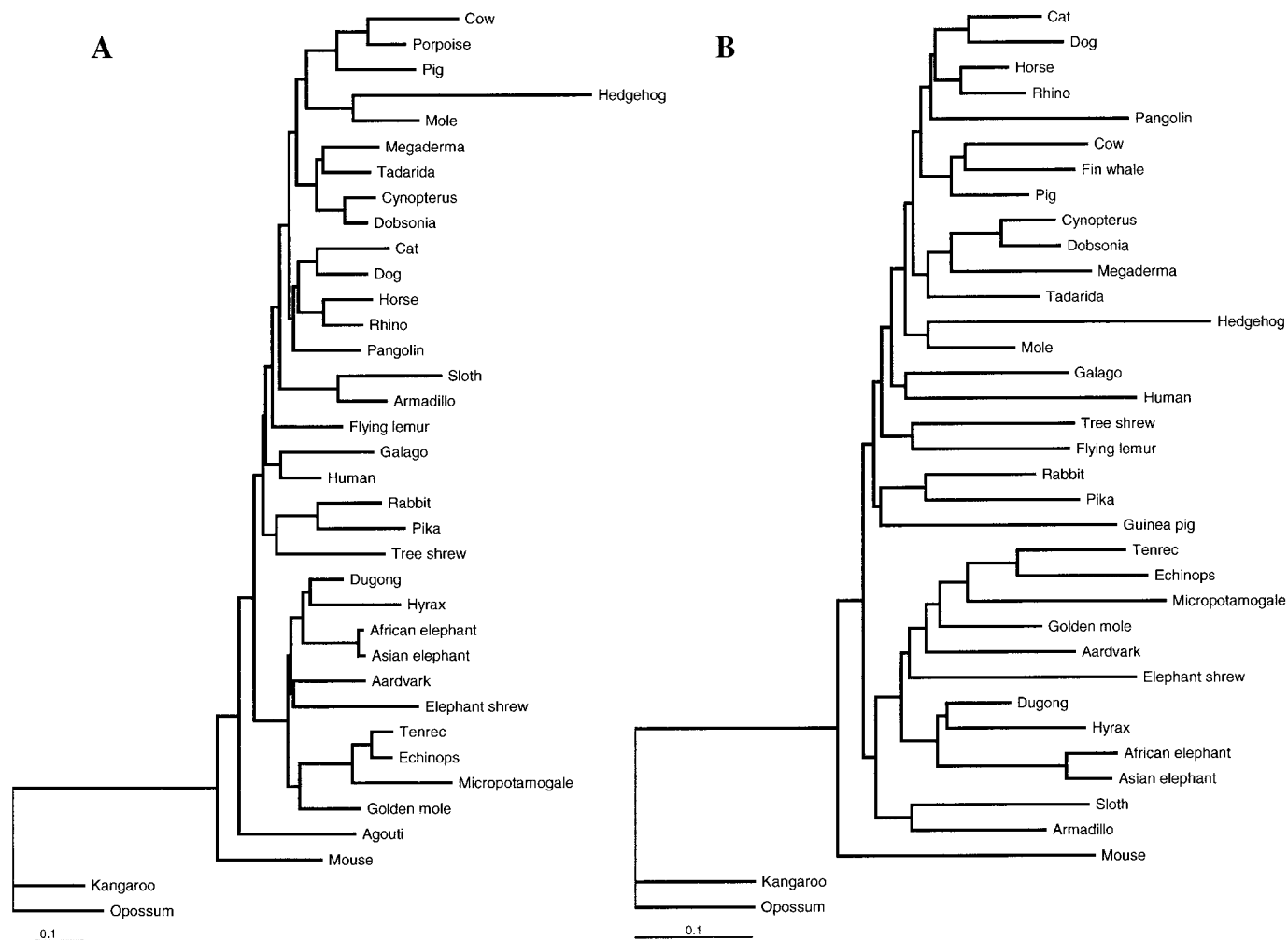


FIG. 1. Maximum-likelihood trees based on vWF (A) and 12S–16S (B) data sets. Topologies and branch lengths were determined under a TN model assuming rate variation across sites and employing a proportion of invariant sites.

strap and puzzle) for this clade ranged from 77 to 100%, with only the amino acid puzzle support value below 94% (Table 2). For 12S–16S rRNA, bootstrap support ranged from 80 to 95% (Table 2). Combining the two sets of data into a single concatenated alignment resulted in 100% bootstrap support for the monophyly of Tenrecidae. With the exception of KH-ML for vWF, statistical tests involving a single locus did not provide significant support for Tenrecidae monophyly (Table 2). However, the concatenated data set did provide significant support for tenrecid monophyly, both with MP and ML (Table 2). Asher's (1999) hypothesis of a paraphyletic Tenrecidae, wherein tenrecines are more closely related to golden moles than to other tenrecids, was rejected with the vWF and concatenated data sets (Table 2). Although statistical tests with the 12S–16S rRNA data set did not reject the Asher hypothesis, bootstrap support was minimal and ranged from 3 to 10%.

All of Asher's (1999) analyses recovered a sister

group relationship between *Limnogale* and potamogalines, which implies paraphyly of Malagasy tenrecs. This conclusion was mainly supported by a close Potamogalinae–*Limnogale* (subfamily Oryzorictinae) relationship, but also by an association of the remaining Oryzorictinae with this clade to the exclusion of tenrecines. The recent publication of a 12S rRNA gene sequence for *Oryzorictes* (Emerson *et al.*, 1999) allowed us to examine the naturalness of a Potamogalinae–Oryzorictinae grouping. After addition of this taxon to our 12S sequences (same selection of taxa as the 12S–16S analyses), MP, ME, and ML 12S rRNA bootstrap trees supported the monophyly of Tenrecidae (bootstrap support ranged from 47 to 71%) and an *Oryzorictes*–Tenrecinae clade (bootstrap support ranged from 65 to 80%). Data sets of 12S rRNA sequences confined to afrotherian species (Table 1), with two different sets of outgroups (two Xenarthra or two Chiroptera), recovered additional support for this *Oryzorictes*–Tenrecinae clade (Table 3). All methods of phylogenetic

TABLE 2

Tenrecidae Monophyly versus Asher's Paraphyletic (Tenrecinae + Chrysochloridae) Hypothesis

	Tenrecidae			Tenrecinae + Golden mole		
	12S-16S	vWF	Concat.	12S-16S	vWF	Concat.
Bootstrap						
DNA						
ME (GTR)	94	100	100	5	0	0
(Logdet)	95	100	100	3	0	0
MP	80	99	100	8	0	0
ML (TN)	85	94	100	10	1	0
ML (TN GI)	93	95	100	6	0	0
Protein						
MP	NA	99	NA	NA	0	NA
QP (Blosom 62)	NA	77	NA	NA	<50	NA
QP (Blosom 62 GI)	NA	77	NA	NA	<50	NA
Decay and statistical tests						
DNA						
MP Decay	8	19	31	-8	-22	-38
KH	0.588-0.634	0.189	0.026	0.588-0.634	0.046-0.050	<0.0001
T	0.495-0.520	0.223	0.025	0.495-0.520	0.049-0.052	<0.0001
WS	0.402-0.545	0.292	0.049	0.402-0.545	0.024-0.032	<0.0001
ML (TN) KH	0.399	0.000	0.000	0.509	0.005	0.000
ML (TN GI) KH	0.204	0.290	0.019	0.538	0.015	0.019
Protein						
MP Decay	NA	9	NA	NA	-13	NA
KH	NA	0.061	NA	NA	0.007-0.028	NA
T	NA	0.061	NA	NA	0.007-0.028	NA
WS	NA	0.093	NA	NA	0.011-0.043	NA

Note. Statistical test results for Tenrecidae correspond to acceptance of that hypothesis, whereas test results for Tenrecinae + Golden mole, refer to rejection.

reconstruction, regardless of the root, recovered this association with bootstrap support ranging from 86 to 100%. Bootstrap support for *Oryzorictes-Micropotamogale* was <1% in all analyses and a subset of statistical tests rejected this hypothesis (Table 3).

Timing and Biogeographic Events

The basal split between Potamogalinae and Tenrecinae was estimated at 53, 52, 53, and 51 MYA with vWF, 12S-16S, 12S, and 16S, respectively. Estimates

TABLE 3

Malagasy Tenrecid Monophyly versus Asher's Paraphyletic (Potamogalinae-Oryzorictinae) Hypothesis

	<i>Oryzorictes</i> + Tenrecinae		<i>Oryzorictes</i> + <i>Micropotamogale</i>	
	Xenarthra	Chiroptera	Xenarthra	Chiroptera
Bootstrap				
ME (GTR)	96	97	0	0
(Logdet)	96	98	0	0
MP	86	91	0	0
ML (TN)	99	99	0	0
ML (TN GI)	99	100	0	0
Decay and statistical tests				
MP Decay	+6	+6	-9	-12
KH	0.4605	0.2280	0.2078	0.0072-0.0640
T	0.4602	0.2278	0.6143	0.0073-0.0641
WS	0.2076	0.2913	0.2626	0.0118-0.0896
ML (TN)	0.2665	0.0548	0.1314	0.0340
ML (TN GI)	0.0371	0.0995	0.0379	0.0995

Note. Statistical test results for *Oryzorictes* + *Tenrecinae* correspond to acceptance of that hypothesis, whereas test results for *Oryzorictes* + *Micropotamogale*, refer to rejection.

for the *Tenrec-Echinops* split within Tenrecinae were more variable (18, 38, 30, 44 MYA). Adding *Oryzorictes* to the 12S data set resulted in a similar estimate for the split between Potamogalinae and other tenrecs at 55 MYA and placed the Oryzorictinae–Tenrecinae split at approximately 37 MYA. These dates are much older than the fossil record would suggest (i.e., the oldest tenrecids are Miocene; McKenna and Bell, 1997).

The separation of Madagascar from mainland Africa occurred between 120 and 165 MYA (Rabinowitz *et al.*, 1983), well before the origin of tenrecids. One or more dispersal events between Africa and Madagascar are thus required to explain the biogeographic history of tenrecids. Given that molecular data are compatible with both tenrecid monophyly and the monophyly of Malagasy tenrecs, the minimal requirement is a single dispersal event from Africa to Madagascar. Further, this event would have occurred after the potamogaline–Malagasy tenrec divergence (51 to 55 MYA) but before the radiation of Malagasy tenrecs (37 MYA based on the 12S rRNA data set that includes tenrecine and oryoricline representation). The mechanism by which tenrecids arrived in Madagascar remains unclear. McCall (1997) argued for a land bridge across the Mozambique Channel. According to this hypothesis, uplift along the Davie fracture zone created a filter bridge by which some terrestrial mammals were able to cross a deep channel with difficult currents, some time between the mid Eocene and the late Oligocene (45 to 26 MYA). The duration of the land bridge thus exhibits partial temporal overlap with the tenrecid dispersal window suggested by our molecular data.

Although molecular phylogenies only require a single dispersal event for living tenrecids, fossil data suggest the possibility of a more complex dispersal history. Specifically, the subfamily Geogalinae includes the living *Geogale* (Madagascar) and the extinct genus *Parageogale* from the early Miocene of Africa (McKenna and Bell, 1997). Butler (1985) proposed a sister taxon relationship between *Geogale* and *Parageogale*. If these taxa are indeed close relatives, then a second dispersal event is required to account for the biogeographic history of the Geogalinae. Depending on the relationship of Geogalinae to other tenrecids, the second dispersal may have been from Africa to Madagascar or vice versa. At present, molecular data do not address this hypothesis.

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