

Multi-gene phylogeny of Madagascar's plated lizards, *Zonosaurus* and *Tracheloptychus* (Squamata: Gerrhosauridae)

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A B S T R A C T

We analyzed the phylogenetic relationships of the Malagasy plated lizards in the family Gerrhosauridae based on DNA sequence fragments of four mitochondrial and five nuclear genes. Various clades were strongly supported by the concatenated data set and also recovered by separate analyses of mtDNA and nucDNA. In particular, two clades here named the *Z. rufipes* group (containing *Z. bemaraha*, *Z. brygooi*, *Z. rufipes*, *Z. subunicolor*, *Z. tsingy* and an undescribed candidate species from northern Madagascar) and the *Z. ornatus* group (containing *Z. anelanelany*, *Z. laticaudatus*, *Z. karsteni*, *Z. ornatus*, *Z. quadrilineatus*, and *Z. trilineatus*) were resolved with strong support. A third clade named the *Z. madagascariensis* group contains *Z. madagascariensis* with a nested *Z. haraldmeieri*; the status of that species requires further investigation. Tentatively we also include *Z. aeneus* in this species group although its phylogenetic relationships were poorly resolved. A fourth clade with less support included *Z. boettgeri* and *Z. maximus*. The phylogenetic position of the genus *Tracheloptychus* remains uncertain: whereas in the species tree it was recovered as the sister group to *Zonosaurus*, other methods indicated that it was nested within *Zonosaurus*, albeit alternative topologies were rejected with only marginal statistical support.

Keywords:

Zonosaurus
Tracheloptychus
Madagascar
Biogeography
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1. Introduction

Madagascar has a rich biota characterized by a high degree of endemism, which extends beyond the species level and often to the level of genera or even families (Goodman and Benstead, 2003). Among the terrestrial vertebrates of the island, there are taxa whose closest evolutionary relationships are to Asian and South American species (Noonan and Chippindale, 2006; Warren et al., 2010; Samonds et al., 2012), but the majority of colonizations probably originated from ancestors rafting over the Mozambique Channel from mainland Africa (Yoder and Nowak, 2006). Such out of Africa rafting is particularly obvious in cases where the Malagasy clades are deeply nested within exclusively African groups, e.g., in frogs of the family Hyperoliidae (Vences et al., 2003; Wollenberg et al., 2007), in lamprophiid snakes (Nagy et al., 2003), or in plated lizards of the family Gerrhosauridae (Crottini et al., 2012b).

Malagasy plated lizards are represented by two genera of Gerrhosauridae: *Tracheloptychus*, with two species inhabiting the sub arid south and south west, and *Zonosaurus*, with 17 species distributed across the different biomes of the island. Gerrhosauridae is the sister group of the exclusively African girdle tailed lizards (family Cordylidae) and both families together comprise the unranked clade Cordyliformes (Lang, 1991; Mouton and Van Wyk, 1997; Frost et al., 2001; Lamb et al., 2003; Townsend et al., 2004; Conrad, 2008). Crown group cordyliforms are restricted to sub Saharan Africa and Madagascar, though fossils related to these lizards have been recovered from Asia and Europe (Conrad, 2008). A Cretaceous era Malagasy cordyliform fossil has been discovered (Krause et al., 2003) but was tentatively attributed to the Cordylidae and thus probably is not closely related to the island's extant gerrhosaurids.

The monophyly of the Cordyliformes (Cordylidae + Gerrhosauridae) is not disputed (Conrad, 2008), yet the few published molecular studies to date (Frost et al., 2001; Odierna et al., 2002; Lamb et al., 2003; Stanley et al., 2011) focus on either one or the other of the families and, hence, a comprehensive molecular assessment

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of cordyliform relationships is wanting. Karyological analyses indicated a high uniformity of chromosomal number among cordyli forms, especially among gerrhosaurid taxa, and therefore were not informative regarding cordyliform phylogeny (Odierna et al., 2002). The first analysis of molecular phylogenetic relationships within Cordylidae was based on mitochondrial data (Frost et al. 2001). More recently Stanley et al. (2011) conducted a more exhaustive study of Cordylidae, including mitochondrial (mt)DNA and nuclear (nuc)DNA, and proposed 10 monophyletic genera in this sub-Saharan family. For the Gerrhosauridae, the only morphological phylogenetic analysis is that of Lang (1991) who found the Malagasy genera (*Tracheloptychus* and *Zonosaurus*) to be monophyletic and sister to a clade of African genera (*Angolosaurus*, *Cordylus*, *Gerrhosaurus*, *Tetradactylus*). Lamb et al. (2003) included representatives of all gerrhosaurid genera in their analysis of four mitochondrial genes and synonymized *Angolosaurus* with *Gerrhosaurus*. They also found moderate support for the reciprocal monophyly of African and Malagasy taxa.

For Malagasy gerrhosaurids, based primarily on external morphological data Lang (1990) proposed that *Tracheloptychus* was sister to a monophyletic *Zonosaurus*. Within *Zonosaurus*, a basal trichotomy separated clades containing (i) *Z. maximus*, *Z. ornatus* and *Z. boettgeri*, (ii) *Z. trilineatus* and *Z. quadrilineatus*, and (iii) all remaining species. In the latter clade (iii), two exemplars of *Z. karsteni* and *Z. laticaudatus* split off in a further trichotomy, followed by a clade containing *Z. madagascariensis* and *Z. haraldmeieri*, which was sister to a clade containing all species with three supralabial scales anterior to the subocular (at that time, *Z. aeneus*, *Z. rufipes* and the yet unnamed *Z. brygooi*). Taxonomic revisions have since demonstrated the existence of additional species in *Zonosaurus* (e.g., Vences et al., 1996; Raselimanana et al., 2000, 2006) and molecular studies (Odierna et al., 2002; Yoder et al., 2005; Raselimanana et al., 2009) have challenged the relationships within Malagasy plated lizards, despite only low support for most of the basal relationships within this group of lizards.

In order to provide a better resolved phylogenetic hypothesis for Malagasy Gerrhosauridae, we assembled a data set of four mitochondrial and five nuclear loci (4.7 k bp total) for most species in this group. Our results confirm Malagasy gerrhosaurids (*Zonosaurus* + *Tracheloptychus*) and the genus *Tracheloptychus* as monophyletic groups. The monophyly of *Zonosaurus* relative to *Tracheloptychus* remains ambiguous, but we identify several highly supported main clades within the genus *Zonosaurus*.

2. Materials and methods

2.1. Sampling

Samples and specimens were obtained during fieldwork in Madagascar from 2000 to 2010 (see Supplementary materials for a Table of all voucher specimens and a map of collecting localities, Fig. S1). Lizards were collected by diurnal opportunistic searches and pitfall trapping, euthanised with an overdose of MS222 or chlorobutanol, fixed in formalin and preserved in 70% ethanol. Tissue samples from femur muscle or tail were taken before fixation and preserved separately in 95–99% ethanol or EDTA. Specimens were deposited in the collections of the Université d'Antananarivo, Département de Biologie Animale (UADBA), the Zoological Museum Amsterdam (ZMA), and the Zoologische Staatssammlung München (ZSM). In some cases, tissue samples were taken from autotomized tails and the specimens released after unambiguous identification by morphology. Additional acronyms used: ZCMV, FGZC, MVDNA, FG/MV, field numbers of M. Vences and F. Glaw;

APR, field numbers of A.P. Raselimanana, and AM, a field number of M. Anjeriniaina.

2.2. DNA sequencing

DNA was extracted from alcohol and EDTA preserved muscle tissue using a Dneasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. Fragments from the following four mtDNA genes were amplified: 12S rRNA (12S), 16S rRNA (16S), cytochrome *b* (COB) and NADH dehydrogenase subunit 1 (ND1). Fragments of the following five nuclear genes were also amplified: brain derived neurotrophic factor (BDNF), recombination activating gene (RAG2), phosphatidylinositol 3-OH kinase (PI3K), oocyte maturation factor (CMOS) and neurotrophin 3 (NT3). PCR reactions contained 0.5 µl of each 10 µM primer, 0.8 µl of 10 mM dNTPs, 0.4 µl Taq polymerase (Genaxxon), 1.0 µl 10X PCR buffer and 1 µl of DNA. Amplification followed standard cycling protocols. Primer sequences and detailed PCR conditions can be found in Supplementary materials Table S2.

PCR products were cleaned with a SAP/CIAP enzyme protocol. The product was then cycle sequenced in both directions using the same primers as in PCR amplification and electrophoresed on an ABI 3130xl after ethanol precipitation.

Forward and reverse sequences were assembled with Sequencher v 4.2.2. Multiple sequence alignment for each gene separately was conducted in ClustalX (Thompson et al., 1997) using default settings. Reading frames for coding genes were inferred in Mac Clade v. 4.07 (Maddison and Maddison, 2003).

All newly determined DNA sequences were submitted to Genbank (Accession Numbers KC515098–KC515339, Table S1).

2.3. Phylogenetic analysis

The model of molecular evolution was inferred per gene, per type of gene (i.e. coding/non coding, nuclear/mtDNA), and per codon position (each separately and first and second positions combined) in MrModeltest v 2.3 (Nylander, 2002) and the best model chosen by AIC (Supplementary materials Table S3). Hypervariable sites in the ND1, 12S and 16S rRNA genes prone to multiple substitutions and gaps in 12S and 16S rRNA were excluded from the analysis after running GBLOCKS using default parameters (Castresana, 2000).

Bayesian phylogenetic analyses (Bayesian Inference, BI) of partitioned data sets were executed in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). The combined data set (all mtDNA and nuclear genes) was analyzed with three alternative partition strategies: 5 partitions (non coding mtDNA, mtDNA 1st and 2nd position, mtDNA 3rd position, nuclear 1st and 2nd position, nuclear 3rd position), nine partitions (each gene separately), or 15 partitions (mtDNA non coding, each coding gene separately with 1st and 2nd position combined, 3rd position coding separately). Statefreq, revmat, shape, pinvar and tratio were unlinked across partitions. Branch lengths prior was set to Unconstrained: Exponential (100), which had been found to improve chain mixing in preliminary runs (Marshall, 2010). The temp parameter was set to 0.025, 0.04 or 0.05, after being decreased stepwise as needed to improve mixing. Four simultaneous chains were run for 10 million generations sampled every 500 generations. The first 5000 or 6000 samples were discarded as burn in after assessing MCMC convergence. Convergence was assumed when the chain swap information for both runs was between 0.4 and 0.8, the average standard deviation of split frequencies was minimized, the harmonic means for run 1 and 2 at stationarity were almost identical ($\pm 0.001\%$), and the PSRF value was ~ 1.001 .

Harmonic mean likelihood values from different partition strategies were compared using the Bayes Factor [$2 \times (\text{null hypothesis} / \text{alternative hypothesis})$] in order to determine the

partition model strategy that showed the highest improvement relative to all other models (Brandley et al., 2005). This was selected as the final and most appropriate phylogenetic analysis.

Additional phylogenetic analyses were carried out under the Maximum Likelihood (ML) and Maximum Parsimony (MP) optimality criteria. For ML the data was partitioned into non coding mtDNA and codon positions for each coding mitochondrial and nuclear gene. The analysis was run on a complete, a mitochondrial and a nuclear dataset in RaxML 7.2.8 (Stamatakis, 2006). A bootstrap search with 1000 replicates using a GTRCAT model was performed followed by ML search using GTRGAMMA. Support values were drawn on the best scoring ML tree.

For MP, bootstrapping with 2000 replicates was carried out in PAUP* (Swofford, 2002) using TBR branch swapping and with ten random addition sequence replicates for each bootstrap replicate.

Alternative tree topologies were compared using Shimodaira Hasegawa tests (SH tests) (Shimodaira and Hasegawa, 1999) as implemented in PAUP*, and Approximately Unbiased tests (AU tests) as implemented in Consel (Shimodaira and Hasegawa, 2001; Shimodaira, 2002) (Supplementary materials Table S4).

A species tree phylogeny was calculated in BEAST 1.7.4 (Heled and Drummond, 2010) using 15 partitions run for 200 million generations with sampling every 40,000 generations. Sequences were grouped according to current taxonomy, with *Z. sp. 1* from Daraina added as additional terminal taxon. The tree was calculated under the uncorrelated relaxed, lognormal clock option with fixed means and the Yule tree prior. The run was repeated four times and convergence was assessed using Tracer v 1.5 (Rambaut and Drummond, 2007). Such coalescent based approaches to reconstruct species phylogenies give reliable results compared to other methods, as they account better for incongruence between gene trees (Heled and Drummond, 2010).

Additional analyses including girdle tailed lizards and plated lizards from Africa and Madagascar were performed using previously published 12S and 16S rRNA gene sequences available on GenBank (Supplementary materials Table S5) combined with our rRNA data generated for this study. Sequences of the lizard species *Androngo trivittatus* and *Plestiodon fasciatus* (family Scincidae) were used as outgroups. The data set was partitioned by gene and the best fitting model of evolution was inferred to be GTR + G. Bayesian analyses were run in MrBayes v. 3.1.2 using the same parameters mentioned above, except that 15 million generations were performed and burn in was set to 7500 samples. MP and ML analyses were run as described above.

3. Results and discussion

3.1. Cordyliform family phylogenetic relationships

All plated lizards (Gerrhosauridae) were recovered as sister to a monophyletic African Cordylidae (Platysaurinae and Cordylinae). Our analysis recovered, with strong support, the split within Cordylidae between the *Platysaurus* group and all other cordylids reported by Stanley et al. (2011). Our BI, MP and ML phylogenetic analyses found all Malagasy plated lizards (Zonosaurinae; genera *Zonosaurus* and *Tracheloptychus*) to be monophyletic and sister to the African plated lizards (Gerrhosaurinae); this relationship is robustly supported in all analyses (Fig. S2). The Zonosaurinae are notable for the short internal branch lengths relative to the other three subfamilies of cordyliforms, especially compared to the sister group Gerrhosaurinae and its long internal branches. We suggest this may represent an initial burst of diversification in Malagasy gerrhosaurids after their colonization of the island, though this requires further testing and is beyond the scope of the current paper. The phylogenetic relationships within the well supported Zonosaurinae are the focus of our current study.

3.2. Zonosaurinae phylogenetic relationships

The combined dataset of mitochondrial and nuclear loci consisted of 4708 nucleotides. A total of 3989 nucleotides were retained for analysis after conservatively excluding all variable parts of the 12S and 16S loci that required gaps for alignment, and excluding terminal portions of each partition due to the prevalence of missing data. In the full data set, 3031 characters were constant and 615 were parsimony informative. The final mtDNA data set had 1852 characters of which 1167 were constant and 521 were parsimony informative. The nucDNA data set had 2059 characters of which 1864 were constant and 94 were parsimony informative.

The phylogenetic tree (BI) inferred using the combined data set contained many well supported nodes (Bayesian posterior probabilities (PP) of 1.0) (Fig. 1). The species tree analysis (Fig. 2) resulted in a topology largely congruent with the BI tree of the concatenated sequences, with a remarkable although only weakly supported difference (i.e., the placement of *Zonosaurus* and *Tracheloptychus* as reciprocally monophyletic; see below). However, because effective sample size (ESS) values of some parameters in the BEAST analysis remained low (<200) despite repeating the analysis four times, this result needs to be seen with caution. As expected, separate analyses of mtDNA and nucDNA alone resulted in less robustly supported topologies and some basal nodes were not congruent among analyses (Fig. 1). Nonetheless, several taxonomic relationships were resolved by both data sets.

In particular, our analysis strongly supports the monophyly of the genus *Tracheloptychus*, with *T. madagascariensis* and *T. petersi*, and four major clades within *Zonosaurus* that we here define as species groups (Fig. 1). The following three species groups were strongly supported and recovered by all analyses: (i) the ***Z. madagascariensis* group** that contains *Z. haraldmeieri* clustering within a paraphyletic *Z. madagascariensis*, (ii) the ***Z. rufipes* group** containing *Z. bemaraha*, *Z. brygooi*, *Z. rufipes*, *Z. subunicolor*, *Z. tsingy*, and an undescribed candidate species from northern Madagascar (*Z. sp. 1*), (iii) and the ***Z. ornatus* group** containing *Z. anelanelany*, *Z. laticaudatus*, *Z. karsteni*, *Z. ornatus*, *Z. quadrilineatus*, and *Z. trilineatus*. Furthermore the combined analyses also recovered (iv) a ***Z. boettgeri* group**, with *Z. maximus* and *Z. boettgeri* but with less support, and without support in the MP analysis, presumably due to fewer data available (PP: BEAST = 1.0, MrBayes = 0.72; ML = 63).

Most of these findings are consistent with the phylogenetic analyses of Raselimanana et al. (2009), but there are some substantial improvements to the resolution of relationships, especially for some of the deeper evolutionary nodes (Fig. 2). *Tracheloptychus* and our *Zonosaurus* clades (i) and (ii) were also supported in the tree of Raselimanana et al. (2009), whereas the *Z. ornatus* group and the *Z. boettgeri* group were not recovered in their analyses, likely due at least in part to the absence of *Z. maximus* and the reduced phylogenetic information of their smaller data set.

3.3. Relationships of *Tracheloptychus*

As in previous studies (e.g., Lang, 1991; Raselimanana et al., 2009), the monophyly of the genus *Tracheloptychus* is strongly supported by our analysis. This group consists of the two Malagasy gerrhosaurid species, *T. madagascariensis* and *T. petersi*, possessing keeled dorsal scales (in contrast *Zonosaurus* have smooth scales), and keeled scales underneath digits (rounded in *Zonosaurus*). These two species inhabit the subarid south and south west of Madagascar. Morphological studies (e.g., Lang, 1990, 1991) have so far not reported any clear synapomorphy shared by all *Zonosaurus* to the exclusion of *Tracheloptychus*, despite the obviously different general appearance of these two groups of lizards. The molecular data are ambiguous as well. *Tracheloptychus* is consistently placed

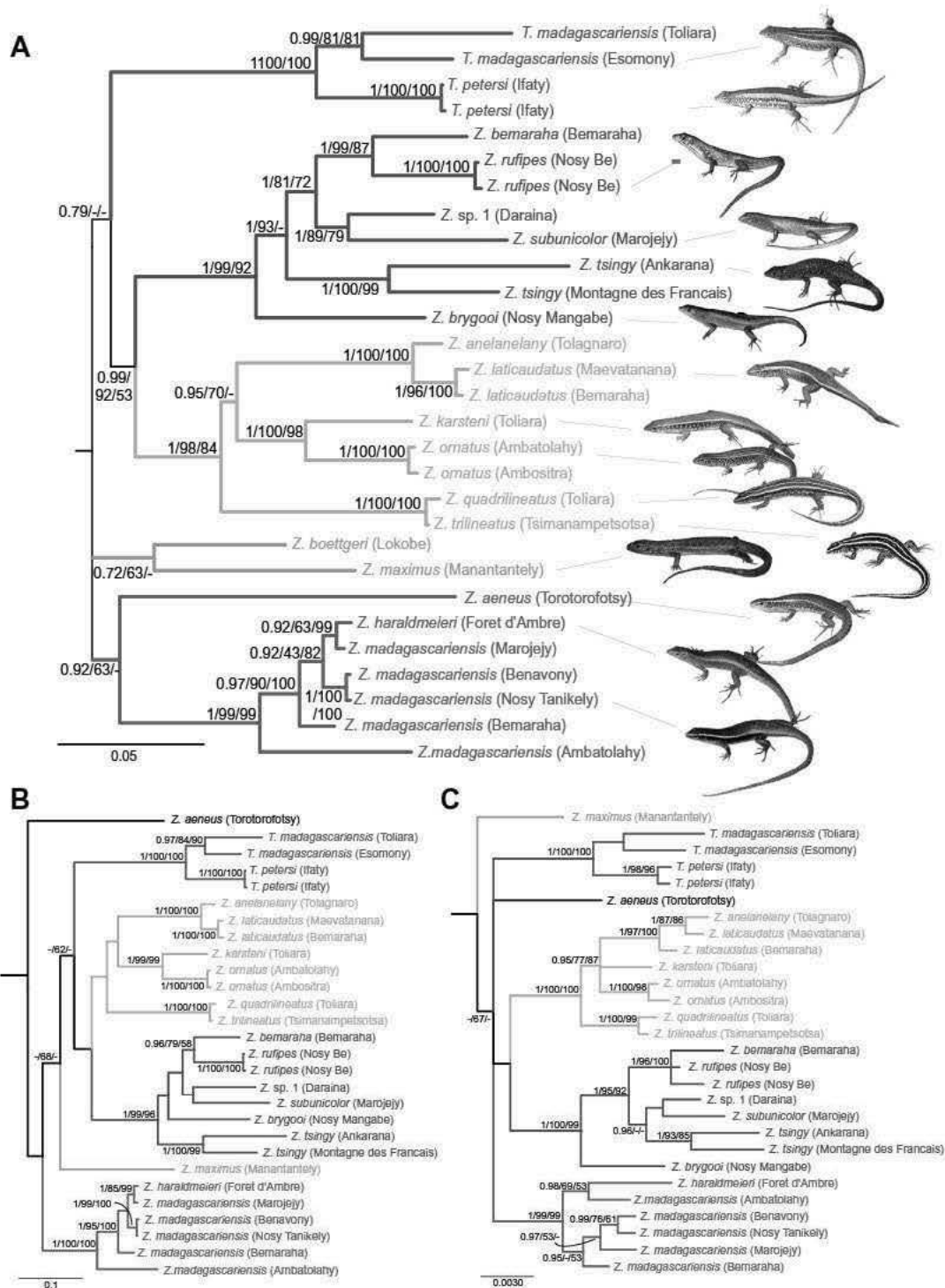


Fig. 1. Phylogenetic trees of species of Malagasy gerrhosaurids (*Zonosaurus* and *Tracheloptychus*) based on an analysis of DNA sequences of mitochondrial and/or nuclear genes. The trees are 50%-majority rule consensus trees from a Bayesian analysis of (A) the whole set of concatenated genes, (B) the mitochondrial gene fragments only (12S, 16S, ND1, COB), and (C) the nuclear gene fragments only (BDNF, PDC, RAG2, CMOS, NT3). Note that *Z. boettgeri* was excluded from calculations based on only either mtDNA or nucDNA due to limited data. Support values are Bayesian posterior probabilities, and bootstrap proportions from ML and MP analyses respectively. Different colors mark species groups congruently suggested by the mtDNA and the nucDNA analysis, as discussed in the text (except the conflicting position of *Z. aeneus* in the *Z. madagascariensis* group). The tree was rooted using an African gerrhosaurid (*Gerrhosaurus* cf. *nigrolineatus*) as the outgroup. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

within *Zonosaurus* in the concatenated analyses, but mitochondrial and nuclear data do not resolve the relationships among the main clades within Malagasy plated lizards, including *Tracheloptychus* (indicated by low support for the topology of deep nodes, Fig. 1).

The species tree analysis placed *Tracheloptychus* sister to a monophyletic *Zonosaurus* (Fig. 2) with low support (PP = 0.62) and this position was not significantly excluded by AU (near significant, $p = 0.054$) and SH tests (Supplementary materials Table S4 and

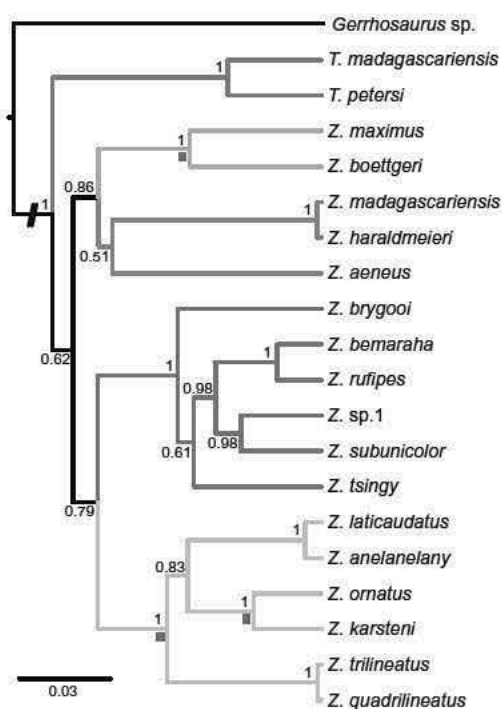


Fig. 2. Species tree phylogeny of Malagasy gerrhosaurids as obtained by the BEAST species tree reconstruction method. Colors refer to main clades (as in Fig. 1). Bayesian posterior probabilities are indicated on each node. Nodes with high support values (PP > 0.95) that were not resolved in previous studies are indicated with a gray square. Four independent runs of the species tree analysis resulted in identical topologies and effective sample size (ESS) values >200 in the majority of parameters, including the likelihood values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. S3). Although it is possible that *Tracheloptychus* should be considered a synonym of *Zonosaurus*, more data are needed before formalizing this conclusion taxonomically.

3.4. Relationships within *Zonosaurus* species groups

The *Z. boettgeri* group as defined herein consists of *Z. boettgeri* and *Z. maximus* in our present phylogeny. The clade was well supported in the species tree analysis (PP = 1.0) (Fig. 2), and less well supported in the combined analysis (PP = 0.72, ML = 63) (Fig. 1). A third species, *Z. maramaintso*, could not be included in our analysis because we had no sample available and there are no sequence data for it available in public databases, but is presumably closely related to *Z. boettgeri* (Raselimanana et al., 2006). Lang (1990) also placed *Z. boettgeri* and *Z. maximus* together in a clade that additionally contained *Z. ornatus*. He based this grouping on obliquely keeled plantar scales found in *Z. ornatus* and *Z. maximus* (and *Z. karsteni*), and fused prefrontal scales in *Z. boettgeri* and *Z. maximus* (plus several other species for which convergent evolution of this character state was assumed). Hence, he did not identify any synapomorphy exclusively shared by *Z. boettgeri* and *Z. maximus*. Statistical tests of the molecular data (Fig. S3) rejected *Z. ornatus* belonging to this clade, indicating morphological convergence of the keeled plantar scales in *Z. maximus* on one hand and the *Z. ornatus/karsteni* clade on the other hand. *Z. boettgeri* and probably *Z. maramaintso* are specialized arboreal lizards with a very long tail while *Z. maximus* is a large sized semi aquatic lizard with a laterally compressed tail. One possible synapomorphy of these taxa is their comparatively low number of dorsal scale rows (14–16 in *Z. boettgeri* and *Z. maramaintso*,

and 18–21 in *Z. maximus*, vs. reaching more than 21 in all other species; Brygoo and Böhme 1985).

Our data are in agreement with previous molecular studies (Yoder et al., 2005; Raselimanana et al., 2009) in suggesting that *Z. haraldmeieri*, a species from the extreme north of Madagascar, is phylogenetically deeply nested within the widespread *Z. madagascariensis*, making *Z. madagascariensis* paraphyletic (Fig. 1). We suggest naming this clade the *Z. madagascariensis* group (into which we also tentatively place *Z. aeneus*; see discussion on that species below). The paraphyly of *Z. madagascariensis* is supported by both mitochondrial and nuclear markers, though the relationships within this group are not consistent across markers; nucDNA analyses placed *Z. haraldmeieri* sister to the southern most *Z. madagascariensis* included (from Ambatolahy) that in mtDNA was most divergent from all other individuals. Given that morphological differences between these two species are restricted to color (uniform greenish in *Z. haraldmeieri* vs. distinct dorsolateral stripes in *Z. madagascariensis*; see Lang, 1990), the species status of *Z. haraldmeieri* is doubtful (Raselimanana et al., 2009). The differentiation of *Z. haraldmeieri* in at least some nuclear genes (see also Raselimanana et al., 2009) suggests that the status of this taxon also requires more detailed population genetic work. The occurrence of a green colored isolated population (*Z. haraldmeieri*) in northernmost Madagascar, nested within the widespread brown colored *Z. madagascariensis*, bears a conspicuous similarity to the pattern encountered in the frogs *Mantella viridis* and *M. eburni*; in these, populations with these different color patterns in northern Madagascar are divided by a barrier to gene flow despite widespread haplotype sharing, and this barrier might be related to bioclimatic differences (Crottini et al., 2012a).

The phylogenetic position of *Z. aeneus* remains unsolved. It forms the sister group of the *Z. madagascariensis/haraldmeieri* clade in the species tree and the concatenated analyses, but with little congruence across genes and rather low support (Figs. 1 and 2). We here include this species tentatively in the *Z. madagascariensis* group considering also its striking similarity to *Z. madagascariensis* in color pattern (Glaw and Vences, 2007), but we are aware that this classification might require revision once that new data become available.

The *Z. rufipes* group as defined herein contains small sized, forest dwelling *Zonosaurus* species that are characterized by the presence of three rather than four supralabials anterior to the subocular (Vences et al., 1996). MtDNA and nucDNA supported close relationships between *Z. bemaraha* and *Z. rufipes*, and of *Z. subunicolor* with *Z. sp. 1* from Daraina. Either *Z. tsingy* (in the mtDNA data set) or *Z. brygooi* (in the nucDNA and complete data set) resolved as sister to those species in this clade (Fig. 1).

Besides species of the *Z. rufipes* group and both species of the genus *Tracheloptychus*, two other species of Malagasy plated lizards are characterized by three supralabials anterior to the subocular and smaller body size: *Z. aeneus* and *Z. anelanelany*. Phylogenetic arrangements that place all taxa with this morphological character in a monophyletic group or otherwise reduce the homoplasy for this character could in most cases be rejected by our analyses as detailed in the following: None of our phylogenetic analyses place *Z. aeneus* or *Z. anelanelany* in the *Z. rufipes* group, nor are these two species recovered as sister taxa (Figs. 1 and 2). Alternative topologies in which these two species are constrained hierarchically as sister to the *Z. rufipes* group (per Lang, 1991) or sister to the rest of the Malagasy gerrhosaurids are significantly rejected by SH and AU tests ($p < 0.001$; alternative trees 2 and 3 in Supplementary materials, Fig. S3 and Table S4) and none of them is supported by the species tree (Fig. 2). Another alternative topology in which only *Z. aeneus* is sister to the rest of the *Z. rufipes* group is only marginally rejected by the AU test ($p = 0.056$) and is not rejected by the SH test (Table S4). Furthermore, the alternative

topologies of *Tracheloptychus* as the sister group to all *Zonosaurus*, or similarly, an alternative with *Z. aeneus* (but not *Z. anelanelany*) sister to the *Z. rufipes* group and *Tracheloptychus* moved to become the sister group of all *Zonosaurus* are not rejected by any of the tests (trees 5 and 6 in Supplementary materials).

In previous assessments (e.g., Lang, 1990; Vences et al., 1996; Vences et al., 1999), *Z. aeneus* was included with *Z. brygooi*, *Z. rufipes*, and *Z. subunicolor* in a clade named the “*Z. aeneus* group”. Yet our current analyses clearly place *Z. aeneus* phylogenetically distant from these other species (Figs. 1 and 2). *Z. aeneus* inhabits rather open areas within forest or at forest edges, while species of the *Z. rufipes* group are typically found in sunlit spots within rather dense forest (Vences et al., 1996; Raselimanana et al., 2000; Glaw and Vences, 2007). This difference in the species' ecology is thus in agreement with the lack of close relationships among *Z. aeneus* and the *Z. rufipes* group. Interestingly, *Z. sp. 1* from Daraina, which unambiguously resolves sister to *Z. subunicolor*, bears a strong morphological similarity to *Z. aeneus* while occurring in more dense parts of the forest (A.P. Raselimanana, unpublished data), highlighting that morphology alone is a poor indicator of phylogenetic relatedness in these lizards.

Homoplasy in the evolution of body size and supralabial configuration in Malagasy plated lizards is furthermore suggested by the strongly supported position of *Z. anelanelany* sister to the included samples of *Z. laticaudatus*. The data of Raselimanana et al. (2009) indicated that *Z. anelanelany* is phylogenetically nested within *Z. laticaudatus*, being more closely related to those *Z. laticaudatus* populations occurring in the south of Madagascar. Single gene analysis of our data revealed that, besides mtDNA, three nuclear genes (BDNF, CMOS, RAG2) support close relationships of *Z. anelanelany* to *Z. laticaudatus* (not shown). A further gene (NT3) had too little variability to reveal any clear grouping, and no *Z. anelanelany* sequence was obtained for PDC. The congruent placement of *Z. anelanelany* with *Z. laticaudatus* by four independent markers (combined mtDNA, BDNF, CMOS, RAG2) unambiguously suggests close phylogenetic relationships among these taxa rather than a confounding pattern of mtDNA introgression. In our analysis, only a single sample of *Z. anelanelany* was included, but the same pattern was found based on multiple specimens (but fewer markers) studied by Raselimanana et al. (2009) and sample confusion is therefore unlikely. *Z. laticaudatus* and *Z. anelanelany* occur sympatrically in the extreme south east of Madagascar, but use different habitats (rocky substrate and open areas vs. almost closed canopy humid forest) and maintain their clear morphological distinctness. Our analysis did not include samples of southern *Z. laticaudatus*, which share CMOS haplotypes with *Z. anelanelany* (Raselimanana et al., 2009). More fieldwork in the contact zones of these two species is needed to understand their population genetic, morphological and ecological differentiation.

The ***Z. ornatus* group** as defined herein mostly contains species specializing in arid and subarid habitats in western and southern Madagascar plus *Z. ornatus*, which inhabits montane ericoid and thicket vegetation as well as rainforest edges in the middle and southern central east, and *Z. anelanelany*, which occurs in south eastern humid environments. The molecular study by Raselimanana et al., (2009) did not detect this clade; however, all our analyses strongly support its monophyly (Figs. 1 and 2). *Zonosaurus laticaudatus* is the most generalist species in this group with regard to habitat (wet or arid, gallery forest, dry forest, rocky or limestone), while *Z. ornatus* occurs over the widest elevational range (from near sea level in Manombo National Park, to above 2000 m a.s.l. in the Andringitra and Ankaratra Massifs). Both mtDNA and nucDNA analyses agree in placing the two closely related and morphologically similar species, *Z. quadrilineatus* and *Z. trilineatus*, as sister to the rest of this clade. Furthermore, *Z. karsteni* is resolved as sister species of *Z. ornatus* and as mentioned above, *Z. anelanelany*

is sister to *Z. laticaudatus*. Previous analyses (Lang, 1990; Raselimanana et al., 2009) unambiguously placed *Z. quadrilineatus* and *Z. trilineatus* together in a clade. A further study based on 16S sequences only and with limited taxon sampling obtained a clade containing *Z. karsteni*, *Z. ornatus* and *Z. trilineatus* (Odierna et al., 2002). Therefore multiple lines of evidence support this biological grouping.

4. Conclusions

While the broad level relationships of the cordyliforms are fairly well established (Conrad, 2008), internal relationships within the Gerrhosauridae have remained conflicted or less well resolved (e.g. Lang, 1991; Odierna et al., 2002; Lamb et al., 2003; Raselimanana et al., 2009). Our present study has contributed information from mitochondrial and nuclear molecular markers and sought to clarify some of the relationships with Zonosaurinae in particular. Our analysis strongly supports five internal groups, of which two have not been detected previously: *Tracheloptychus*, the *Z. rufipes* group, the *Z. madagascariensis* group, and the newly identified *Z. ornatus* and *Z. boettgeri* groups. Questions remain as to the validity of the genus *Tracheloptychus*, which might render *Zonosaurus* paraphyletic. However, the species tree supported *Tracheloptychus* being sister to *Zonosaurus* (Fig. 2) which differs from our combined tree including all specimens (Fig. 1) and suggests that more extensive molecular data will be required to clarify the relationships. Some of the basal nodes within the Malagasy Gerrhosauridae thus remain unresolved and may require even more data to increase resolution. We hypothesize that there has been a fast initial radiation of the Malagasy gerrhosaurids that makes it difficult to resolve the deeper nodes of this phylogenetic group.

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