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Phylogeny and evolution of Malagasy plated lizards

Achille P. Raselimanana^a, Brice Noonan^{b,*}, K. Praveen Karanth^c, Jacques Gauthier^d, Anne D. Yoder^b^a Département de Biologie Animale, Université d'Antananarivo, BP 906, Antananarivo (101), Madagascar and Association Vahatra, BP 3972, Antananarivo (101), Madagascar^b Department of Biology & BAA, Duke University, Box 90338, Durham, NC 27708, USA^c Centre for Ecological Sciences, Indian Institute of Science, Bangalore 560012, India^d Department of Geology and Geophysics, Department of Ecology and Evolutionary Biology, and Peabody Museum of Natural History, Yale University, New Haven, CT 06520, USA

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ABSTRACT

The endemic plated lizards (Gerrhosauridae) of Madagascar are one of the most diverse groups of lizards on the island (19 species) and are found in all ecoregions. On an island that presents so many interesting biological questions, plated lizards are an ideal group for examining patterns of diversification due to their high (but tractable) diversity and wide distribution. To resolve the taxonomy and evolutionary relationships of Malagasy plated lizards, and to explore their biogeographic history, we have generated a molecular phylogeny based on >2 kb of both mitochondrial (cob, 1142 bp) and nuclear (NT-3, 419 bp; c-mos, 542 bp) DNA sequences. Phylogenetic relationships and divergence-time estimates indicate Zonosaurinae are monophyletic, arising from a single colonization event, likely from Africa to Madagascar in the Paleogene (Paleocene/early Eocene), with subsequent radiation. Furthermore, our results reveal widespread mischaracterization of species delimitation and assignment based on phenotypic characteristics. Paraphyly of a number of zonosaurine species was strongly supported by our dataset, revealing not only instances of likely cryptic species (phenotypic conservatism) but also phenotypic/genotypic discordance in the delimitation of species (phenotypic variability). These results clearly demonstrate the complex history of biotic diversification on Madagascar and provide novel insight into biogeographic patterns on the island.

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1. Introduction

The plated lizards (Gerrhosauridae) are a diverse group of scincomorph squamates currently restricted to sub-Saharan Africa and Madagascar. For more than a century, systematic study of the Gerrhosauridae has focused less on scientific questions about phylogenetic interrelationships and more on aspects of species delimitation and Linnaean categorical ranks. On Madagascar, this group is represented by two genera (*Tracheloptychus* [2 species] and *Zonosaurus* [17 species]) that together form the Zonosaurinae (Lang, 1991). Although recent collection efforts have significantly increased the number (+33% since 1989) of recognized zonosaurine taxa (Meier, 1989; Lang and Böhme, 1989; Glaw and Vences, 2007; Raselimanana, 2000; Raselimanana et al., 2000), the relationships among the Malagasy plated lizards and the monophyly of this group have yet to be rigorously tested.

Relationships among zonosaurines, which have traditionally been described and grouped on the basis of plesiomorphic characteristics, have been studied in detail only recently (Raselimanana, 2000) based on 143 morphological characters. The results of that study supported the monophyly of Malagasy gerrhosaurids, as well as that of the two genera *Tracheloptychus* and *Zonosaurus*. Preliminary molecular studies, including only select gerrhosaurids, have also supported zonosaurine monophyly (Odierna et al., 2002; Lamb et al., 2003). This monophyly suggests that the endemic species of Madagascar, an island isolated for more than 160 MY (Rabinowitz et al., 1983), may be the product of a single dispersal event, a biogeographic pattern evident in other Malagasy lizards (Schmitz et al., 2005).

There are, however, also a number of examples of Malagasy squamates whose extant diversity appears to be the product of multiple colonization events of the island (Nagy et al., 2003; Raxworthy et al., 2002; Townsend and Larson, 2002). In this respect, squamates are somewhat unique among Malagasy vertebrates in that all other Malagasy groups thus far examined appear to be the product of single (rather than multiple) colonization events from Africa to Madagascar (Caccone et al., 1999; Douady et al., 2002; Olson and Goodman, 2003; Yoder et al., 2003; Karanth et al., 2005; Poux et al., 2005). It is therefore of great interest to

* Corresponding author. Fax: +1 662 915-5144.

E-mail addresses: araselimanana@vahatra.mg (A.P. Raselimanana), anne.yoder@duke.edu, bnoonan@olemiss.edu (B. Noonan), karanth@ces.iisc.ernet.in (K.P. Karanth), jacques.gauthier@yale.edu (J. Gauthier).¹ Present address: Department of Biology, University of Mississippi, University, MS 38677, USA.

determine whether the extant diversity of Zonosaurinae is the product of one or more dispersal events.

Relationships among zonosaurines and the reality of morphologically delimited species within this group are also of great interest. Surprisingly few characters have been employed in identifying species, as diagnostic traits are scarce. Differentiation frequently relies on a few differences in scalation and in at least one instance (*Z. haraldmeieri*), relies heavily on differences in color. With our nearly comprehensive taxonomic sampling of zonosaurines and the inclusion of numerous populations for widespread species, these data provide an excellent opportunity to explore in depth not only species limits and interrelationships, but also to assess biogeographic patterns and to compare these to other Malagasy groups.

2. Materials and methods

2.1. Sample collection

Our sampling included 123 individuals of 17 of the 19 recognized species (Table 1) with as many as 20 individuals per species and nearly comprehensive geographic sampling for all. The zonosaurine tissues used in this study came from specimens collected by APR as well as tissue grants from Nussbaum, Rakotomalala, Rakotondravony, Ramanamanjato, Mirana and Rocha. Specimens were euthanized by injection of chlorobutanol, and muscle tissue was preserved in concentrated EDTA. Voucher specimens are held either at the Animal Biology Department, University of Antananarivo, Madagascar, or at the Museum of Zoology, University of Michigan, USA. Sequences for non-zonosaurine squamates used in phylogeny and divergence-time estimations were newly acquired or obtained from GenBank (Table 2). When phylogenetic results conflicted with specimen identification, these individuals were reexamined, and verified by APR.

2.2. PCR amplification and sequencing

DNA was extracted from ~25 mg of tissue using Qiagen DNeasy Tissue Kits. The entire mtDNA cytochrome *b* gene (*cob*, about 1142 bp), and subsets of the nDNA loci *NT-3* and *c-mos* (419 and 542 aligned bp, respectively) were amplified using the primers listed in Table 3 and standard amplification protocols for *cob* (below) and *NT-3* (Noonan and Chippindale, 2006). The nDNA markers were chosen to provide phylogenetic signal at both deep (*NT-3*) and shallow (*c-mos*) levels of the zonosaurine tree. The portion of the nuclear *c-mos* locus used in this study was amplified using a semi-nested PCR approach with primers reported by Saint et al. (1998). The primer pair G73 and G78 was used for the initial round of amplification. One microliter of this product was then used as template for the second round of amplification, this time using primer pair G73 and G74. Both the first and second amplifications were performed using a standard cycling profile with 30 cycles and an annealing temperature of 50 °C. PCR products were sequenced using 1/32 BigDye Terminator v3.1 (Applied Biosystems) reactions (total volume 5.0 µl) and analyzed using an automated sequencer (ABI 3700).

2.3. Data analysis

Sequences were edited and aligned in Sequencher 4.1 (Gene Codes Corp) and checked by eye. Amino acid sequences of *cob* data were examined to test for the possibility that nuclear copies of mitochondrial genes (numts) were inadvertently amplified, as might be revealed by the appearance of missense or nonsense mutations (van der Kuyl et al., 1995; Zhang and Hewitt, 1996; Bensasson et al., 2001).

Two different phylogenetic methods, maximum likelihood (ML) and Bayesian inference (MB) were used to infer evolutionary relationships among plated lizards. The program DT-ModSel (Minin et al., 2003) was used to determine the substitution models that best fit the individual and combined mtDNA + nDNA datasets for use in MB and ML analyses respectively. Support for nodes in the ML analysis was determined through 100 bootstrap replications using Garli v.0.951 (Zwickl, 2006) with a two-parameter model of nucleotide substitution and empirical estimation of rates.

Bayesian phylogenetic analysis was conducted with MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) using a mixed model (Nylander et al., 2004; two-parameter model with a gamma-distributed prior on rate variation and a proportion of invariant sites [the latter for *cob* and *NT-3* only]). Three independent Bayesian analyses (each with two independent runs) were initiated with random starting trees for 2×10^7 generations and four chains (three hot). Markov chains were sampled at intervals of 1000 generations. Burn-in was determined by examining plots of parameter values of the cold chain for convergence on stationarity using TRACER v1.3 (Drummond and Rambaut, 2007).

The program MacClade (Maddison and Maddison, 2000) was used to build a constraint tree of the plated lizards that is consistent with the pattern of relationships derived from analysis of morphological data (Fig. 1). The likelihood scores of the morphological, ML and MB trees were compared by implementing the Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999) in PAUP*.

We performed divergence-time estimates using a relaxed Bayesian molecular clock with uncorrelated rates (BEAST 1.4.7; Drummond et al., 2006) on a reduced dataset (24 zonosaurines, all outgroups). Five fossil calibrations from the literature (Table 4) were used to place priors on the ages of nodes within our tree (including the root node). Prior information on clade ages derived from fossil material was implemented as the lower limit of a log-normal distribution (mean = 2; SD = 2) on the age of the node uniting the descendant clade representative of that fossil and its sister clade. In two instances, nodes were constrained with both an upper and lower bound using a normal distribution based on inferences derived from paleontological literature (see Noonan and Chippindale, 2006; Table 1). For this analysis we assumed a GTR+I+G model of nucleotide substitution (see above) and an uncorrelated lognormal model of rate variation. The prior for the rate at the root node was set to the median value of the sum of all root-to-tip branch lengths divided by time. Some clades strongly supported by phylogenetic analysis were constrained for reasons of computational efficiency, and a Yule prior was placed on the branching rates. The results of two independent, 10,000,000-generation analyses were compared and combined in LogCombiner v1.4.5 (Drummond et al., 2006). To ascertain the true “joint prior” of the temporal constraints used in the BEAST analysis, and thus to test the strength of signal in our data, we conducted one analysis with only a single ambiguous character in our data matrix.

3. Results

3.1. Data

The aligned sequences of the *cob* gene region have neither stop codons nor indels. Base frequencies at the three positions of the codon were in accordance with expectations for vertebrate *cob* (Johns and Avise, 1998; Prusak and Grzybowski, 2004). Additionally, the third position of the codon has a very low frequency of G's as reported for mtDNA genes. Thus, these sequences are believed to represent true mitochondrial *cob* sequences rather than numts (Zhang and Hewitt, 1996; Bensasson et al., 2001).

Table 1

Specimen numbers, locality information and GenBank accession numbers.

Taxon	Number (field/ext)	Locality	Coordinates		cob	c-mos	NT-3
<i>T. madagascariensis</i>	APR2321/642	Kirindy Mite	20°47.4'	44°08.8'	DQ004421	DQ100104	EU571550
<i>T. madagascariensis</i>	APR1604/640	Andatabo	23°25.4'	43°44.8'	DQ004419	DQ100102	EU571545
<i>T. madagascariensis</i>	APR1305/641	Tsimanampetsotsa	24°03.0'	43°45.0'	DQ004420	DQ100103	EU571546
<i>T. madagascariensis</i>	APR5429	Antabore	24°23.9'	43°50.8'	EU571481	EU571646	
<i>T. madagascariensis</i>	APR6243	Itampolo	24°44.2'	44°01.8'	EU571482	EU571647	EU571547
<i>T. madagascariensis</i>	APR5602	Behara	24°52.1'	46°23.9'	EU571483	EU571648	EU571548
<i>T. madagascariensis</i>	APR5502	Tranomaro	24°41.2'	46°27.2'	EU571484	EU571649	EU571549
<i>T. petersi</i>	APR4869/643	Mikea -Abrahama	22°48.0'	43°26.0'	DQ004422	DQ100105	EU571551
<i>T. petersi</i>	APR4953/644	Mikea -Andaladomo	22°15.9'	43°28.7'	DQ004424	DQ100107	EU571552
<i>T. petersi</i>	APR5031/645	Mikea -Ankotapike	21°52.5'	43°22.6'	DQ440023	DQ100106	EU571553
<i>Z. aeneus</i>	APR5220/960	Midongy Sud	23°50.1'	46°57.8'	DQ100101	DQ004418	EU571565
<i>Z. aeneus</i>	APR0783/648	Daraina	13°15.3'	49°37.0'	DQ100100	DQ004417	EU571560
<i>Z. aeneus</i>	APR1534/253	Ivohibe	22°25.3'	46°53.9'	DQ100099	DQ004416	EU571563
<i>Z. aeneus</i>	RAN52092/646	Andohahela	37°55.0'	45°92.0'	DQ100098	DQ004415	EU571554
<i>Z. aeneus</i>	JB69/259	Andranomay	18°28.8'	47°57.3'	DQ100097	DQ004414	EU571567
<i>Z. aeneus</i>	RAN44456	Andringitra	22°13.2'	47°01.3'	EU571491	EU571656	EU571562
<i>Z. aeneus</i>	RD1117	Manambolo	22°08.6'	47°01.3'	EU571492	EU571657	EU571564
<i>Z. aeneus</i>	APR5685	Midongy Sud	23°35.4'	47°01.2'	EU571493	EU571658	EU571566
<i>Z. aeneus</i>	APR6520	Anjozorobe	18°25.3'	47°56.6'	EU571494	EU571659	EU571568
<i>Z. aeneus</i>	APR6648	Anjozorobe	18°25.5'	47°57.2'	EU571495	EU571660	EU571569
<i>Z. aeneus</i>	APR6853	Anjozorobe	18°31.3'	47°58.4'	EU571485	EU571650	EU571555
<i>Z. aeneus</i>	APR6987	Anjozorobe	18°18.3'	48°00.9'	EU571486	EU571651	EU571556
<i>Z. aeneus</i>	APR7138	Anjozorobe	18°38.6'	47°56.5'	EU571487	EU571652	EU571557
<i>Z. aeneus</i>	HER1637	Daraina	13°15.1'	49°35.3'	EU571488	EU571653	EU571558
<i>Z. aeneus</i>	HER0166	Daraina	13°15.1'	49°35.3'	EU571489	EU571654	EU571559
<i>Z. aeneus</i>	HER0782	Daraina	13°15.3'	49°37.0'	EU571490	EU571655	EU571561
<i>Z. anelanelany</i>	AM001	Tolagnaro	25°04.4'	46°46.9'	EU571496	EU571661	EU571570
<i>Z. anelanelany</i>	AM002	Tolagnaro	25°04.4'	46°46.9'	EU571497	EU571662	EU571571
<i>Z. bemaraha</i>	RAN54217	Bemaraha	18°41.5'	44°43.0'	DQ090877	DQ100143	EU571572
<i>Z. boettgeri</i>	RAN44099	Lokobe	13°25.0'	48°19.0'	DQ090876	DQ100136	EU571573
<i>Z. brygooi</i>	APR5754	Tampolo	17°17.2'	49°24.5'	EU571498	EU571663	EU571574
<i>Z. brygooi</i>	APR5508/965	Tampolo	17°17.2'	49°24.5'	DQ004452	DQ100135	
<i>Z. brygooi</i>	APR1181	Tampolo	17°17.2'	49°24.5'	EU571500	EU571665	EU571575
<i>Z. brygooi</i>	APR5844	Marotandrano	16°16.8'	48°48.1'	EU571501	EU571666	EU571576
<i>Z. brygooi</i>	APR5996	Marotandrano	16°17.1'	48°48.9'	EU571502	EU571667	EU571577
<i>Z. brygooi</i>	APR6000	Marotandrano	16°17.1'	48°48.9'	EU571503	EU571668	EU571578
<i>Z. brygooi</i>	RAN54392/652	Daraina	13°10.7'	49°42.0'	EU621710	DQ100133	EU571579
<i>Z. brygooi</i>	RAN42557/651	Antalaha	15°15.7'	50°26.1'	DQ004451	DQ100134	EU571580
<i>Z. haraldmeieri</i>	RAN38260/654	Montagne d'Ambre	12°31.0'	49°09.0'	DQ004448	DQ100130	EU571581
<i>Z. haraldmeieri</i>	HER2193/974	Analamerana	13°09.5'	49°7.5'	DQ004450	DQ100132	EU571582
<i>Z. haraldmeieri</i>	APR0001/655	Ankarana	12°55.9'	49°03.4'	DQ004449	DQ100131	EU571583
<i>Z. karsteni</i>	RAN54618/264	Ambatomainy	17°41.0'	45°40.0'	DQ004406	DQ100089	EU571584
<i>Z. karsteni</i>	HER2653	Bemaraha	18°40.0'	44°13.0'	EU571504	EU571670	EU571588
<i>Z. karsteni</i>	HER2873	Bemaraha	18°40.0'	44°13.0'	EU571505	EU571671	EU571589
<i>Z. karsteni</i>	APR2320/656	Kirindy Mite	20°47.4'	44°08.8'	DQ004407	DQ100090	EU571590
<i>Z. karsteni</i>	APR6424	Andranomanintsy	16°31.5'	44°29.4'	EU571506	EU571672	EU571591
<i>Z. karsteni</i>	APR7257	Lambokely	19°52.2'	44°38.7'	EU571507	EU571673	EU571592
<i>Z. karsteni</i>	APR7196	Kirindy (CFPF)	20°08.2'	44°41.0'	EU571508	EU571674	EU571593
<i>Z. karsteni</i>	APR7244	Kirindy (CFPF)	20°08.2'	44°41.0'	EU571509	EU571675	EU571594
<i>Z. karsteni</i>	APR4969	Mikea - Ankindranoka	22°13.0'	43°19.8'	EU571510	EU571676	EU571595
<i>Z. karsteni</i>	APR5044/659	Mikea - Ankotapike	21°52.5'	43°22.6'	DQ004404	DQ100087	EU571585
<i>Z. karsteni</i>	APR4823/657	Mikea - Ankazomifio	22°46.7'	43°31.4'	DQ004403	DQ100086	EU571586
<i>Z. karsteni</i>	RAN34526/660	Petriky	25°04.0'	46°53.0'	DQ004405	DQ100088	EU571587
<i>Z. laticaudatus</i>	APR1215/662	Ankarana	19°08.9'	44°49.7'	DQ004433	DQ100115	
<i>Z. laticaudatus</i>	RAN39043/661	Ambanja	13°59.5'	48°12.4'	DQ004432	EU571677	
<i>Z. laticaudatus</i>	APR7258	Lambokely	19°52.2'	44°38.7'	EU571518	EU721711	EU571604
<i>Z. laticaudatus</i>	APR6183	Sahamalaza	14°18.6'	47°54.9'	EU571519	EU571684	EU571605
<i>Z. laticaudatus</i>	APR6425	Andranomanintsy	16°31.5'	44°29.4'	EU571520	EU571685	EU571606
<i>Z. laticaudatus</i>	APR2206	Namoroka	16°28.2'	45°20.9'	EU571521	EU571686	EU571607
<i>Z. laticaudatus</i>	APR1085/663	Bemaraha	19°45.0'	44°37.0'	DQ004434	DQ100116	EU571608
<i>Z. laticaudatus</i>	APR1188/664	Bemaraha	19°08.9'	44°49.7'	DQ004436	DQ100117	EU571596
<i>Z. laticaudatus</i>	APR1145/665	Bemaraha	19°08.9'	44°49.7'	DQ004435	EU721712	EU571597
<i>Z. laticaudatus</i>	APR7223	Kirindy (CFPF)	20°08.2'	44°41.0'	EU571513	EU571678	EU571598
<i>Z. laticaudatus</i>	APR5454	Antabore	24°23.9'	43°50.8'	EU571514	EU571679	EU571599
<i>Z. laticaudatus</i>	APR6343	Tsimanampetsotsa	24°11.3'	43°45.9'	EU571515	EU571680	EU571600
<i>Z. laticaudatus</i>	APR1283/667	Tsimanampetsotsa	24°03.0'	43°45.0'	DQ004437	DQ100118	EU571601
<i>Z. laticaudatus</i>	APR5686	Ifotaka	24°45.9'	46°09.2'	EU571516	EU571681	EU571602
<i>Z. laticaudatus</i>	APR5528	Tranomaro	24°41.2'	46°27.2'	EU571517	EU571682	EU571603
<i>Z. laticaudatus</i>	APR5123/668	Ankarafantsika	16°19.1'	46°53.4'	DQ004431	DQ100114	
<i>Z. madagascariensis</i>	RD844/299	Anjanaharibe-sud	14°47.0'	49°26.5'	EU721713	DQ100119	
<i>Z. madagascariensis</i>	APR486/973	Marojejy	14°26.0'	49°45.7'	DQ004446	DQ100128	EU571617
<i>Z. madagascariensis</i>	RD698/297	Betaolana	14°32.0'	49°25.0'	DQ004441	DQ100123	
<i>Z. madagascariensis</i>	RAN39226/673	Manongarivo	14°05.0'	48°21.0'	DQ004440	DQ100122	
<i>Z. madagascariensis</i>	RAN43649/672	Lokobe	13°25.0'	48°19.0'	DQ004445	DQ100127	
<i>Z. madagascariensis</i>	RAN42479/670	Antalaha	15°15.7'	50°26.1'	DQ004444	DQ100126	EU571618
<i>Z. madagascariensis</i>	APR5846	Marotandrano	16°16.8'	48°48.1'	EU571529	EU571695	EU571619

Table 1 (continued)

Taxon	Number (field/ext)	Locality	Coordinates	<i>cob</i>	<i>c-mos</i>	NT-3
<i>Z. madagascariensis</i>	APR6117	Marotandrano	16°17.1'	48°48.9'	EU571530	EU571620
<i>Z. madagascariensis</i>	RAN45134	Zahamena	17°30.0'	48°38.0'	EU571531	EU571697
<i>Z. madagascariensis</i>	APR5803	Tampolo	17°17.3'	49°25.8'	EU571522	EU571688
<i>Z. madagascariensis</i>	RAN37489/674	Mantadia	18°53.2'	48°26.9'	DQ004447	DQ100129
<i>Z. madagascariensis</i>	APR7040	Anjozorobe	18°31.3'	47°58.4'	EU571523	EU571689
<i>Z. madagascariensis</i>	APR7123	Anjozorobe	18°38.6'	47°56.5'	EU571524	EU571690
<i>Z. madagascariensis</i>	RD1275/307	Ambohijanahary	18°15.7'	45°25.2'	DQ004443	DQ100125
<i>Z. madagascariensis</i>	RAN54373/263	Bemaraha	18°41.5'	44°43.0'	DQ004442	DQ100124
<i>Z. madagascariensis</i>	RD1878/669	Analavelona	22°38.5'	44°10.3'	DQ004439	DQ100121
<i>Z. madagascariensis</i>	HER1558	Daraina	13°15.4'	49°36.3'	EU571525	EU571691
<i>Z. madagascariensis</i>	HER1636	Daraina	13°15.4'	49°36.3'	EU571526	EU571692
<i>Z. madagascariensis</i>	CM13	Seychelles		EU571527	EU571693	EU571616
<i>Z. madagascariensis</i>	APR0782	Daraina	13°15.3'	49°37.0'	EU571528	EU571694
<i>Z. ornatus</i>	JB50/270	Andranomay	18°28.8'	47°57.3'	DQ004409	DQ100092
<i>Z. ornatus</i>	JB259/271	Andranomay	18°28.8'	47°57.3'	DQ004410	DQ100093
<i>Z. ornatus</i>	APR6936	Anjozorobe	18°31.3'	47°58.4'	EU571532	EU571698
<i>Z. ornatus</i>	APR7124	Anjozorobe	18°38.6'	47°56.5'	EU571533	EU571699
<i>Z. ornatus</i>	RD499/296	Itremo	20°38.0'	46°34.0'	DQ004408	DQ100091
<i>Z. ornatus</i>	RD1078/303	Manambolo	22°08.6'	47°01.3'	DQ004412	DQ100095
<i>Z. ornatus</i>	RD1816/677	Vinanintelo	21°46.0'	47°28.0'	DQ004413	DQ100096
<i>Z. ornatus</i>	RAN45120/678	Vohipaha	22°11.4'	46°58.2'	DQ004411	DQ100094
<i>Z. quadrilineatus</i>	APR04834	Mikea - Ankazomifio	22°46.7'	43°31.4'	EU571534	EU721714
<i>Z. quadrilineatus</i>	APR 04928	Mikea - Andalandomo	22°15.9'	43°28.7'	EU571535	EU721715
<i>Z. quadrilineatus</i>	APR 05045/683	Mikea - Ankotapike	21°52.5'	43°22.6'	DQ004425	DQ100108
<i>Z. rufipes</i>	HER2277	Andavakoera	13°17.0'	49°40.5'	EU571536	EU571701
<i>Z. rufipes</i>	APR0002/966	Ankarana	12°55.9'	49°03.4'	DQ004453	DQ100137
<i>Z. rufipes</i>	RAN42714/684	Antalaha	15°15.7'	50°26.1'	DQ004457	DQ100141
<i>Z. rufipes</i>	HER0760	Daraina	13°06.2'	49°42.4'	EU571537	EU571702
<i>Z. rufipes</i>	HER0499	Daraina	13°04.0'	49°41.3'	EU571538	EU571703
<i>Z. rufipes</i>	HER1812	Daraina	13°12.4'	49°46.2'	EU571539	EU571704
<i>Z. rufipes</i>	RAN55179/686	Daraina	13°10.7'	49°42.0'	DQ004455	DQ100139
<i>Z. rufipes</i>	HER0636/967	Daraina	13°06.2'	49°42.4'	DQ004456	DQ100140
<i>Z. rufipes</i>	RAN37989/688	Marojejy	14°26.2'	49°46.5'	DQ004458	DQ100142
<i>Z. rufipes</i>	RAN43672/687	Lokobe	13°25.0'	48°19.0'	DQ004454	DQ100138
<i>Z. subunicolor</i>	RAN43937/691	Lokobe	13°25.0'	48°19.0'	DQ004430	DQ100113
<i>Z. subunicolor</i>	RAN39093/690	Manongarivo	14°05.0'	48°21.0'	DQ004429	DQ100112
<i>Z. subunicolor</i>	APR0308/692	Marojejy	14°26.2'	49°46.5'	DQ004428	DQ100111
<i>Z. trilineatus</i>	APR1323/694	Tsimanampetsotsa	24°03.0'	43°45.0'	DQ004426	DQ100109
<i>Z. trilineatus</i>	APR1382/695	Tsimanampetsotsa	24°00.5'	43°53.9'	DQ004427	DQ100110
<i>Z. trilineatus</i>	APR5403	Antabore	24°23.9'	43°50.8'	EU571542	EU571707
<i>Z. trilineatus</i>	APR6258	Itampolo	24°44.2'	44°01.8'	EU571543	EU571708
<i>Z. tsingy</i>	RAN38884/696	Ankarana	12°55.8'	49°05.7'	DQ004459	DQ100144
<i>Z. tsingy</i>	APR0972/697	Ankarana	12°59.5'	49°06.6'	DQ004460	DQ100145
<i>Z. tsingy</i>	APR0039/698	Ankarana	12°55.9'	49°03.4'	DQ004461	DQ100146
<i>Z. tsingy</i>	APR6395	Nosy Hara	12°14.9'	49°00.5'	EU571544	EU571709
<i>Z. sp. 1</i>	HER2561	Bemaraha	18°47.8'	44°52.9'	EU571540	EU571705
<i>Z. sp. 2</i>	HER2636	Bemaraha	18°30.9'	44°39.6'	EU571541	EU571706

Specimen numbers correspond to field collection numbers of A. Raselimanana (APR), Hery Rakotondravony (HER), Ronald Nussbaum (RAN), Sara Rocha (CM) Jean-Baptiste Ramanamanjato (JB), Anjeriniaina Mirana (AM), and Domoina Rakotomalala (RD).

Table 2

Outgroup species used in phylogenetic and divergence time estimation. Group corresponds to terminal annotation of Fig. 2.

Taxon	Voucher	Group	<i>cob</i>	<i>c-mos</i>	NT-3
<i>Agama</i> sp.	AMB 6966	Iguania	AF355559	AF137530	EU636219
<i>Boa constrictor</i>	GenBank	Serpentes	BCU69746	AF471115	AY988047
<i>Coleonyx variegatus</i>	None	Gekkonidae	AB114446	AY172924	EU636220
<i>Chamaeleo</i> sp.	GenBank	Iguania	CFU69759	AY987990	AY988006
<i>Cordylus namaquensis</i>	GenBank	Cordylidae	AY217848	AY217848	AY988010
<i>Gerrhosaurus flavigularis</i>	YPM 13011	Gerrhosauridae	DQ090883	DQ100151	EU636221
<i>Gerrhosaurus major</i>	YPM 13012	Gerrhosauridae	DQ090881	DQ100149	EU636222
<i>Gerrhosaurus validus</i>	YPM 13658	Gerrhosauridae	DQ090882	DQ100150	EU636223
<i>Gerrhosaurus nigrolineatus</i>	No voucher	Gerrhosauridae	AY217794	AY217846	EU636224
<i>Oplurus</i> sp.	GenBank	Iguania	AF020259	AF315391	AY988000
<i>Phrynosoma cornutum</i>	GenBank	Iguania	AY141087	AY987989	AY988004
<i>Platysaurus</i> sp.	YPM 13037	Cordylidae	DQ090884	DQ100152	EU636225
<i>Xantusia</i> sp.	YPM 13239	Xantusiidae	EU129533	DQ100153	EU636226

AMB, Aaron Bauer; YPM, Yale Peabody Museum.

3.2. Phylogenetic relationships of Zonosaurinae

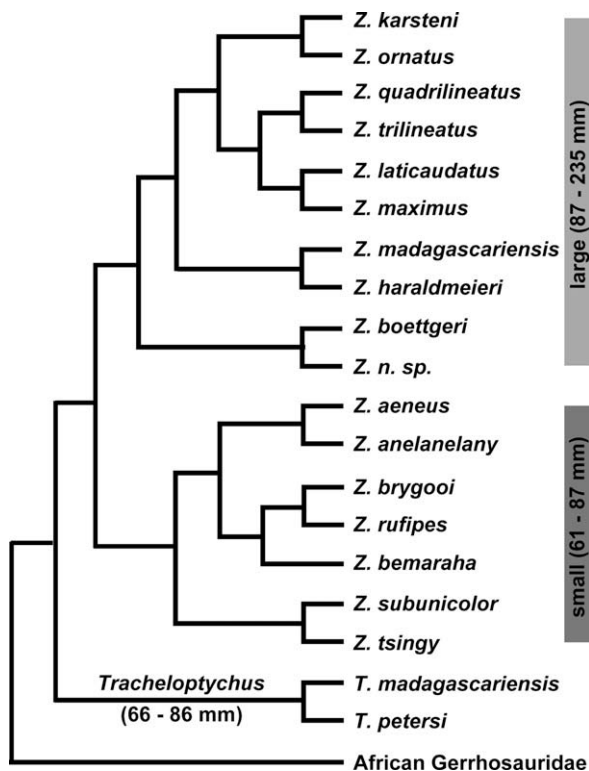
All analyses (ML, MB) strongly support the monophyly of Malagasy plated lizards (Zonosaurinae) (Fig. 2) with respect to African

mainland gerrhosaurids. Within the zonosaurines, monophyly of the genus *Tracheloptychus* is well supported, though its placement within Zonosaurinae is not, and the single most likely tree (–ln L = 18661.7) recovers *Zonosaurus* as paraphyletic with respect to

Table 3
Primer sequences.

Name	Locus	Primer sequence 5' → 3'
L14724 ^a	<i>cob</i>	CGAAGCTTGATATGAAAAACCATCGTT
L14973	<i>cob</i>	AACGGGGCCTCAATATTCTT
L15171	<i>cob</i>	CATGAGGACAAATATCATTCTGAGG
L15625	<i>cob</i>	CGATCYATYCCYAAAYAACTAGGAGG
H15149 ^a	<i>cob</i>	GCCCTCAGAATGATATTTGCTCTCA
H15261	<i>cob</i>	AAGAATCGTGTAGKGTGCTT
H15673	<i>cob</i>	GAATACCATACTTCGTTGTTGGC
H15915	<i>cob</i>	AACTGCAGTCATCTCCGTTTACAAGAC
GerrL393 ^a	<i>cob</i>	TAGTMATAGCMACCGCYTTGTWGG
GerrL786 ^a	<i>cob</i>	CCAGAAAATYTYACMCCMGCAAACCC
GerrH810 ^a	<i>cob</i>	GCAAATAGGAARTAYCAATCTGG
GerrHthr ^a	<i>cob</i>	ATCTCCGGTTTACAAGACCGGTGC
NT3-F3	NT-3	ATATTTCTGGCTTTTCTCTGTGGC
NT3-R4	NT-3	GCGTTTCATAAAAATATTGTTGACCGG
G73	c-mos	GCGGTAAAGCAGGTGAAGAAA
G74	c-mos	TGAGCATCCAAAGTCTCCAATC
G78	c-mos	AGRCTGATRWCAAANGARTARATGTC

^a primers used to amplify African gerrhosaurs and cordylids, and *Xantusia henshawi*.

**Fig. 1.** Phylogeny of Malagasy plated lizards based on non-molecular data (adapted from Raselimanana, 2000).

this genus. Most interspecific relationships are not well supported by these data. These phylogenetic analyses supported a clade comprising six of the 17 included species of *Zonosaurus* previously identified largely by scutellation and color pattern characteristics (Glaw and Vences, 2007), and there are a number of instances of recognized taxa being rendered both paraphyletic (e.g. *Z. madagascariensis*/*Z. haraldmeieri*) and polyphyletic (e.g. *Z. aeneus*) by our data.

3.3. Divergence-time estimation

Divergence-time estimates place the origin of Zonosaurinae in the Paleogene (TMRCA of Zonosaurinae + African gerrhosaurs median = 66 MYA, 95% CI 53–85 MYA).

4. Discussion

All phylogenetic analyses, including different tree-building methods and different data partitions, support the monophyly of Malagasy plated lizards (Zonosaurinae) with high support (Fig. 2). As the sister group to Zonosaurinae and successively deeper sister lineages are absent from Madagascar, this result suggests a single colonization of Madagascar from Africa with subsequent radiation. This island radiation is therefore similar to those seen in other terrestrial vertebrates from Madagascar such as lemurs (Yoder et al., 1996; Yoder and Yang, 2004; Karanth et al., 2005), tenrecs (Olson and Goodman, 2003), carnivorans (Yoder et al., 2003; Poux et al., 2005), scincid lizards (Whiting et al., 2004), tortoises (Caccone et al., 1999), and amphiglossine scincids (Schmitz et al., 2005).

Within Zonosaurinae, *Tracheloptychus* is supported as monophyletic, but monophyly of *Zonosaurus* is not conclusive due to the poor support for interspecific relationships. While a number of morphological characters (largely components of scutellation) support the differentiation of these two genera, our molecular data alone are not sufficient to test this hypothesis. Nor do these data provide sufficient evidence to test the hypotheses of Raselimanana (2000) that body-size (large vs. small) defines two distinct clades of *Zonosaurus* (Fig. 1). This evolutionary pattern was predicted by phylogenetic analysis of morphological characters, but was considered somewhat surprising given the evolutionary lability of body size, and the potential for correlated allometries to confuse phylogenetic relationships of characters influenced by size and age. Our findings do support the monophyly of the dwarf species group (see Fig. 1) with the exception of the placement of *Z. anelanelany* outside this group. It is noteworthy that this taxon is the only species of the dwarf lineage not restricted in distribution to the northern portion of the island (Fig. 3a). Although the poorly supported pattern of interspecific relationships precludes conclusive determination of the ancestral body size, it is worth noting that large-bodied lizards tend to have wider distributions and are usually ecological generalists, whereas dwarf species have restricted distributions and tend to be specialists. For example, *Z. tsingy* is a rock-dwelling specialist that is entirely restricted to karstic limestone formations on the northern tip of the island (Fig. 3b).

Tracheloptychus is strongly supported as a monophyletic group in our analyses, supporting previous morphological work. Within this genus, *T. madagascariensis* is rendered paraphyletic with respect to a single individual of *T. petersi* (Fig. 3a). While *T. petersi* is restricted to transitional dry forest on sandy soil, *T. madagascariensis* is a generalist found even on calcareous soil and in rocky areas. In general appearance, both species are very similar except with respect to color pattern and some fine meristic characteristics. Though retained ancestral polymorphism is equally plausible, it is noteworthy that within the range of *T. petersi*, many *T. madagascariensis*-like individuals have been recovered suggesting the possibility of hybridization.

Within Zonosaurinae, the widely distributed *Z. madagascariensis* is paraphyletic with respect to *Z. haraldmeieri*, which has a very restricted distribution in the northern tip of the island (Fig. 3a). Glaw and Vences (2007) report the occurrence of phenotypically intermediate forms in Ankarana and mention unpublished data indicating these two species are closely related with possible paraphyly of *Z. madagascariensis*. These two species are morphologically very similar (Brygoo and Böhme, 1985; Raselimanana, 2000), with *Z. haraldmeieri* long being considered a subspecies of *Z. madagascariensis*. Arguments for recognizing these two species as distinct have been based primarily on their geographic distributions and habitat, rather than on morphologic differences. Whereas *Z. madagascariensis* is widely distributed across the island, and largely confined to

Table 4

Calibration points (priors) used in BEAST analysis of divergence time.

Node	Prior	Ref.
TMRCA ex.- <i>Coleonyx</i>	Normal. $M = 197$; $SD = 17$	Evans et al., 2002; Noonan and Chippindale 2006
TMRCA Iguania	Normal. $M = 103$; $SD = 40$	Norrell and de Queiroz, 1991; Evans et al., 2002; Noonan and Chippindale 2006
TMRCA <i>Boa</i> + Iguania	LogNormal. $M = 2$; $SD = 2$; $os = 75$	Rage, 1984; Albino, 1996, 2000
TMRCA Cordylus + Gerrhosauridae	LogNormal. $M = 2$; $SD = 2$; $os = 65$	Krause et al. (2003)
TMRCA Scincoidea + Gerrhosauridae	LogNormal. $M = 2$; $SD = 2$; $os = 75$	Gao and Norell (2000); Gauthier personal communication

M, mean; SD, standard deviation; os, = zero offset.

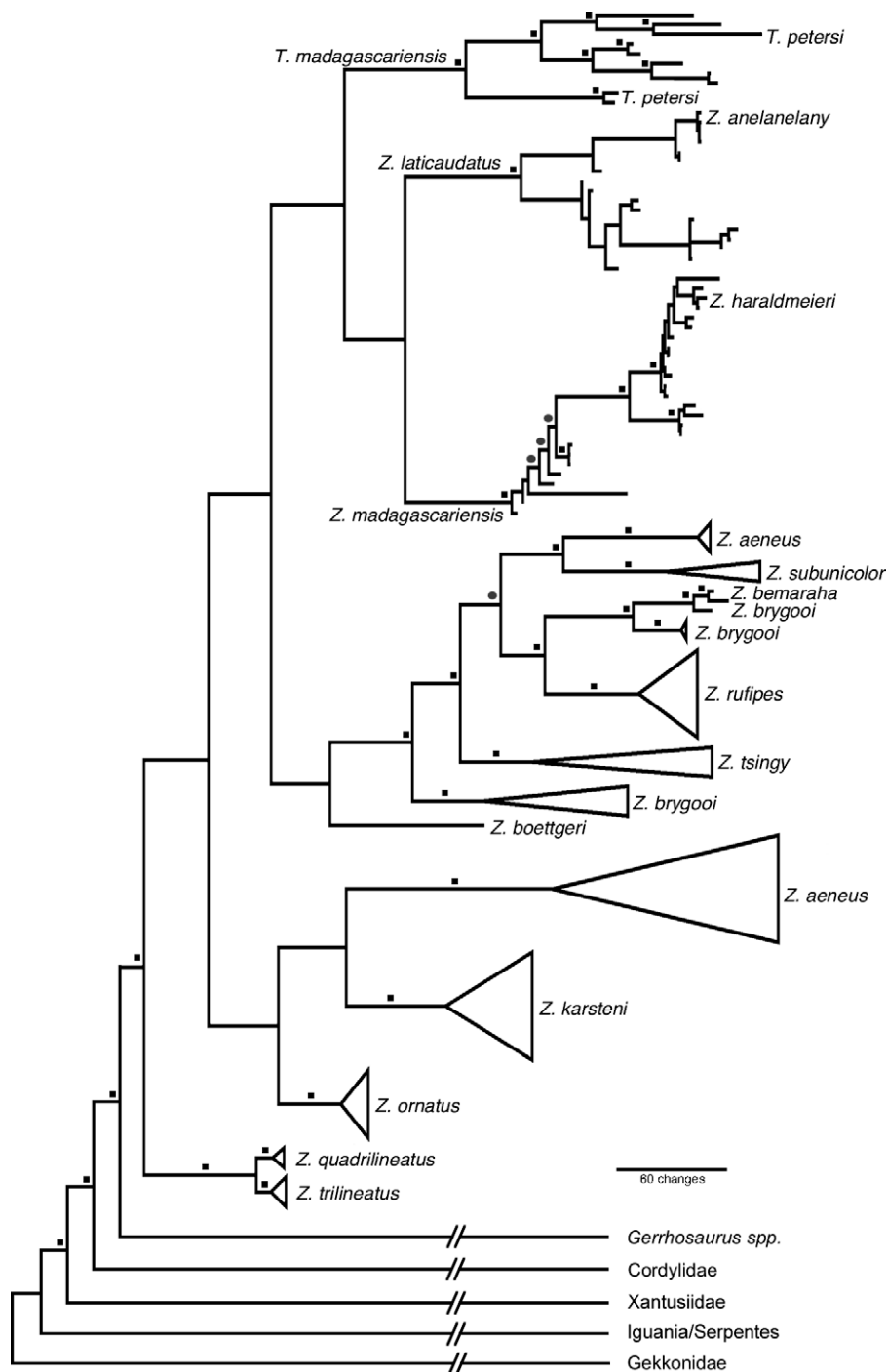


Fig. 2. Single most likely tree produced by ML analysis of combined nDNA + mtDNA dataset. Terminals of monophyletic units and outgroup lineages have been collapsed for ease of viewing (see Fig. 3 for detail). Nodes receiving >70 and 0.95 support from ML bootstrap and MB analyses respectively are indicated by solid squares. Gray circles indicate nodes receiving significant support from MB analysis only.

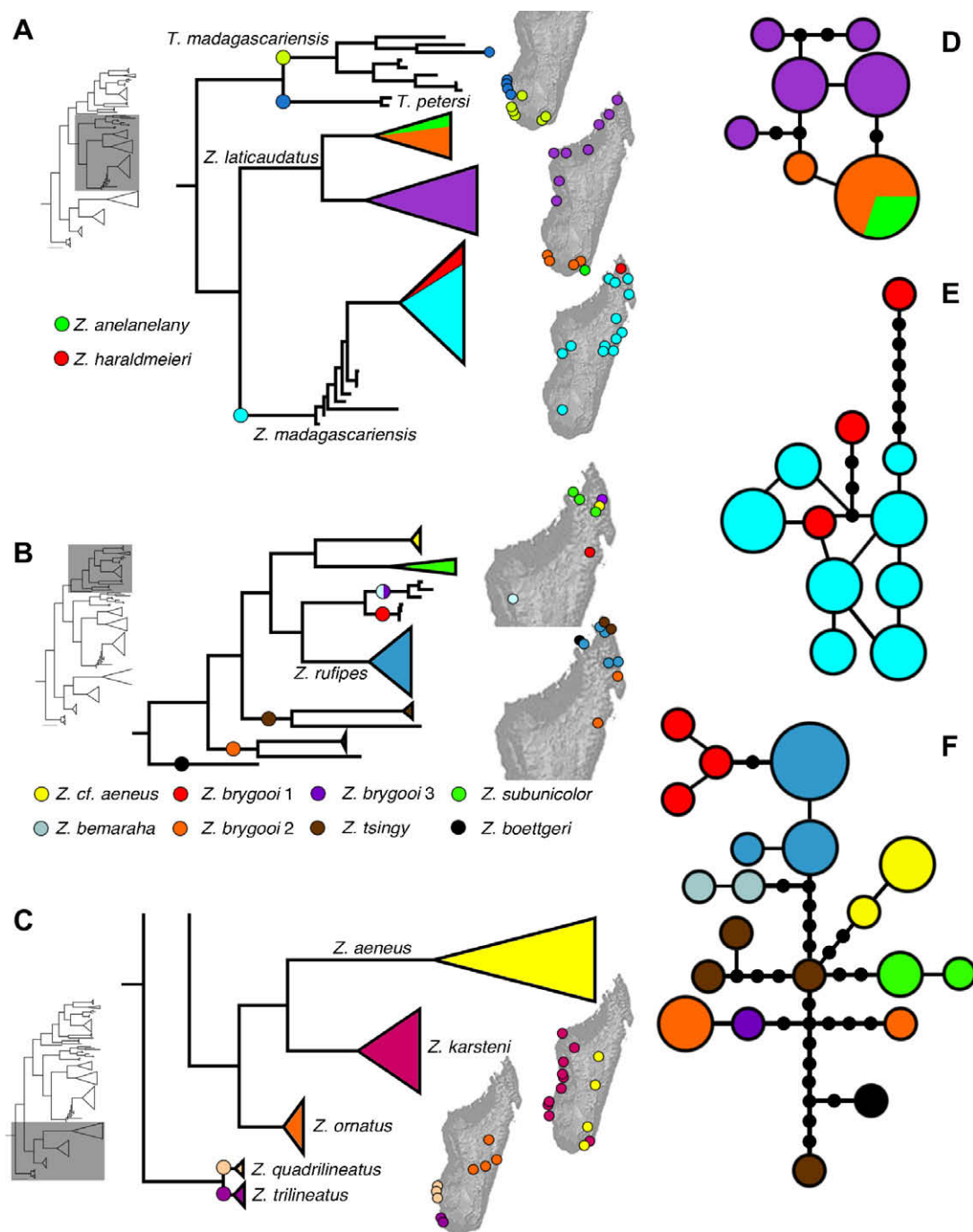


Fig. 3. Phylogeographic patterns of individual samples included in this study. For each subtree, the shaded portion of the inset tree (identical to Fig. 2) indicates the portion illustrated. Colored circles on internodes, fill of collapsed terminals and haplotype networks indicate geographic distribution on adjacent maps. *c-mos* haplotype networks illustrate interrelationships among groups discussed in the text: (D) *Z. laticaudatus*/*Z. anelanelany*; (E) *Z. madagascariensis*/*Z. haraldmeieri*; (F) northern small-bodied species group.

low-elevation humid forest, *Z. haraldmeieri* is restricted to a small area of transitional forest near Antsiranana (Raselimanana, 2000). Given the significant phenotypic polymorphism present in *Z. madagascariensis* and that *Z. haraldmeieri* is genetically nested within *Z. madagascariensis*, it seems that *Z. haraldmeieri* is merely an isolated population of *Z. madagascariensis*. However, examination of *c-mos* data alone (Fig. 3E) reveals a highly divergent nuclear allele present in the lone *Z. haraldmeieri* individual sampled from Mt. d'Ambre. The two other putative samples of this species were collected near Ankarana, an area from which intermediate forms have been reported (Glaw and Vences, 2007). Phylogenetic analyses of this marker alone do not provide substantial support for relation-

ships of this clade. Thus, while mtDNA alone clearly suggests that all three specimens attributed to *Z. haraldmeieri* are nested within *Z. madagascariensis* (not shown), it is possible that this is the product of mtDNA introgression and that the two species are in fact distinct. A similar pattern (northern isolate of widespread lineage exhibiting phenotypic and ecological differentiation) has been reported in the anuran genus *Mantella* (Rabemananjara et al., 2007a; Nagy et al., 2007; Boumans et al., 2007) report the presence of distinct genetic lineages in the northernmost portion of the island for a number of reptile groups. Additional sampling of nDNA markers and individuals in the north (including Mt. d'Ambre) are needed before the validity of *A. haraldmeieri* can be rigorously tested.

Additionally, within *Z. madagascariensis*, there is strong support for the differentiation of a mtDNA lineage restricted in distribution to the northern half of the island (all populations north of Toamasina, collapsed shaded terminus of Fig. 3a). Phylogeographic relationships of individuals from localities south of Toamasina are poorly resolved, and monophyly is not supported and indeed contradicted by MB analyses.

The only species of *Zonosaurus* other than *Z. madagascariensis* to span the length and breadth of the island is *Z. laticaudatus*. This large-bodied species was found to be paraphyletic, in this case with respect to the dwarf species *Z. anelanelany*, which is itself known only from the extreme southwest near Tolagnaro (Fig. 3a). Similar to *Z. madagascariensis*, the phylogeographic pattern within *Z. laticaudatus* indicates strongly supported, reciprocally monophyletic north-south lineages (and *Z. anelanelany* as a component of the southern lineage). While the geographic area separating these two lineages is a relatively large expanse of land on the west coast, spanning the entire region between the Onilahy and Mangoky rivers, our sampling does not include specimens from this area, and it is unknown where the transition occurs. *Zonosaurus anelanelany* itself is phenotypically (size and coloration) quite distinct from *Z. laticaudatus*, and the two 'species' are reported to occur syntopically in the south-east of Madagascar (M. Vences, pers com). In the south, there is little support for the differentiation of the two species. Individuals of *Z. laticaudatus* sampled from the southeast, near *Z. anelanelany*, are genetically indistinguishable from this taxon at both mtDNA and nDNA loci (Fig. 3D). As these two species are quite distinct morphologically and our sample of *Z. anelanelany* is limited to two specimens from a single locality, we hesitate to suggest any taxonomic changes at this time. Intensive sampling of the area inhabited by both species in the southeast is sure to reveal either a rapid speciation/adaptation of *Z. anelanelany* or dramatic polymorphism that persists despite significant gene flow. Notably, the latter possibility would support recognition of all *Z. laticaudatus* populations south of the Onilahy River as a distinct species (Fig. 3a,d, orange).

In the case of *Z. aeneus*, a species long believed to span the length of the eastern mountain range, two extremely divergent genetic lineages are recovered (Fig. 3b,c). A recent biological survey of the extreme northeast portion of Madagascar (Daraina forest) revealed the existence of an isolated population attributed to *Z. aeneus* (Raselimanana, unpublished report). Geographically, Daraina lies in the extreme north of the distribution of *Z. aeneus*, and ecologically this forest belt is much drier than the typical humid rainforest habitat of *Z. aeneus*. A similar geographic distribution of a genetically contiguous lineage (*Mantella baroni*/*M. nigricans* group) has been reported previously (Rabemananjara et al., 2007b). The molecular data analyzed here clearly indicate that the population near Daraina is not conspecific with *Z. aeneus* and is strongly supported as nested within the "dwarf" species of the northern region (Fig. 3b). Morphologically, individuals from the Daraina population have fewer ventral scales than the typical form as well as a post-subocular scale divided into two sections (not divided in typical *Z. aeneus*). These molecular and scutellation characters suggest that the "*Z. aeneus*" population from Daraina may constitute a new species unrelated to *Z. aeneus*. Haplotypes from all other populations of *Z. aeneus*, ranging from Antananarivo south to Tolagnaro, form a strongly supported monophyletic group (Fig. 3c).

Zonosaurus karsteni, *Z. quadrilineatus*, *Z. trilineatus*, *Z. ornatus*, *Z. subunicolor* and *Z. rufipes* are all recovered as monophyletic groupings of haplotypes (Fig. 2). With the exception of *Z. karsteni*, these species are not widely distributed (Fig. 3b,c). Among all species mentioned immediately above, *Z. karsteni* is the most broadly distributed, genetically continuous (i.e. little geographic structure to observed genetic variation) taxon and is the only such lineage to

occur both north and south of the Onilahy River. This river serves to delimit the northern and southern borders of the sister-species pair *Z. trilineatus* and *Z. quadrilineatus*, respectively, as well as potentially playing a role in the biogeographic structure of the species of *Tracheloptychus* and *Z. laticaudatus* (see above).

As has been described for other faunal groups, the northern region is here recovered as possessing a diverse assemblage of endemic plated lizards. This northern portion of Madagascar has been recognized as a center of diversity and endemism for a number of lizard species (Raxworthy and Nussbaum, 1995, 1997; Boumans et al., 2007). Within this region we find evidence to support the recognition of *Z. subunicolor*, *Z. rufipes*, *Z. tsingy*, *Z. boettgeri*, *Z. brygooi*, *Z. bemaraha* and the Daraina population previously assigned to *Z. aeneus*. While known from very few specimens, our sample of *Z. boettgeri* from the off-shore island of Nosy Be does appear distinct from all other taxa sampled (Fig. 3b). Both *Z. subunicolor* and *Z. rufipes* span the breadth of the northern one-third of the island from Nosy Be to Antalaha and are strongly supported as monophyletic groupings of haplotypes. Although sampling of *Z. tsingy* from the off-shore island of Nosy Hara is limited to a single individual, the genetic differentiation between this individual and the three samples from the mainland (Ankarana) is extensive for both mtDNA (>15% uncorrected divergence in *cob*) and nDNA (Fig. 3f), suggesting the presence of an unrecognized species on Nosy Hara. The phylogeographic pattern presented by *Z. brygooi* is remarkably complex. Specimens assigned to this taxon based on morphological characteristics are resolved into no less than three population lineages, a pattern supported by both mtDNA and nDNA (Fig. 3b,f). Perhaps most surprising is the well supported grouping of an individual referred to this taxon from the Daraina forest (Fig. 3b, purple) with individuals of *Z. bemaraha* from the distant Bemaraha region of western Madagascar (though this pattern is not apparent in the nDNA alone; Fig. 3f). This disjunct clade was in turn supported as the sister taxon to a population of "*Z. brygooi*" from the Marotrandrano Reserve in the western foothills of the eastern mountain range. Yet another strongly supported "*Z. brygooi*" clade composed of two highly divergent (~12% uncorrected divergence in *cob*; 7 substitutions in *c-mos*) lineages was recovered from two eastern coastal populations from Tampolo and Antalaha (Fig. 3b,f, orange). Thus it appears that *Z. bemaraha* may be more widespread than previously thought, and the delimitation of *Z. brygooi* needs to be further investigated with potential for the recognition of additional species.

Despite the thorough sampling of molecular markers, zonosaurine taxa, and populations in this study, we are unable to resolve a number of questions regarding the validity of certain taxa. If anything, our results highlight additional populations whose classification must be reexamined. However, these data do provide some very useful information in disentangling the process of biotic diversification in Madagascar. Particularly notable is the recurring theme of significant, and frequently cryptic diversity in the north of the island. While this region has been recognized as an area of endemism for some time, the cause of this has prompted many explanations (Boumans et al., 2007). Species of southern Madagascar also hold great promise in the study of the role of rivers as biogeographic barriers as at least two species pairs (*Z. quadrilineatus*/*Z. trilineatus* and *T. madagascariensis*/*T. petersi*) have their distributional limits associated with this drainage.

Overall, our results clarify some very important aspects of zonosaurine evolution, but they raise significantly more interesting questions. Unlike other Malagasy vertebrates that have been studied (primarily mammals and anurans) zonosaurines are relatively abundant, easy to sample, not subject to stringent protection/regulation, and are not known to be experiencing noticeable declines in population size and diversity. They are however, widespread, suitably diverse, and as has been demonstrated here, a Malagasy

radiation presenting numerous instances of regional endemism, cryptic species, biogeographic structure, and possible incipient speciation. Further detailed investigation of zonosaurine evolution will undoubtedly contribute substantially to the understanding of Malagasy biotic diversification, and may also yield novel insight into mechanisms of speciation in the tropics.

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