Tenrec Phylogeny and the Noninvasive Extraction of Nuclear DNA

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Abstract.—Due in part to scarcity of material, no published study has yet cladistically addressed the systematics of living and fossil Tenrecidae (Mammalia, Afrotheria). Using a noninvasive technique for sampling nuclear DNA from museum specimens, we investigate the evolution of the Tenrecidae and assess the extent to which tenrecids fit patterns of relationships proposed for other terrestrial mammals on Madagascar. Application of several tree-reconstruction techniques on sequences of the nuclear growth hormone receptor gene and morphological data for all recognized tenrecid genera supports monophyly of Malagasy tenrecids to the exclusion of the two living African genera. However, both parsimony and Bayesian methods favor a close relationship between fossil African tenrecs and the Malagasy Geogale, supporting the hypothesis of island paraphyly, but not polyphyly. More generally, the noninvasive extraction technique can be applied with minimal risk to rare/unique specimens and, by better utilizing museum collections for genetic work, can greatly mitigate field expenses and disturbance of natural populations. [Afrotheria; DNA; fossils; Madagascar; museums; phylogeny; Tenrecs.]

With the exception of a single genus of shrew (Suncus), insectivoran-grade mammals from Madagascar are members of the family Tenrecidae (Eisenberg and Gould, 1970; Olson and Goodman, 2003). This group of placental mammals consists of eight genera endemic to Madagascar and two from equatorial Africa and is remarkably diverse, occupying terrestrial, semi-terrestrial, fossorial, and semi-aquatic niches. Other Malagasy groups are similarly diverse; previous morphological investigations of its primates (Cartmill, 1975; Yoder, 1992), carnivorans (Veron, 1995), and rodents (Ellerman, 1940, 1941), as well as its tenrecs (Butler, 1984; Asher, 1999, 2000), have indicated multiple sister-group relationships with mainland taxa within each group.

Given the absence of modern taxa from the Malagasy Cretaceous and the isolation of Madagascar from other landmasses over the past ca. 80 to 90 million years (Krause, 2003), dispersal has become the primary hypothesis for explaining the arrival of many of Madagascar’s inhabitants (cf. Raxworthy et al., 2002; Zakharov et al., 2004). Phylogeny can further illustrate the biogeographic history of a given group. Monophyly (Fig. 1A) and paraphyly (Fig. 1B) of island taxa are compatible with a single dispersal event leading to island colonization, whereas polyphyly (Fig. 1C) implies multiple colonization events.

The aforementioned morphological studies noting the diversity of Malagasy mammalian groups have to varying degrees implied island polyphyly (Fig. 1C); i.e., that each of the modern groups has undergone multiple dispersal events across water barriers in order to colonize the island. In contrast, recent molecular phylogenies of terrestrial Malagasy mammals have supported island monophyly (Fig. 1A) for living primates (Yoder et al., 1996), carnivorans (Flynn et al., 2005), tenrecs (Olson and Goodman, 2003), and possibly rodents (Jansa and Weksler, 2004; Steppan et al., 2004; see discussion below).

Many tenrecid species are rare and/or endangered (Vogel, 1983; Benstead and Olson, 2003) and are difficult to obtain for research purposes. For example, the semi-aquatic Limnogale mergulus is known from barely over a dozen museum specimens in Europe and North America. Destructive sampling of such material (e.g., for DNA sequencing) is generally not possible. Because it is so difficult to obtain tissues, most molecular studies sampling this group (e.g., Emerson et al., 1999; Mouchaty et al., 2000; Douady et al., 2002; Malia et al., 2002) have included between one and five of the over two dozen species. Olson and Goodman (2003) described a much better sample and were the first to publish a study with representatives of all living tenrec genera, including sequences from one nuclear (vWF) and three mitochondrial (12S, tRNA-Valine, ND2) genes. However, as of this writing (August 2005), their DNA sequences and alignments are unavailable from public sources (e.g., GenBank). No published study has yet cladistically analyzed the three recognized fossil tenrecs, Erythrozootes, Protenrec, and Parageogale (Butler, 1984; McKenna and Bell, 1997). Jacobs et al. (1987) named a fourth fossil genus, Ndamathaia. However, we follow Morales et al. (2000) in regarding this taxon as a non-tenrec.

In this article, we provide new DNA sequence data from the nuclear growth hormone receptor (GHR) gene using a noninvasive procedure applied to museum specimens. We also include a morphological data set, enabling us to sample all recognized living and extinct tenrec genera. To reconstruct phylogenetic trees, we apply both maximum parsimony (MP) and a Markov k (Mk) model (Lewis, 2001) in a Bayesian framework (Nylander et al., 2004). Using these data we estimate the fit of living and fossil tenrecs to phylogenetic and biogeographic patterns proposed for other Malagasy groups.

Materials and Methods

The Noninvasive Extraction Method

We obtained between 756 and 855 base pairs from exon 10 of the growth hormone receptor (GHR) gene from crania accessioned at the Zoologisches Museum Berlin (ZMB), Harvard Museum of Comparative Zoology (MCZ), and the Department of Ecology and Evolution, University of Lausanne (IZEA). Specifically, we used skulls of Hemicyclorhynchus semispinosus (ZMB 71999),...
FIGURE 1. Biogeographic implications of (A) monophyly, consistent with a single dispersal event to colonize Madagascar (cf. Eisenberg, 1975; Olson and Goodman, 2003); (B) paraphyly, consistent with a single dispersal event coupled with limited back-migration from Madagascar to Africa (cf. Butler, 1985); and (C) polyphyly, consistent with multiple dispersal events between Africa and Madagascar (cf. Asher 2000: fig. R1-12). Dotted lines in B and C indicate uncertainty in the positions of Erythrozootes and Protenrec.

FIGURE 2. Lateral view of crania in Micropotamogale (top, IZEA 4975), Limnogale (middle, ZMB 35258), and Setifer (bottom, ZMB 44586), used for noninvasive extraction of nuclear GHR sequences. Images were taken after DNA extraction. Boxes highlight patent lacrimal foramen in Setifer, and absence thereof in Micropotamogale and Limnogale.

Limnogale mergulus (ZMB 35258; Fig. 2), Potamogale velox (ZMB 46588), Setifer setosus (ZMB 44586; Fig. 2), Geogale aurita (MCZ 45044), and Micropotamogale lamottei (IZEA 4975; Fig. 2). New sequences were aligned with previously published GHR sequences (Malia et al., 2002; Adkins et al., 2001; Pantel et al., 2000; van Garderen et al., 1999; Zogopoulos et al., 1999; Wang et al., 1995; Adams et al., 1990; Baumbach et al., 1989; Smith et al., 1989; Leung et al., 1987). Table 1 shows GenBank accession numbers for extant taxa, including DQ202287 to DQ202292, for our new sequences.

Expanding upon the method of Rohland et al. (2004) for mitochondrial DNA, we obtained nuclear GHR sequences from museum crania, leaving the treated specimens completely intact. We incubated either lower jaws or rostra in 20 mL of a buffer containing 5 M guanidinium isothiocyanate, 50 mM Tris, pH 8.0, 25 mM NaCl, 1.3% Triton-X, 20 mM EDTA, and 50 mM DTT. To minimize the possibility of damage, we incubated the specimens at room temperature and rotated them in near-vertical tubes that permitted flow of the buffer but kept specimens stationary. DNA was then eluted from the buffer and the specimens washed and dried as described in Rohland et al. (2004). The DNA was eluted in a final volume of 200 μL 1×TE. PCR amplification was done using 2 units of Taq Gold and 60 cycles under the conditions described in Hofreiter et al. (2002). Depending on the taxon, we used seven to nine primer pairs to amplify GHR sequences (Tables 2, 3). When possible, we designed at least one primer per primer pair that selected against human GHR sequence to avoid amplification of contaminating sequences.
TABLE 1. Taxon sample and accession numbers of taxa used in our sample of nuclear GHR sequences. Boldface indicates new GHR sequences; daggers indicate extinct taxa. For nomenclature we follow Nowak (1999) and Asher (2005).

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<th>Genus</th>
<th>Accession number</th>
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<td>Geogale</td>
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<tr>
<td></td>
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<td></td>
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</table>

The Netherlands) and multiple clones sequenced.

TABLE 2. Primer pairs for the amplification of the seven fragments used to determine GHR sequences in the six tenrecid species. Primer pairs are listed in Table 3. The length of the products is given in base pairs, including primers, n.p.: no product obtained.

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<thead>
<tr>
<th>Primer fragment</th>
<th>Sequence</th>
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<td>G AAT TCACAAGTATGACT CTG GG</td>
</tr>
<tr>
<td>R1g</td>
<td>GAAT GTG CATT ACAAC TAC TGG TAC</td>
</tr>
<tr>
<td>R1gap</td>
<td>AT CAT CCT CTC TCT CCA</td>
</tr>
<tr>
<td>R2a</td>
<td>GCT TCAATCT CAT TGC CTG</td>
</tr>
<tr>
<td>R2</td>
<td>CTG T AAT GAC TAC GAA TAC CT</td>
</tr>
<tr>
<td>R2g</td>
<td>G ATC RGA CAC ACA CAG RCT TCTAA</td>
</tr>
<tr>
<td>R2.2s right</td>
<td>TGT GAC TAT CTC GTC AAC GCA G</td>
</tr>
<tr>
<td>F3</td>
<td>GTGACATG TGGAT GTACCT CAG AGG TG</td>
</tr>
<tr>
<td>R3</td>
<td>A RGA CTC GAC TCA GAY CCA</td>
</tr>
<tr>
<td>F3g</td>
<td>ACA RAG GTT RAAAGG GAAAG</td>
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</tr>
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<td>R6gap2</td>
<td>R7 T GAT CAG TGT GTC TGT CAC</td>
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<tr>
<td>R7</td>
<td>R7 F7 GAAGAC CTC TAC TAC TGG T</td>
</tr>
<tr>
<td>R7 gap</td>
<td>R7 R7 short a</td>
</tr>
<tr>
<td>R7 gap2</td>
<td>T GAT CAG TGT GTC TGT C</td>
</tr>
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</table>

TABLE 3. Primers used to obtain tenrec GHR sequences.

<table>
<thead>
<tr>
<th>Primer fragment</th>
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</thead>
<tbody>
<tr>
<td>Flg</td>
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<td>GAAT GTG CATT ACAAC TAC TGG TAC</td>
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<tr>
<td>R1gap</td>
<td>AT CAT CCT CTC TCT CCA</td>
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<tr>
<td>R2a</td>
<td>GCT TCAATCT CAT TGC CTG</td>
</tr>
<tr>
<td>R2</td>
<td>CTG T AAT GAC TAC GAA TAC CT</td>
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<tr>
<td>R2g</td>
<td>G ATC RGA CAC ACA CAG RCT TCTAA</td>
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<td>R2.2s right</td>
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<tr>
<td>F3</td>
<td>GTGACATG TGGAT GTACCT CAG AGG TG</td>
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<tr>
<td>R3</td>
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<tr>
<td>R3g</td>
<td>TG GTC ATAAAA GTC GAT GTG T</td>
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<tr>
<td>Fl</td>
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<tr>
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<td>F4agtgagc GAGACC GAGAC GAGG</td>
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<td>CA CTG GAATAT CCC TGC TTAAAG</td>
</tr>
<tr>
<td>F5</td>
<td>F5 GAC TAT TAT GAC CAG GATACG GAG</td>
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<td>R5</td>
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<td>R7</td>
<td>R7 F7 GAAGAC CTC TAC CAC TAC TGG T</td>
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<tr>
<td>R7 gap2</td>
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</tr>
</tbody>
</table>

human DNA, ubiquitous in the environment (Hofreiter et al., 2001). Amplification of human sequences occurred regularly when it was not possible to select against human DNA, showing that not only mitochondrial but also nuclear human DNA is an abundant contaminant. Due to the variability of the GHR sequences, different primer pairs were used for the different species for some of the amplified fragments (Table 3). Amplification products were cloned using the TOPO TA cloning kit (Invitrogen, The Netherlands) and multiple clones sequenced.
The challenges confronting ancient DNA studies (Hofreiter et al., 2001; Olson and Hassanin, 2003) are relevant to this work, as we used museum specimens collected nearly 100 years ago. Hence, we used appropriate laboratory techniques at a dedicated ancient DNA facility at the Max Planck Institut for Evolutionary Anthropology, Leipzig.

Figure 3 shows sequence overlap between adjacent fragments for all taxa. Except for Pottergale, which has a 6-bp gap between fragments 3 and 4, all species have continuous sequences when the amplified fragments are concatenated after trimming the primers. Table S1 details sequence overlap across our amplified fragments, all of which are identical within species, both between different fragments and alternative amplicons that span homologous regions. Moreover, each amplified fragment excluding primers is unique in our data set, making it highly unlikely that our sequences are chimaeric (see Olson and Hassanin, 2003). When compared to the available sequences in GenBank by Blast searches (Table S2), all fragments show closest matches to members of the Afrotheria, and 38 out of 47 fragments are closest to published GHR sequences of the Tenrecidae. Given the occasionally short length of the fragments, slightly closer matches to other members of Afrotheria are not surprising. Finally, except for two fragments from Setifer setosus (which match corresponding sequences from Echinops AF392889), all others differ slightly from previously published sequences available in GenBank.

Sequence Alignment

Using MacClade 4.07 (Maddison and Maddison, 2000) and Clustal X (Thompson et al., 1997), we concatenated GenBank files, added new sequences, and constructed alignments that preserved reading frames and contained few indels. GHR shows several conserved regions that facilitate a priori homology assessment. Nevertheless, some ambiguity remains regarding the positions of certain indels and adjacent nucleotides. Exploration of alignment ambiguity has occasionally (e.g., Messinger and McGuire, 1998), but not always (e.g., Douady et al., 2003), led to revised phylogenetic interpretations. For this reason, we explore a limited number of alternative alignments, differences across which are summarized in Table S3. Confidence indices mentioned in the text, as well as statistical comparisons of alternative topologies, are based on the first alignment (with the addition of the morphological partition, as indicated below), unless stated otherwise. Topological results were not significantly altered by using the other three alignments.

Each series of internal (i.e., not leading or trailing), contiguous gap characters was assumed to represent a single insertion and/or deletion event (indel). For all of our analyses including sequence data, we coded indels for each alignment, adding them as binary characters following the aligned nucleotides. Actual gap characters interspersed among the aligned nucleotides were treated as missing data. In Bayesian analyses, indels were treated using the binary (restriction site) model without assuming that all presence/absence characters have been observed (MrBayes command “LSET CODING=VARIABLE”). Sequence alignments and other supplementary data are available online at http://systematicbiology.org.

Morphological Data Collection

We used an anatomical dataset consisting of 126 characters, 20 of which are from the soft-tissues of the rostrum and cranial arterial supply, 46 from the cranium, 30...
from the jaw and dentition, and 30 from the postcrania.

Olive and Goodman (2003) questioned two coding de-
cisions made by Asher (1999, 2000): occurrence of the fen-
estrate basisioccipital in Microgale and morphology of the
nasolacrimal duct (also known as the “lacrimal canal”) in
Limnogale. Olson and Goodman stated that Asher (1999)
coded both as absent, whereas they noted that a fen-
estrate basisioccipital occurs in some species of Micro-
gale (Asher [1999] sampled only M. talazaci) and stated
that Limnogale possesses a nasolacrimal duct. As of this
writing, M. talazaci remains the only species of Micro-
gale with nuclear DNA sequences available to us (Malia
et al., 2002). Hence, we still have a limited sample of
this genus, but accept Olson and Goodman’s (2003) ob-
servation and code the genus Microgale as polymorphic
for the nasolacrimal duct (character no. 35) in this
study.

Concerning the presence of a nasolacrimal duct in
Limnogale, this was in fact not the character cited as a
potential semiaquatic tenrec synapomorphy by Asher
(1999, 2000). Rather, absence of an external lacrimal for-
amen (character no. 53) was coded in both studies, as
depicted here in Figure 2. There is a clear ostego-
logical difference in the expression of a single, conspi-
cuous lacrimal foramen at the anterior margin of the orbit,
dorsal to the infraorbital canal, in most tenrecs (e.g.,
Setifer, Fig. 2). This region is smooth and without a major
foramen in both potamogalines and Limnogale (Fig. 2).
Hence, we retain the coding of Asher (1999, 2000) for
the present study (see also Sánchez-Villagra and Asher,
2002). Expression of a nasolacrimal duct was coded sepa-
ately from the lacrimal foramen in Asher (2000) based on
observations of soft tissue anatomy in histologically pre-
pared anatomical sections (Asher, 2001). To our know-
edge, no histological preparation of Limnogale has ever
been made, so we cannot compare the patent, partly
soft tissue nasolacrimal duct in most tenrecs and other
mammals with any such structure in Limnogale. Hence,
we code this character (no. 15) “missing” for Limno-
gale in our morphological data matrix. Coding these
two characters (duct, foramen) independently is justi-
fied by the variable expression of the lacrimal foramen
in taxa with a patent nasolacrimal duct (e.g., Frahnert,
1999).

Phylogenetic Inference

The search strategies described below were applied to
each of the four alignments summarized in Table S3 using
a 43-taxon data set sampling only GHR and a 23-taxon
data set sampling GHR plus morphology.

MP analyses were undertaken with PAUP 4.0b10
(Swofford, 2002); Bayesian algorithms were applied with
MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). For
the full GHR taxon sample, our MP analyses searched
heuristically with at least 100 random addition replicates
with TBR branch swapping, multiple states treated as
polymorphic, and branches with a zero length under any
optimization collapsed. For our Bayesian and likelihood
bootstrap analyses, we used the HKY+I+G model for
the full GHR taxon sample (Fig. 4) and GTR+I+G as the
optimal model for the smaller GHR sample (Fig. 5), as
indicated by the AIC in MrModelTest 2.1 (Nylander,
2004). We used the default PAUP commands given in MrMod-
test (“Dist stability”=JC objective=ME base=equal
rates=equal pinv=0 subst=all negbrlen=setzero; NJ
showtree=no breaks=radius=asinh”) to obtain an initial
tree, used by PAUP to estimate maximum likelihood
(ML) parameters for the likelihood bootstrap analysis of
the 43-taxon GHR data set (which resulted in the values
“LSet Base=[0.2547 0.3078 0.2299] Nst=2 TRatio=2.1473
Rates=gamma Shape=1.7732 Pinvar=0.1990”). In addi-
tion, the ML bootstrap analysis excluded indels, used 143
pseudoreplicates of an “as-is” addition sequence with
TBR branch swapping, and obtained starting trees with
stepwise addition.

Bayesian analyses of the larger GHR dataset were
based on at least four independent runs, each using a
random starting tree and 1,000,000 generations with one
cold and three heated chains, sampling trees every 100
generations. Bayesian runs of the 43-taxon GHR and
the 23-taxon combined morphology-GHR data sets both
reached stationarity between approximately 10,000 to
14,000 generations, as determined by visually inspecting
asymptotic graphs of likelihood scores across genera-
tions. Our phylogenetic conclusions are based on only
succeeding generations, starting at 15,000, discarding the
first 14,900 (sampling every 100th generation) as “burn-
in.” Each run of 1,000,000 generations converged on a
single, consistent result.

The taxon sample for the smaller, combined GHR-
morphology data set was chosen based on availability
of GHR sequences as well as osteological and soft tis-
sue data from Asher (2000, 2001; see also Appendix 1).
This sample included all genera of tenrecs, including fos-
sils, plus Orctopus afer, Procavia capensis, Elephantulus
brachyrhynchus, a composite golden mole (Chrysosopala
trevelyani GHR, Chryoschoris stuhlmanni and C. asiatica
morphology), Erinaceus europaeus, three soricids, and Can-
is latrans, and was rooted with a composite didelphid
(Monodelphis domestica GHR, Didelphis sp. morphology).
Most soft tissue characters remain missing for two of the
extant tenrecs: Limnogale and Oryzorictes.

Combined and GHR-only MP analysis of the smaller
dataset used the same search parameters as described
above, leaving DNA entries missing for the three fos-
sil taxa. MP bootstrap support values were generated
with 1,000 pseudoreplicates, each with 10 random ad-
dition replicates and TBR branch swapping. Bayesian
search parameters for the smaller GHR dataset were
as described above. In the combined Bayesian anal-
ysis of morphology and sequences we used different
models for each partition: the GTR+I+G for
sequences following the AIC in MrModelTest (Nylander
2004), the binary (restriction site) model for indels
(with LSET CODING=VARIABLE), and Mk for mor-
phology following Lewis (2001) and Nylander et al.
RESULTS

Affinities of Living Tenrecs

Parsimony, likelihood, and Bayesian methods applied to our GHR data consistently supported the monophyly of Malagasy tenrecs to the exclusion of the two living African genera with high support indices (Fig. 4). In each case, regardless of the alignment (Table S3) or algorithm used, and in agreement with Olson and Goodman (2003), *Limnogale* was closely related to *Microgale*, and potamogalines were reconstructed as the sister group to other living tenrecs. The extant Malagasy tenrec clade consisted of two radiations: spiny tenrecs (Tenrecinae) and soft tenrecs (Oryzorictinae plus *Geogale*). Less clear were the positions of *Geogale* and *Oryzorictes* within the soft-tenrec clade, and of *Hemicentetes* and *Tenrec* within the spiny tenrec clade.

Bayesian analysis of sequence data alone favors *Oryzorictes* at the base of a soft-tenrec clade, contradicting oryzorictine monophyly (Fig. 4). However, Bayesian support for a soft-tenrec clade excluding *Oryzorictes* ranged from 54 to 58 across the four alignments; and trees produced by MP for each of the four alignments left *Oryzorictes* and *Geogale* unresolved at the base of this clade (Fig. 4). Furthermore, we cannot statistically reject a monophyletic Oryzorictinae with *Geogale* as its sister taxon (Table 4). In contrast, statistical comparisons based on the GHR-only and combined datasets reject any sister-group relation between the semiaquatic Malagasy *Limnogale* and African potamogalines (Table 4).

Application of MP to the morphological dataset yields optimal trees similar in some regards to those generated by sequences alone, such as monophyly of tenrecids and potamogalines, support for a spiny tenrec clade, and a
sister taxon relationship between *Echinops* and *Setifer*. However, in contrast to the GHR signal, morphological data support the position of African potamogalines near *Limnogale* (Fig. 6). This relationship appears in most of the optimal trees in the combined MP analysis, but is unresolved in the strict consensus. Nevertheless, a *Limnogale*-potamogaline clade is supported by MP applied to the living taxa alone with a bootstrap value of 71 (not figured; see also Asher, 1999), and by Mk applied to the morphological dataset including fossils (Fig. 6). Using the morphological dataset alone, the alternative topologies summarized in Table 4, including variants that preserve monophyly of Malagasy tenrecs and a *Limnogale*-Microgale clade, are rejected by Templeton and winning sites tests.

**Affinities of Extinct Tenrecs**

Application of MP to the combined dataset including the three fossil tenrecs, regardless of alignment (Table S3) or analysis parameters, supports a *Parageogale-Geogale* clade with relatively high confidence, with MP-bootstrap support values (89) comparable to that for potamogalines (87; see Fig. 5). Bayesian analyses of the combined dataset also supported this clade, but with a posterior probability (80) weaker than that for potamogalines (98).

*Erythrozootes* and *Protenrec* are also reconstructed together, in turn adjacent to *Geogale-Parageogale*, regardless of alignment or tree-building technique. However, support indices for this clade are much lower (posterior probability 67, MP bootstrap below 50), as are the supports for a clade joining the three fossil taxa with *Geogale* (posterior probability 76, MP bootstrap below 50; see Fig. 5).

Several morphological characters support a *Parageogale-Geogale* clade, which, following Butler (1984) may be referred to the Geogalinae. First, the reduction of its upper molar metacone (character no. 73, state 2), protocone (no. 74, state 1), and of the lower molar talonid (no. 85, state 1) favor its placement with other dentally zalambdodont taxa (i.e., in this sample, tenrecs and golden moles; see Asher and Sánchez-Villagra [2005] for a definition of anatomical zalambdodonty). *Parageogale* and *Geogale* share a highly reduced maxillary process of the zygoma (no. 59, state 1; also present in soricids). Geogalines also possess a broad
gap between the anterior central incisors (character no. 126, state 1), a condition also seen in Erinaceus and in some specimens of Setifer (here coded as polymorphic). They also have two premaxillary teeth (no. 67, state 2; also present in some tenrecines and Erythrozoozoa). In contrast to the other nine tenrecid genera, fossil tenrecs plus Geogale possess a relatively long infraorbital canal (no. 60, state 0).

Templeton and winning sites tests based on MP reject alternative hypotheses placing all three fossils either outside of living Tenrecidae or together as the sister-clade to African potamogalines (Table 4). However, another alternative, placing Parageogale as the sister-taxon to a (potamogaline (Protenrec Erythrozoozoa)) clade, again with all African tenrecs outside of the Malagasy radiation (Fig. 1A), cannot be rejected.

Additional Tests of Fossil Tenrec Phylogeny

All three fossil tenrec genera were first described from the Kenyan Miocene (Butler and Hopwood, 1957) and remain known only from a few craniodental fragments (Butler, 1984). Published reviews including these taxa have generally supported their affinity to modern tenrecids (Butler, 1969, 1978, 1984, 1985; McKenna and Bell, 1997; Mein and Pickford, 2003; but see Poduschka and Poduschka, 1985). As is the case for other fossils over 1 million years in age, sequence data cannot be obtained from these specimens (Hofreiter et al., 2001). Of the 126 characters sampled in our morphological matrix, Erythrozoozoa and Protenrec are 24% complete and Parageogale is ca. 18% complete. Nevertheless, the most poorly known taxon in this study, Parageogale, shows a relatively well-supported position, consistent with the hypothesis originally presented by Butler and Hopwood (1957) that it is the sister-taxon to the living Geogale aurita, and contradicting the monophyly of the Malagasy radiation (Fig. 1A).

To test the hypothesis that the 22 characters sampled for Parageogale can accurately reconstruct its phylogeny, we used these same characters to reconstruct the phylogeny of other tenrecs in our study. That is, for each of the 10 living tenrecid genera, we replaced GHR data and all morphological characters, except for the 22 known for Parageogale, with missing entries and ran the modified morphology + GHR dataset using MP, as described above in Materials and Methods. Stated differently, if a living tenrecid genus had gone extinct in the early Miocene, and its phylogenetic position? If the respective extant taxon was sampled only for the 22 Parageogale characters appears in a different part of the tree relative to its position in the full analysis, we would have less confidence in the placement of Parageogale.

In fact, the reduced dataset did not greatly change the position of any extant tenrec (Fig. 7). Out of the 10
FIGURE 7. Results of reduced-character MP analyses of each of the 10 living tenrecid genera (as identified in boldface), with all GHR sequences and morphological characters, except for the 22 known for Parageogale, coded as missing. Matrices with either Echinops or Setifer coded in this fashion yield the same topology (top left), also identical to the combined-data topology depicted in Figure 5. Each tree represents either a strict consensus or a single, most-parsimonious result, as follows:

- **Echinops**: 1 tree 1,539 steps.
- **Geogale**: 3 trees 1,491 steps.
- **Hemicentetes**: 4 trees 1,517 steps.
- **Limnogale**: 3 trees 1,521 steps.
- **Microgale**: 4 trees 1,521 steps.
- **Micropotamogale**: 1 tree 1,520 steps.
- **Oryzorictes**: 7 trees 1,515 steps.
- **Potamogale**: 1 tree 1,511 steps.
- **Setifer**: 1 tree 1,543 steps.
- **Tenrec**: 2 trees 1,522 steps.

Numbers adjacent to nodes represent MP bootstrap support values (100 pseudoreplicates of a simple addition sequence). Bootstrap values in tree at top left for Echinops are listed above nodes, Setifer below.
modified datasets (1 for each living tenrecid genus), 2
(Echinops and Setifer) yielded the same tree as the full
sample, and 6 of the remaining 8 yielded varying de-
grees of nonresolution in multiple shortest trees, con-
sensuses of which (Fig. 7) were still compatible with
the topology supported by the full dataset. Only two
cases (Tenrec and Potamogale) yielded optimal trees with
a slightly different topology. The former altered relations
within spiny tenrecs (supporting Tenrec-Setifer rather
than Tenrec-Hemicentetes), and the latter reconstructed
Oryzorictes adjacent to Microgale-Limnogale to the ex-
clusion of Geogale, preserving oryzorictine monophyly.
However, Tenrec bootstrap resampling still supports a
spiny-tenrec clade with a value of 79; and the Tenrec-
Setifer clade has an MP bootstrap support value under 50.
Similarly, for the run using a reduced sample for Potamo-
gale, oryzorictine monophyly is supported with an MP
bootstrap of just 57, and the unmodified, combined-data
sample cannot reject this hypothesis (Table 4). In these
and other cases, bootstrap resampling generally yielded
lower support values compared to the full sample (cf.
Fig. 5 versus Fig. 7), but in no case did a clade pro-
duced by a reduced-sample analysis contradict a well-
supported clade in the full sample.

Discussion

Data Combination and Tenrec Phylogeny

A previous morphology-based investigation of tenre-
cid phylogeny published by one of us (Asher, 1999) ar-
gued for a clade of semiaquatic tenrecs, placing Malagasy
Limnogale as the sister-taxon to continental African pota-
mogalines. Character support for this clade was primarily
from the skull, including a fenestrate basioccipital (no.
35 in this study), a shortened frontal bone (no. 61), and a
reduced lacrimal foramen (no. 53). Importantly, none of
the other character states are consistently found in nontenre-
cid, semiaquatic, faunivorous, small mammals (Sánchez-
Villagra and Asher, 2002), a factor that had previously
led Asher to view the “semiaquatic” tenrec clade with
increased confidence.

As discussed above, morphological data analyzed
alone still yield some support for a semiaquatic clade, al-
though recoding fenestration in the basioccipital to ac-
count for polymorphism in Microgale (as recommended
by Olson and Goodman, 2003) has eliminated this char-
acter from optimizing unambiguously as a Limnogale-
potamogaline synapomorphy. Furthermore, compared
to the study of Asher (1999), the larger number of char-
acters and sampled tenrecs in this study yields reduced
support for a semiaquatic tenrec clade (Fig. 6).

However, the key reason for the nonrecovery of
a semiaquatic clade in this study is the very strong
sequence-based signal favoring a Limnogale-Microgale
clade. Indeed, with their sample of different loci for mul-
tiple species of Microgale, Olson and Goodman (2003)
found that Limnogale actually nests within that genus,
comprising the sister-taxon to an M. dobsoni-M. talazaci
clade to the exclusion of other Microgale species. The
strength of the signal supporting a Microgale-Limnogale
clade in our study (100 MP bootstrap, 100 ML bootstrap,
and 100 Bayesian posterior probability in the GHR-only
analysis [Fig. 4]; 95 MP bootstrap and 94 Bayesian poste-
rior probability in the combined analysis [Fig. 5]) has con-
vincing both of us that the previous interpretation of
the morphological signal as indicative of a semiaquatic ten-
rec clade (Asher, 1999) is incorrect. Due to this unambigu-
ous support from GHR sequences, which is considerably
stronger than that from morphology alone for a semi-
aquatic clade and which prevails in the combined analy-
sis, the cranial characters supporting the “semiaquatic”
clade cited above must be reinterpreted as homoplastic.

If the morphological data used here are misleading
regarding a semiaquatic tenrec clade, why do we then
combine them with our GHR data? The most important
reason for retaining morphology in our dataset is one
of principle: most individual datasets are not in their en-
tirety either “true” or “false”; but are themselves mosaics
of variable character-data that may provide resolution at
different levels in any given tree (Gatesy et al., 2003).
Combined data sets enable recognition of phylogenetic
signals that would remain obscure with the analysis of
subdivisions thereof (Gatesy et al., 1999, 2005). Further-
more, including morphological data in the combined
analysis remains the best means to sample fossil ten-
recs. We cannot be completely sure that the morphology
known for these fossils enables us to accurately under-
tend their phylogenetic history. However, as discussed
above, when used in simulations to replace the complete
morphology-GHR dataset for each of the 10 living ten-
recid genera, the morphological characters known for
the most incomplete of the fossils (Parageogale) yield re-
results that are largely congruent with the combined-data
topology.

Character Assessment and Hindlimb Function

in Potamogale

One recent study of hindlimb characters (Salton and
Szalay, 2004) has also argued for the inclusion of
Limnogale within the Malagasy radiation. By assess-
ing characters of the tarsal complex in an “ecological
and evolutionary framework,” Salton and Szalay pro-
posed to identify phylogenetically informative charac-
ters: “traits with clear species-specific adaptations are
a potential interference in cladistic analyses and can-
not be meaningfully used without ecology-based charac-
ter assessment” (Salton and Szalay, 2004:73). In regards
to the “semiaquatic” clade, their procedure resulted in
the identification of anatomical differences (e.g., astrag-
alar neck-head transition) and similarities (e.g., medi-
ally directed tibial-fibular malleoli) between Limnogale
and Potamogale (they did not include Micropotamogale
in their analysis). In their opinion, the former comprise phy-
logenetic data in support of the “family level distinction”
of Potamogale from other tenrecs, and the latter are inter-
preted as homoplastic.

However, we are concerned that Salton and Szalay
(2004) did not identify a replicable optimality criterion
(e.g., MP, ML) by which they reached their conclusions
on homology. Furthermore, we believe that Salton and Szalay have not fully appreciated the function of the hindlimb in *Potamogale*. Regarding its locomotion, Salton and Szalay refer to its "heavy foot thrusts" (p. 90), and note that "heavy loading in the UAJ [upper ankle joint]... and UAJ stabilization play an important role... in the aquatic locomotion of *Potamogale*" (p. 86). In regards to calcaneal morphology, Salton and Szalay state that "*Potamogale* has an extremely long and narrow calcaneus with a long tuber, appropriate for strong, dorsolateral aquatic propulsion" (p. 93). In fact, these inferences of locomotion run counter to published descriptions of locomotor behavior in *Potamogale* (e.g., DuChaillu, 1860; Kingdon, 1974), which indicate that it uses its massive tail, not its feet, for aquatic propulsion. As in the other two potamaline species (*Microptamogale lamottei* and *M. neumsmori*), digits II and III of the hindfoot in *Potamogale* are syndactyly, and their use in grooming has been documented (Nicoll, 1985; Kingdon, 1997). As summarized by Nowak (1999), the relatively small, nonwebbed pes of *Potamogale* is tucked under its pelvic region during swimming and is not used for propulsion. Dobson (1883:97–98) infers from its anatomy that during locomotion, "the sole [of the foot] lies so evenly against the [pelvic ventrum] as to present the least possible projection and interfere in the least degree with the rapid passage of the body through the water, propelled by the powerful tail... [The tail] is doubtless the sole organ of propulsion." Based on field observations, Kingdon observed that in the water, "the animal is propelled entirely by lateral movements of the back and tail" (Kingdon, 1974:15). In contrast, *Limnogale* (the "web-footed" tenrec), has been observed to use its hindlimbs for semi-aquatic propulsion (Benstead and Olson, 2003:1272). Despite this, and without presenting new behavioral data for either taxon, Salton and Szalay (2004:100) propose the opposite: "...the tarsal complex indicates that [*Limnogale*']s hind limbs are less important for propulsion than those of *Potamogale*.'

Hence, we remain skeptical about Salton and Szalay's method for distilling phylogenetically informative data from their morphological observations. Although we agree with them that *Limnogale* is not more closely related to *Potamogale* than to other Malagasy tenrecs, contra Asher (1999), we do not believe they presented in their paper a basis for reaching this conclusion, independent of the sequence data analyzed by Olson and Goodman (2003), and confirmed with additional data in this article.

**Tenrec Biogeography**

Considering the living radiation alone, Malagasy tenrecs show substantial morphological diversity, yet are recognized as a single radiation by sequence data, as observed for primates (cf. Yoder, 1992, versus Yoder et al., 1996) and carnivorans (cf. Veron, 1995, versus Flynn et al., 2005). Similarly, our results support a cohesive Malagasy tenrec radiation and argue against Malagasy tenrec polyphyly. However, the living tenrecid radiation is not a complete picture of this group's diversity. Although its paleontological record is meager, fossil African tenrecids appear to have a close relationship with living *Geogale*. This relationship makes the Malagasy tenrec radiation paraphyletic (Figs. 1B, 5).

A similar phylogenetic scenario was presented by Jansa et al. (1999) for Malagasy nesomyine rodents. Based on cytochrome b sequences for multiple representatives of all genera of this group, Jansa et al. (1999) disputed previous interpretations of polyphyly (Ellerman, 1940, 1941), but argued that two mainland African genera (*Steatomys* and *Tachyoryctes*) nested within the Malagasy radiation. They suggested that this phylogenetic pattern would be consistent with colonization of Madagascar by nesomyines via a single founder event, followed by dispersal to Africa from Madagascar. The inclusion of mainland African muroids within the Malagasy radiation has subsequently been questioned (Steppan et al., 2004; Jansa and Weksler, 2004); and nesomyine monophyly remains possible. A definitive conclusion must await a study that synthesizes the taxon and character samples discussed by Jansa et al. (1999), Jansa and Weksler (2004), and Steppan et al. (2004).

Monophyly of Malagasy tenrecs is also possible. We have at present no way of knowing how the missing GHR nucleotides for *Parageogale*, or characters from its still unknown skeleton, would affect our estimate of its relationships. Some uncertainty regarding our results supporting paraphyly (Fig. 1B) is reflected in the nonrejection of at least one alternative topology that preserves Malagasy tenrec monophyly (Table 4); and we eagerly anticipate how this result is affected by future discoveries of better-preserved fossil tenrecid material. Nevertheless, the current hypothesis of a *Parageogale-Geogale* clade has support from both MP and Bayesian methods (Fig. 5). Furthermore, as discussed above, the limited morphological sample available for *Parageogale* appears to perform fairly well when these same characters are used to reconstruct the phylogeny of each of the ten living tenrec genera, a result that slightly increases our confidence in the placement of this fossil.

As stated in the introduction, the absence of modern mammalian orders from Madagascar (and elsewhere) during the Mesozoic (Krause, 2003), during which time land connections existed with mainland Africa (until the late Jurassic) and India (until the early Late Cretaceous), has led many to favor dispersal as the prime mechanism by which modern mammals colonized Madagascar (e.g., Olson and Goodman, 2003; Yoder et al., 2005). Repeated monophyly of Madagascar's endemic radiations is consistent with dispersal, as individual colonization events are hypothesized to be rare, and a previously unpopulated island may have open adaptive zones into which a founder can radiate into a diverse clade.

Nonmonophyly is also compatible with dispersal, but requires more (potentially unparsimonious) crossings of a geographic barrier, in this case the Mozambique channel. No one will ever know exactly how or why the tenrec crossed the channel; but based on our phylogeny we can estimate how often such an event took place. Given the combined-data tenrec phylogeny presented in
Figure 5, we hypothesize that a single founder event of Madagascar by the common ancestor of Malagasy tenrecs took place at some point after the Maastrichtian, during which time a diverse vertebrate fauna shows no sign of Madagascar’s modern inhabitants (Krause, 2003). Prior to the Miocene, when fossil tenrecs were present in east (Butler, 1984) and southwest (Mein and Pickford, 2003) Africa, an additional dispersal of an animal related to the geogaline common ancestor took place from Madagascar to continental Africa. The position of Erythrozooidea and Protenrec (in the Protenrecinae of Butler [1984]) as sister taxa to Geogale-Parageogale implies that a protenrecine relative made this back-migration yet again.

However, we note that a Protenrec-Erythrozooidea clade to the exclusion of geogalines has an unimpressive MP bootstrap value below 50 and a Bayesian posterior probability of 3 (Fig. 5). An alternative hypothesis, placing protenrecines as the sister clade to Parageogale within Geogalinae, would require just a single Madagascar-Africa dispersal event postdating the initial Madagascar colonization. This alternative is just two steps longer in MP analyses and cannot be statistically rejected (Table 4). As stated above, we are cognizant of yet another alternative that preserves Malagasy tenrec monophyly (Fig. 1A), also statistically unrejected in Table 4. This scenario would require only a single colonization event of Madagascar by tenrecs, with no back-migration, again at some point after the Late Cretaceous.

Nevertheless, the optimal explanation of the data presented in this article supports paraphyly of Malagasy tenrecs relative to their mainland relatives (Figs. 1B, 5), not monophyly (Fig. 1A) or polyphyly (Fig. 1C). DNA sequence data for extinct, pre-Pleistocene tenrecs will probably never be available; and even for certain living taxa, in particular Geogale and Limnogale, such data are very difficult to obtain. Our technique for sequencing nuclear DNA from museum specimens without damaging them eases this constraint, can be applied to other groups, and greatly reduces fieldwork expense and disturbance of living populations otherwise necessary for obtaining research material. This highlights yet further the value of museum collections for basic science (Suárez and Tsutsui, 2004).

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APPENDIX 1

List of osteological specimens examined. The geographic provenance of specimens is listed in parentheses following the taxon name. Crosses denote extinct taxa; asterisks denote taxa sampled for soft tissue characters using an uncataloged collection of histological specimens (see table 2 of Asher, 2001). Institutional abbreviations are as follows:

AMNH, American Museum of Natural History, New York
BMNH, The Natural History Museum, London
FMNH, Field Museum of Natural History, Chicago
IZEA, Institut de Zoologie et d’Ecologie Animale, Lausanne
MCZ, Museum of Comparative Zoology, Harvard University
MHNH, Museum Nationale d’Histoire Naturelle, Paris
USBA, USA University at Stony Brook, Department of Anatomical Sciences
USNM, United States National Museum, Washington
ZMB, Zoologisches Institut, Universität Tübingen

1. *Didelphis* sp. (Mexico, Nicaragua, USA): AMNH 28408, 28962, 29255, 70082, 145630, 146551, 148959, 201327; USBA MMr1, MMr4, MMr5


3. **Erinaceus europaeus** (France, Italy, England, Germany): AMNH 3770, 42561, 42563, 57219, 70613, 140469, 140470, 160470, 201230, 215299; USNM 251763, 251764, ZIUT M140

4. **Pentorrecus capensis** (Kenya, Zaire, Central African Republic, South Africa): AMNH 53777, 53781, 53784, 53785, 83411, 83412, 80997, 80998, 80999, 88418; USBA Mhy1, Mhy4, Mhy5

5. **Setifer setosus** (Kenya, Zaire, Cameroon, Congo, Gabon): AMNH 51161, 51162, 51164, 51165, 51319, 51322, 51324, 51334, 51344, 51348, 51368, 55203, 55204, 120250, 240968; USNM 266897, FMNH 5637, 5639, 5640, 5641, 5642, 5643, 5644, 5645, 5646, 5647, 5648, 5649, 5650, 5651, 5652, 5653, 5654, 5655, 5656, 5657, 5658, 5659, 5660, 5661, 5662, 5663, 5664, 5665, 5666, 5667, 5668, 5669, 5670, 5671, 5672, 5673, 5674, 5675, 5676, 5677, 5678, 5679, 5680, 5681, 5682, 5683, 5684; ZIUT 3860, 3861

8. **Erinaceus europaeus** (France, Italy, England, Germany): AMNH 3770, 42561, 42563, 57219, 70613, 140469, 140470, 160470, 201230, 215299; USNM 251763, 251764, ZIUT M140

9. **Pentorrecus capensis** (Kenya, Zaire, Central African Republic, South Africa): AMNH 53777, 53781, 53784, 53785, 83411, 83412, 80997, 80998, 80999, 88418; USBA Mhy1, Mhy4, Mhy5

10. **Setifer setosus** (Kenya, Zaire, Cameroon, Congo, Gabon): AMNH 51161, 51162, 51164, 51165, 51319, 51322, 51324, 51334, 51344, 51348, 51368, 55203, 55204, 120250, 240968; USNM 266897, FMNH 5637, 5639, 5640, 5641, 5642, 5643, 5644, 5645, 5646, 5647, 5648, 5649, 5650, 5651, 5652, 5653, 5654, 5655, 5656, 5657, 5658, 5659, 5660, 5661, 5662, 5663, 5664, 5665, 5666, 5667, 5668, 5669, 5670, 5671, 5672, 5673, 5674, 5675, 5676, 5677, 5678, 5679, 5680, 5681, 5682, 5683, 5684; ZIUT 3860, 3861

NOTE: We wish to acknowledge the recent study of Poux et al. (2005), published after the completion of this paper, on the colonization of Madagascar by terrestrial mammals. Poux et al. sampled the most Recent genera of tenrecs (except Potamogale and Geogale), plus a large sample of other endemic Malagasy genera, and report a tenrec phylogeny congruent with that discussed here and by Olson and Goodman (2003), for example in supporting a Limnogale-Microgale clade. However, they did not address the phylogeny of fossil taxa.