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Short Communication

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

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ABSTRACT

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. quadrilineaus*, 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.

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 subarid 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 cordyliforms, 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*, *Cordylosaurus*, *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), phosducin (PDC), 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 materialsTable 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 materialsTable 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 (±<0.001%), and the PSRF value was ~1.001.

Harmonic mean likelihood values from different partition strategies were compared using the Bayes Factor [2 * (null hypothesis – alternative hypothesis)] in order to determine the