Opening the black box: phylogenetics and morphological evolution of the Malagasy fossorial lizards of the subfamily “Scincinae”

A. Schmitz, M.C. Brandley, P. Mausfeld, M. Vences, F. Glaw, R.A. Nussbaum, T.W. Reeder

Abstract

The island of Madagascar harbors a highly endemic vertebrate fauna including a high diversity of lizards of the subfamily “Scincinae,” with about 57 species in eight genera. Since limb reduction seems to have been a common phenomenon during the evolution of Malagasy “scincines,” diagnosing evolutionary relationships based on morphology has been difficult. Phylogenetic analyses of multiple mitochondrial DNA sequences including the entire ND1, tRNA<sub>LEU</sub>, tRNA<sub>ILE</sub>, tRNA<sub,GLN</sub> genes, and fragments of the 12S and 16S rRNA and tRNA<sub>MET</sub> genes were conducted to test the monophyly of the largest genus Amphiglossus, and to evaluate the various formal and informal species groupings previously proposed for this species-rich group. A further objective was to determine the phylogenetic placements of the several greatly limb-reduced and limbless Malagasy “scincines” and ascertain whether any of these are derived from within the morphologically plesiomorphic Amphiglossus. As limb reduction in skinks is mostly associated with body elongation via an increase in the number of presacral vertebrae, we evaluate the pattern of evolution of the numbers of presacral vertebrae in the context of our phylogeny. We demonstrate that Amphiglossus as currently diagnosed is non-monophyletic, and the species fall into two major groups. One of these groups is a clade that contains the included species of the subgenus Amphiglossus (Madascincus) among other species and is a member of a larger clade containing Paracontias and Pseudoacontias. In the second group, the nominate subgenus Amphiglossus (Amphiglossus) forms several subclades within a larger clade that also contains Androngo crenni and Pygomeles braconnieri, and is sister to Voeltzkowia. All analyses provide strong support for the monophyly of Paracontias and Voeltzkowia. Based on the preferred phylogenetic hypothesis and weighted squared-change parsimony we show that the ancestor of the Malagasy clade was already elongated and had a moderately high number of presacral vertebrae (46–48), which is hypothesized to be the ancestral condition for the whole Malagasy “scincine” clade. We further demonstrate that both multiple increases and reductions of presacral vertebrae evolved in many clades of Malagasy “scincines” and that the use of presacral vertebrae as a major character to diagnose supraspecific units is dubious. Based on our results and published morphological evidence we consider Scelotes waterloti Angel, 1930 to be a junior synonym of Amphiglossus reticulatus (Kaudern, 1922).

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1. Introduction

Within Squamata, scincid lizards (skinks) are a particularly diverse group with four recognized subfamilies: the Acontinae, Feylininae, Lygosominae, and paraphyletic “Scincinae.” The family contains ~1260 species, making it the most speciose lizard family, and among squamates is only outnumbered by the snake family “Colubridae” (>1800 species) (Pough et al., 2004). However, scincid diversity extends beyond pure numbers of species. The family is distributed worldwide and displays a remarkable array of morphological variation including the presumably repeated, convergent evolution of body elongation and extreme limb reduction. The lack of phylogenies for many skink clades has prohibited researchers from examining these phenomena in a phylogenetic context. This is particularly true for the diverse fauna of skinks from Madagascar. Due to their secretive lives and the scarcity of available material in collections, very little is known about the diversity, geographic distribution, and phylogenetic affinities of these lizards. This situation prompted Greer (1970), in his analysis of the subfamily “Scincinae” (=“scincines”), to describe the island as a “...’black box’ of our analysis of scincine evolution....” Until relatively recently, only 44 species from seven different “scincine” genera were known from Madagascar (Glaw and Vences, 1994), but the number of described Malagasy “scincines” has risen substantially in the last 10 years. Currently, there are no less than 57 species from eight genera known from Madagascar (e.g., Andreone and Greer, 2002; Nussbaum and Raxworthy, 1995; Sakata and Hikida, 2003a,b; see Appendix).

Because of their fossorial/semi-fossorial nature (many species burrow in leaf litter, sand, soil, or rotten wood), several taxa in the different “scincine” genera have partially or completely lost their limbs (Andreone and Greer, 2002; Nussbaum and Raxworthy, 1995). As limb reduction has presumably evolved multiple times within these skinks, diagnosing evolutionary relationships based on morphology has been difficult. Coupled with the scarcity of museum specimens, this widespread morphological convergence has hindered phylogenetic studies of these lizards. Greer (1970) hypothesized that the Malagasy “scincines” are part of a more inclusive “scincine” group inhabiting sub-Saharan Africa, the Seychelles, and Mauritius. Extensive taxonomic revisions of Malagasy “scincines” were published by Brygoo between 1979 and 1987 (Brygoo, 1979, 1980a,c, 1981a,b,c,d, 1983, 1984a,b,c,d,e, 1985, 1987). He provided a re-definition of the largest genus Amphiglossus and distinguished two subgenera (Amphiglossus and Madascin-}

Keywords: Scincinae; Molecular phylogeny; Presacral vertebrae; Partitioned Bayesian analyses; Mixed-models; Weighted squared-change parsimony; Madagascar; Bayes factors
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2. Materials and methods

2.1. Choice of terminal taxa

In all, 29 Malagasy “scincine” species were included in this study (with several species represented by multiple individuals; Table 1): *Amphiglossus* (19 species), *Androngo crenni*, *Paracontias* (4), *Pseudoacontias menamainty*, *Pygomeles braconnieri*, and *Voeltzkowia* (3). Additional non-Malagasy “scincines” (*Eumeces* sensu lato [Brandley et al., 2005; Schmitz et al., 2004], *Proscelotes, Scincus*) as well as one acontine (*Acontias meleagris*) were sampled as outgroups. Because of the uncertain higher-level relationships among skinks and the fact that the Malagasy “scincines” may not represent a clade, the overall skink phylogeny was simultaneously rooted with one cordylid and one gerrhosaurid (*Cordylus* and *Zonosaurus*, respectively). The Cordylidae and the Gerrhosauridae are generally thought to be closely related to the Scincidae, with these three families forming the Scincoidea (Estes et al., 1988; Lee, 1998; Townsend et al., 2004). General locality and voucher information is provided in Table 1.

2.2. DNA amplification, sequencing, and alignment

DNA was extracted from tissue using QiaAmp kits (Qiagen) or a standard phenol/chloroform/proteinase-K protocol (Hillis et al., 1996). Multiple mitochondrial DNA fragments were amplified including the entire ND1, tRNA LEU, tRNA ILE, tRNA GLN genes, and partial fragments of the 12S and 16S rRNA and tRNA MET genes. PCR and sequencing primers are given in Table 2. Sufficient PCR product was generated after 33–40 cycles (12S and 16S fragments: 94°C for 45–60 s, 50–55°C for 30–45 s, and 72°C for 60–90 s; ND1 fragment: 94°C for 60 s, 50–58°C for 30 s, and 72°C for 60–90 s). PCR products were purified using Qiaquick purification kits (Qiagen) or PEG/NaCl precipitation. Purified PCR templates were sequenced using dye-labeled dideoxy terminator cycle sequencing on an ABI 377 automated DNA sequencer.

The ND1 protein-coding sequences were aligned by eye. The 12S, 16S, and tRNA data were aligned with reference to published secondary structure maps (12S: Titus and Frost, 1996; 16S: Gutell and Fox, 1998; and tRNAs: Kumazawa and Nishida, 1993). To assess positional homology in the 12S and 16S loops, each data set was aligned under varying pairwise and multiple gap costs (6, 9, and 12) using ClustalX (Thompson et al., 1997). Nucleotide positions that changed under one or more different gap costs were considered ambiguously aligned and were excluded from the phylogenetic analyses (Gatesy et al., 1993; Milinkovitch and Lyons-Weiler, 1998). In some regions of the 12S and 16S data, the ability to align the data for the skinks was improved if the
sequences for Cordylus and Zonosaurus were removed (their corresponding data replaced with ‘‘?’’ in these regions). Because our explicit goal is to test the relationships of Amphisidius and its relatives, and not the monophyly of Scincidae, we feel the exclusion of these two taxa is justified. All DNA sequences have been deposited in GenBank (Table 1).

2.3. Phylogenetic analyses

Phylogenetic analyses were conducted using maximum parsimony (MP), maximum likelihood (ML), and partitioned Bayesian methods. MP and ML analyses were implemented in PAUP* 4.0b10 (Swofford, 2002). The MP heuristic search consisted of 1000 random addition sequence replicates, TBR branch swapping, and gaps coded as missing data. The ML phylogeny was estimated following a successive approach similar to that described by Swofford et al. (1996) and Wilgenbusch and de Queiroz (2000), with Modeltest 3.0 (Posada and Crandall, 1998) being used to test alternative models of sequence evolution. An initial ML tree was constructed using the JC model (Jukes and Cantor, 1969; as-is stepwise addition, TBR branch swapping). The best model (and model parameters) estimated by Modeltest from this initial tree were used in a subsequent ML heuristic tree search (20 random addition sequence replicates, TBR branch swapping). If the resulting ML tree differed from the initial starting tree, then all models were re-tested on the new tree, followed by a new ML tree search. This process was iterated until the $-\ln L$ stabilized.

All partitioned Bayesian analyses were implemented with MrBayes 3b4 (Huelsenbeck and Ronquist, 2001). Because different genes and gene regions may be under very different biochemical constraints, they may also evolve under very different models of evolution. It has been demonstrated previously that applying different models to different subsets of the data (i.e., partitioned or mixed-model analyses) may yield better estimates of phylogeny (as measured by $-\ln L$) and, in some cases, improved estimates of posterior probabilities (Brandley et al., 2005; Nylander et al., 2004). Thus, we took advantage of the ability of MrBayes 3b4 to perform partitioned analyses.

Because numerous partitioning strategies are possible, we employed the method of Brandley et al. (2005) and used the Bayes factor to select among a priori selected partitioning strategies. Our goal was to choose a partitioning strategy that modeled the data well, but did not include extraneous partitions. We selected six partitioning strategies ranging from six total partitions to no partitions (i.e., a traditional, single-model analysis) (Table 3). All partition strategies are denoted with a capital P and a numerical subscript identifying the number of data partitions (e.g., $P_1$, $P_6$, etc.). Additional subscript letters identify multiple partitioning strategies that have the same number of data partitions but partition the data differently (e.g., $P_{AB}$, $P_{AB}$, etc.). We then used the Bayes factor to compare the results of the most-partitioned analysis to the alternative strategies with fewer partitions. If a strategy using fewer partitions was not strongly different from the most partitioned, then this strategy was chosen as the best partitioning scheme (i.e., the one that best modeled the data, but with the fewest partitions). Bayes factors were estimated by calculating the difference of the ln-transformed harmonic means of the posterior likelihoods between the two analyses being tested (Newton and Raftery, 1994). Harmonic means were estimated using the $smp$ command in MrBayes. We used a 2ln Bayes factor $>10$ as the criterion.
for strong support (Brandley et al., 2005; Huelsenbeck and Imenov, 2002; Kass and Raftery, 1995).

Models for each partition were determined using the likelihood-ratio test implemented by MrModeltest (Nylander, 2002). All partitioned Bayesian analyses consisted of $2 \times 10^7$ generations (started on random trees) and four incrementally heated Markov chains (using default heating values), sampling the Markov chains at intervals of 1000 generations. The first $4 \times 10^6$ generations were discarded as “burn-in” and we confirmed stationarity by tracking the posterior probabilities of individual clades through time using the `cump` and `slide` command in Converge v0.1 (Warren et al., 2003). Stationarity was assumed when the cumulative posterior probabilities of all clades stabilized. To ensure the Bayesian analyses were not trapped on local optima, three separate analyses were performed (per partitioning strategy), mean $-\ln L$ scores were compared for each of the three runs, and posterior probability estimates for each clade were compared between the three analyses using scatterplots created by the `compare2trees` command in Converge. If apparent convergence on the same optimum was determined for all three analyses, the postburn-in trees for the three analyses were combined.

The percentage of samples (pooled for a given data set) recovering any particular clade represents that clade’s posterior probability (Huelsenbeck and Ronquist, 2001; Huelsenbeck et al., 2001). Unlike non-parametric bootstrap proportions which are known to be conservative estimates of clade confidence (Hillis and Bull, 1993), recent simulation studies (e.g., Alfaro et al., 2003; Erixon et al., 2003; Wilcox et al., 2002) have demonstrated that Bayesian posterior probabilities are less biased estimators of confidence and thus generally represent much closer estimates of true clade probabilities (referred to as “$P_p$” throughout). Also, whereas the Bayesian approach may be more sensitive to signal in the sequence data (i.e., provide higher confidence for short internodes; Alfaro et al., 2003), there is also an increased chance of the Bayesian method assigning higher confidence to incorrectly inferred short internodes because of the stochastic nature of the underlying model of evolution (Alfaro et al., 2003; Erixon et al., 2003). Given this, clades with $P_p \geq 0.95$ were generally considered strongly (significantly) supported, but with the caveat that relatively high posterior probabilities for short internodes (particularly those that might receive low bootstrap values) may be overestimates of confidence.

Nodal support for the MP analyses was inferred using the non-parametric bootstrap (5000 pseudoreplicates, 100 random addition sequences/pseudoreplicate, and TBR branch swapping). Clades with bootstrap values (referred to as “$BS$” throughout) of $\geq 70\%$ were considered strongly supported (Hillis and Bull, 1993).

Table 4
Number of presacral vertebrae (PSV) for the Malagasy “scincines” and Proscelotes eggeli (sub-Saharan “scincine”)

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</tr>
<tr>
<td>Voeltzkowia fierinensis</td>
<td>47; 46–50</td>
<td>Brygoo (1981c)</td>
</tr>
<tr>
<td>Voeltzkowia lineata</td>
<td>50; 48–54</td>
<td>Brygoo (1981c)</td>
</tr>
</tbody>
</table>

a Modal number of presacral vertebrae (except where noted), followed by range (when present).
b Mean number of presacral vertebrae.
2.4. Ancestral reconstruction of number of presacral vertebrae

Evolutionary changes in the number of presacral vertebrae were evaluated by mapping these attributes onto the preferred partitioned Bayesian phylogeny (with branch lengths estimated from the mean posterior density; see Section 3). Data on the number of presacral vertebrae are available in the literature for all the described Malagasy “scincine” species included in this present study (see Table 4). Ancestral character state reconstructions were performed in Mesquite v1.0 (Maddison and Maddison, 2003).

When multiple individuals of a given species are examined, a range of presacral vertebrae numbers (Table 4) is often present, but usually a “common” number is evident. Thus, in our analysis we used the modal number of presacral vertebrae. The evolution of presacral vertebrae number was reconstructed using the method of weighted squared-change parsimony (Maddison, 1991) which weights the minimized sum of the squared-changes by dividing this measure by a given branch length (implemented in Mesquite). Also, for those species represented by multiple individuals, the “single” species branch length was based on the summed branch lengths of all individuals. This weighted squared-change parsimony method of evaluating the evolution of continuous data was preferred over a more traditional parsimony approach of mapping discrete attributes onto a phylogeny. A discrete parsimony approach could be implemented by assigning a unique character state for each possible number of vertebrae between 30 and 67 and ordering the array. However, Mesquite (as well as MacClade v4; Maddison and Maddison, 2000) can handle only 26 discrete character states. Though information is lost, it was possible to recode the presacral vertebrae numbers into ordered character state bins of two vertebrae; thus, reducing the effective number of unique character states to 19. The overall general patterns and results of such a discrete parsimony approach were essentially the same (results not shown) as those from the weighted squared-change parsimony approach; however, the ancestral reconstructions at many of the internal nodes were equivocal. Presacral vertebrae numbers will be abbreviated “PSV” throughout the text. All reported ancestral reconstructions are rounded to the nearest whole number.

3. Results

3.1. Phylogenetic analyses

The complete alignment consisted of 2732 bp. In total, alignments for 370 positions were ambiguous; thus, the analyzed sequences constituted a matrix of 2362 characters. Of these, 1130 sites were variable and 935 were parsimony-informative.

The maximum parsimony analysis inferred one most parsimonious tree (tree length = 6438; Fig. 1). The single optimal tree from the single-model ML analysis (TVM + 1 + Γ; −ln L = 29899.195; parameters provided in Table 5; tree not shown) and the majority-rule consensus tree from the partitioned Bayesian analysis (Fig. 2) were essentially the same. The only difference involved the placement of Amphiglossus tsaratananensis, with the ML analysis placing this species as the sister lineage to the clade containing A. tanysoma, A. ornati-ceps, A. melanurus, and Androngo crenni. All phylogenetic analyses (MP, ML, and Bayesian) clearly show that Amphiglossus is non-monophyletic. On the other hand, all analyses provide strong support for the monophyly of Paracondrias and Voeltzkowia.

The results of the partitioned Bayesian analyses and estimated Bayes factor comparisons are provided in Table 6. Partitioning the ND1 data by codon position had a dramatic effect on the mean −ln L (compare P4 and P4A to the other strategies), a result consistent with Brandley et al. (2005). Employing six partitions resulted in an improvement of mean −ln L that was very strongly better than any of the alternative strategies according to the Bayes factor. Thus, the six-partition analysis is our preferred partitioning strategy and all discussion of Bayesian phylogeny and clade posterior probabilities will be limited to the results of this analysis.

From this point, we will primarily present the specific relationships inferred by the partitioned Bayesian analysis employing six partitions (Fig. 2) since it better models the evolution of the molecular data (though we also mention the MP support values for the different clades). The partitioned Bayesian analysis strongly supports two separate monophyletic groups that together contain all species of “Amphiglossus” (Fig. 2), with Androngo and Pygomeles being nested within one of these major clades. One major clade contains the included species of the subgenus Madascincus (“A.” melanopleura and “A.” morou-undavae), as well as “A.” intermedius, “A.” stumpffi, “A.” ignecaudatus, and “A.” namus. We will refer to this clade as the Madascincus group hereafter. The second major clade contains the three large semi-aquatic species of the nominate subgenus Amphiglossus (“A.” astrolabi, “A.” reticulatus, and “A.” waterloti), the remaining as yet unclassified “Amphiglossus” species, Androngo crenni, and Pygomeles braconnieri. We will refer to this clade as the nominate Amphiglossus group hereafter.

“Amphiglossus” splendidus is placed as sister taxon to Pygomeles, though without strong support (BS < 50; PP = 0.90). Additionally “A.” splendidus and Pygomeles form a clade exclusive of the rest of the nominate Amphiglossus group, though the level of support for this specific placement within the nominate Amphiglossus group is weak. The only included member of Androngo is nested
within the nominate *Amphiglossus* group and is consistently placed as the sister taxon to "A." *tanyosoma* (BS = 63; PP = 1.0). *Voeltzkowia* is strongly supported as monophyletic (BS = 100; PP = 1.0) and is placed as the sister clade to the nominate *Amphiglossus* group (BS = 67; PP = 0.99). *Paracontias* is also a well-supported monophyletic group (BS = 99; PP = 1.0) and is placed as the sister clade to the *Madascincus* group.

The partitioned Bayesian analysis places *Pseudoacontias menamainty* as the sister taxon of the *Paracontias + Madascincus* group clade (Fig. 2). However, this specific placement of *Pseudoacontias* is not strongly supported in the partitioned Bayesian analysis and the MP analysis weakly supports a relatively more basal position within the Malagasy "scincine" clade, as sister taxon to "A." *nanus* (Fig. 1).
3.2. Evolution of presacral vertebrae

Based on the preferred phylogenetic hypothesis (Fig. 2) and weighted squared-change parsimony, 47 presacral vertebrae (PSV) is hypothesized to be the ancestral condition for the Malagasy “scincine” clade (Fig. 3). While the placement of the relatively basal Pseudoacontias is weakly supported (Figs. 1 and 2), there is relatively strong support for its exclusion from the two major basal clades (i.e., Clade A = Paracontias + Madascincus group [PP = 0.92] and Clade B = Voeltzkowia + nominate Amphiglossus group [PP = 1.0]) (Fig. 2). The potential alternate placements of the greatly elongated Pseudoacontias (∼67 PSV) as the sister taxon of Clade B (Fig. 3) or the sister taxon of all remaining Malagasy “scincines” does not greatly change the hypothesized ancestral number of presacral vertebrae (46–48 PSV). Thus, evidence appears strong that the ancestral Malagasy “scincine” possessed a relatively high number of presacral vertebrae compared to the
lowest numbers exhibited by some “Amphiglossus” species nested well within different parts of the phylogeny (e.g., ~30 PSV in “A.” melanopleura and “A.” mouroundavae; ~32 PSV in “A.” punctatus).

According to the scenario suggested by our results, the ancestor of the Malagasy “scincines” was elongate with a moderate number of presacral vertebrae (46–48 PSV) and evolution to the extremes exhibited within this clade (high and low PSV) has occurred independently multiple times. The highest numbers of presacral vertebrae have independently evolved in the following lineages or clades: Pseudoacontias menamainty (67 PSV), Pygomeles braconnieri (62 PSV), Paracontias (51–63 PSV), and Androngo crenni (56 PSV). The evolution of an elevated number of presacral vertebrae has also occurred in Voeltzkowia (e.g., to 50 PSV in V. lineata). And finally, the highest number of PSV exhibited by any of the “Amphiglossus” species is 52 and is exhibited by “A.” tanysoma, which is strongly placed as the sister taxon of the elongate Androngo crenni (BS = 63; PP = 1.0).

There is strong phylogenetic evidence for the evolution to a relatively low number of presacral vertebrae (30–40) occurring independently within the major Clades A and B (Fig. 3). This conclusion is based on the assumption that the ancestor of the overall Malagasy clade possessed a moderately high number of presacral vertebrae (46–48 PSV; further elaboration on this assumption in Section 4). Within Clade A, all the “Amphiglossus” represent a strongly supported clade, with some of the basal-most members exhibiting the lowest numbers of presacral vertebrae among Malagasy “scincines” (i.e., “A.” melanopleura and “A.” mouroundavae; 30 PSV). The weighted squared-change parsimony reconstruction for the ancestor of this “Amphiglossus” clade is 41 presacral vertebrae, with a subsequent reduction to 30 in “A.” melanopleura. There is also a further hypothesized reduction in the common ancestor of the clade containing the remaining “Amphiglossus” of Clade A (exclusive of “A.” melanopleura), with additional independent reductions to much lower numbers of presacral vertebrae in “A.” nanus and “A.” mouroundavae (30 and 33 PSV, respectively).

Within Clade B, there also appears to have been multiple independent reductions in presacral vertebrae number (as well as multiple increases). However, because many of the inferred relationships within this major clade are weakly supported, identifying the exact number and sequence of reduction events is difficult. Even so, one of the lowest numbers of presacral vertebrae exhibited by a Malagasy “scincine” is found within Clade B (i.e., “Amphiglossus” punctatus with 32 PSV; other “Amphiglossus” species with similar PSV number are members of Clade A). Given the strong support of the nested position of “A.” punctatus within Clade B,
where all the remaining species possess $\geq$ 36 presacral vertebrae (and the basal-most species generally possess many more), it appears there is strong evidence once again that significant and recurrent reduction of presacral vertebrae has occurred among the Malagasy "scincines."

4. Discussion

Relationships among species of "Amphiglossus" have been controversial (Glaw and Vences, 1994; Raxworthy and Nussbaum, 1993) mainly because of their morphological similarity. The data presented in this study provide the first molecular phylogeny of the "scincines" of Madagascar and provide an independent means of testing the different taxonomic schemes.

4.1. "Amphiglossus" phylogeny

One of the most intriguing results from this study is that the genus "Amphiglossus" as currently recognized is not-monophyletic, with two very distinct radiations. The members of the two previously classified subgenera (Amphiglossus Madascincus) are each part of different larger groups containing many additional "Amphiglossus" species of very different morphologies. The subgenus Amphiglossus (sensu Brygoo, 1980a,c; "A." astrolabi, "A." waterloti, and "A." reticulatus) is strongly supported as a clade. However, the taxa of the subgenus Madascincus (sensu Brygoo, 1984b) do not form a clade exclusive of other species of "Amphiglossus"; but these species are members of a larger major clade including some other "Amphiglossus", a group we refer to as the Madascincus group (Fig. 2).

Since the mtDNA provides strong evidence that "Amphiglossus" is not monophyletic, formal generic taxonomic changes are needed in order to have a classification that reflects the evolutionary history of the group. Since all the included members of the two previously recognized subgenera (Amphiglossus and Madascincus) are, respectively, restricted to each of the two strongly supported major clades (i.e., nominate Amphiglossus group and Madascincus group), it is tempting to elevate these subgenera to generic status and apply the names (i.e., Amphiglossus sensu stricto and Madascincus) to these major clades (which will ultimately likely be the case). However, while some informal phenetic groups of species have been proposed (e.g., Glaw and Vences, 1994), widespread convergence in morphological attributes (e.g., color patterns, presacral vertebrae number; see below) reduces our confidence in being able to assess the phylogenetic affinities (i.e., taxonomic allocations) of those species for which we currently lack samples (\~50% of recognized species of "Amphiglossus"). Thus, formal taxonomic recommendations will be postponed until additional "Amphiglossus" species, as well as a couple of other Malagasy "scincines" genera missing in our data set (e.g., Cryptoscincus or the only very recently described Sirenoscincus), can be included in future studies.

Below we further discuss the phylogenetic relationships within and/or among some of the formal and informal species groups previously proposed within "Amphiglossus".

The first of these proposed groups correlates with the large aquatic/semi-aquatic species ("A." astrolabi, "A." reticulatus, and "A." waterloti) placed into the nominate subgenus by Brygoo (1980a,c). Our data strongly support the monophyly of these species and their nested position within the nominate Amphiglossus group. Because of their strong morphological similarities, the validity of the specific status of "A." waterloti with respect to "A." reticulatus has been discussed by several authors (Andreone and Greer, 2002; Brygoo, 1980c; Glaw and Vences, 1994; Raxworthy and Nussbaum, 1993). The main differences between these two species are the number of longitudinal scale rows at mid-body and the number of ventral scales (Brygoo, 1980c). Recent data from several new specimens significantly narrows the gaps for these characters (Andreone and Greer, 2002), but both forms continued to be recognized as distinct taxa. Our data support a very close relationship between the two forms, with "A." waterloti possibly paraphyletic with respect to "A." reticulatus. There is also a relatively low level of genetic differentiation between the three sampled individuals, with the levels of divergence being essentially equal to or less than that observed between multiple conspecific individuals sampled from other "Amphiglossus" species (e.g., "A." morroundatiae, "A." melanurus, and "A." igneocaudatus; Fig. 2). All this recent morphological and molecular evidence leads us to consider the two forms as conspecific; thus, "A." waterloti (Angel, 1930) becomes a subjective junior synonym of "A." reticulatus (Kaudern, 1922).

Besides the previously recognized subgenera, a few other phenetic groups of "Amphiglossus" have been recognized. One of these is characterized by a conspicuous dark lateral stripe (Glaw and Vences, 1994) and contains four recognized species ("A." igneocaudatus, "A." intermedius, "A." polleni, and "A." stumpfii) generally distributed in western and northwestern Madagascar (with one questionable locality of "A." polleni on the east coast). Our analysis strongly supports the monophyly of this group (="A." igneocaudatus species group of Brygoo, 1984d), as well as its placement within the Madascincus group. Previously, there has been doubt as to the taxonomic status of the Ibity population of "A." igneocaudatus (Brygoo, 1984d), with Raxworthy and Nussbaum (1993) noting morphological similarity to "A." intermedius. In our study, the mtDNA strongly groups the Ibity individuals of "A." igneocaudatus with the Itremo "A." igneocaudatus, and the individuals of this species are genetically quite divergent from "A." intermedius (Fig. 2). And finally, our analysis does not support a close rela-
tionship between the two “A.” intermedius individuals from different localities (Fig. 2), with the Ampijoroa individual being more closely related to a sympatric unidentified species. Obviously, the species limits within the “A.” igneoacaudatus group need to be further evaluated.

Brygoo (1981a) informally recognized another phenetic group of “Amphiglossus” for four brownish medium-sized species (“A.” melanurus, “A.” macrocercus, “A.” gastrotrictus, and “A.” poecilopus) from eastern and central Madagascar. Brygoo (1984a) also hypothesized that the northern “A.” tsaratananensis may also be a member of this group. We provide strong evidence that the species of this phenetic group included in our study (“A.” melanurus, “A.” macrocercus, and “A.” tsaratananensis) do not form a clade (Figs. 1 and 2).

A final informal group of “Amphiglossus” was noted by Glaw and Vences (1994) for several species (i.e., “A.” andranovahensis, “A.” arduoinii, “A.” frontoparietalis, “A.” ornaticeps, and “A.” splendidus) possessing transverse markings on the head and/or body. Brygoo (1984a, 1985) also postulated affinities between members of this group (i.e., “A.” andranovahensis and “A.” ornaticeps; “A.” arduoinii, “A.” frontoparietalis, and “A.” splendidus; respectively). However, our data do not support a close relationship between the two species included in our study. “Amphiglossus” ornaticeps is strongly placed as the sister lineage to a small clade containing “A.” melanurus and an unidentified “Amphiglossus” species, and “A.” splendidus is weakly placed with Pygomeles (Figs. 1 and 2).

4.2. Phylogenetic affinities of the limb-reduced genera

The monophyly of the greatly limb-reduced Voeltzkowia is strongly supported by the mtDNA data (Figs. 1 and 2) and it is placed as the sister taxon of the nominate Amphiglossus group. Traditionally, this small clade (containing five described species) confined to the arid regions of southwestern and western Madagascar (Glaw and Vences, 1994) has been divided into two distinctive subgenera, Voeltzkowia (completely limbless; represented by V. lineata) and Grandidierina (forelimbs absent, hindlimbs greatly reduced; represented by V. fierinensis and an undescribed species). The rare limbless Cryptoscincus, probably from southwestern Madagascar, is one of only two Malagasy “scincine” genera not represented in this study, but has been hypothesized to be closely related to Voeltzkowia (Brygoo, 1981c).

The limbless genus Paracontias is also strongly supported as a clade and appears to be most closely related to the “Amphiglossus” of the Madascincus group. Traditionally, three subgenera (i.e., Angelias, Malacontias, and Paracontias) have been recognized to accommodate four species. Recently, Andreone and Greer (2002) described three new Paracontias species, but did not place these into any of the previously recognized subgenera.

Though they did not conduct an explicit phylogenetic analysis, they questioned whether the few characters used to define these subgeneric groups actually diagnosed monophyletic taxa. Andreone and Greer (2002) suggested the possibility that Malacontias or Malacontias + Paracontias group could be a “true lineage” (=clade?). However, though our sampling within the genus Paracontias is limited (only two of the subgenera represented: Malacontias [P. hildebrandti, P. holomelas] and Paracontias [P. brocchii]), our data do not support a monophyletic Malacontias.

Very little is known about the biology of the enigmatic largely limbless Pseudoacontias (Andreone and Greer, 2002; Nussbaum and Rexworthy, 1995), with each of the four species being known only from their holotypes. Glaw and Vences (1994) suggested a possible close relationship between the genera Pseudoacontias and Paracontias. Our data do not provide strong support for such a hypothesis. The partitioned Bayesian analysis weakly places Pseudoacontias as the sister taxon to the Paracontias + Madascincus group clade (Fig. 2), but this taxon could almost as likely be placed as the sister taxon to the Voeltzkowia + nominate Amphiglossus group clade or even as the sister taxon to all the Malagasy “scincines.” Even though our data cannot precisely determine the specific phylogenetic placement of Pseudoacontias, there is support for a relatively basal separation from the other Malagasy “scincine” clade because of the relatively strong support for its exclusion from the large Paracontias + Madascincus and Voeltzkowia + nominate Amphiglossus clades.

The elongated, limb-reduced genera Pygomeles and Androngo are nested within the strongly supported nominate Amphiglossus group. The specific placement of Pygomeles braconnieri (forelimbs absent; short hindlimbs with single toes) within this group is weakly supported, but all analyses suggest a possible close relationship to the pentadactylosus “Amphiglossus” splendidus. Androngo was originally erected to accommodate four “Amphiglossus” species (i.e., “A.” trivittatus, “A.” alluaudi, “A.” crenni, and “A.” elongatus) with varying degrees of limb reduction (but none completely limbless) and greater than 48 presacral vertebrae (Brygoo, 1987). The single Androngo species included in our study, Androngo crenni, was placed as the sister species of “Amphiglossus” tanysoma. This corroborates the taxonomic decision of Andreone and Greer (2002) who transferred three of the four Androngo species (i.e., An. alluaudi, An. crenni, and An. elongatus) back to “Amphiglossus.” We were unable to evaluate the phylogenetic placement of Androngo trivittatus. However, based on two morphological features (i.e., postnasal scale absent and relatively high number of presacral vertebrae [53–56 in An. trivittatus]) and geographic distribution, Andreone and Greer (2002) postulated a possible close relationship between An. trivittatus and Pygomeles braconnieri.
4.3. The evolution of presacral vertebrae number in Malagasy ‘scincines’

Body elongation and limb-reduction are generally correlated phenomena that have occurred repeatedly during scincid lizard evolution. In general, body elongation (=increase in relative snout-vent-length) in skinks is the result of increases in the number of presacral vertebrae. Malagasy ‘scincines’ exhibit great diversity in the degree of body elongation, with the number of presacral vertebrae ranging from a low of 29–30 in the ‘Amphiglossus’ species traditionally placed in the subgenus ‘Madascincus’ (e.g., Brygoo, 1984b) to as high as 82 in Pseudoacontias angelo- rum (Nussbaum and Raxworthy, 1995). The number of presacral vertebrae is known for all the described species in our study, with the modal number ranging from 30 to 67 (see Table 4 and Fig. 3). Given these extensive comparative data, it is possible for us to investigate the evolution of presacral vertebrae number within the Malagasy ‘scincine’ clade from a phylogenetic perspective.

Greer (1989) has suggested that the scincid ancestral number of presacral vertebrae is 26, a number exhibited by many phylogenetically diverse scincid species. As previously mentioned, the lowest presacral vertebrae number exhibited by any Malagasy ‘scincine’ is 29, suggesting the common ancestor of the Malagasy clade likely exhibited slightly more presacral vertebrae than the postulated ancestral scincid. Given this, a major question remains; namely, does the lowest number of presacral vertebrae exhibited by some extant Malagasy ‘scincines’ represent the ancestral condition for the whole group? Andreone and Greer (2002) have hypothesized that 29–30 presacral vertebrae is ancestral for ‘Amphiglossus’ and that ‘Amphiglossus’ contains some of the most ‘primitive members’ of the Malagasy ‘scincine’ clade. Though they also acknowledge that ‘Amphiglossus’ is likely non-monophyletic, their ideas suggest (at least implicitly) that 29–30 presacral vertebrae is the ancestral condition for the Malagasy ‘scincines.’ However, based only on the Malagasy taxa sampled in our study, the weighted squared-change parsimony reconstruction of the ancestral condition is 47–48 presacral vertebrae (depending on the placement of Pseudoacontias menamainty; see Section 3.1). The inclusion of the elongated Prosceletes eggeli (a sub-Saharan ‘scincine’) only slightly lowers the possible range of the ancestral condition to 46–48 presacral vertebrae. MtDNA data from a more extensive study of ‘scincine’ phylogeny (Brandley et al., 2005) strongly supports the phylogenetic placement of the Malagasy ‘scincines’ within a major clade containing other ‘scincines’ from sub-Saharan and North Africa, the Seychelles, and southern Europe and south-southwest Asia. The very elongate and limbless Feylinia (of the subfamily Feylininae) is also a member of this more inclusive major clade. However, the exact relationship between the Malagasy ‘scincines’ and these other skinks is uncertain. Given the importance of closely related outgroup taxa for reconstructing the ancestral condition at the ingroup node (=Malagasy clade; Maddison et al., 1984), it is important to note that all of the non-Malagasy skinks in this more inclusive major clade are elongate, with many species being greatly limb-reduced (e.g., Melanoseps, Sphenops, and Typhlacontias). Of these non-Malagasy skinks, some members of ‘Chalcides’ probably exhibit the lowest number of presacral vertebrae (i.e., ranging between 34 and 65; Caputo et al., 1995; Greer et al., 1998). Even if these ‘Chalcides’ with the lowest number of presacral vertebrae are used as the outgroup to the Malagasy ‘scincines’ (an unlikely hypotheses given ‘Chalcides’ is a member of a relatively strongly supported exclusive clade containing the elongated Sphenops of north-northeast Africa and other elongated sub-Saharan ‘scincines’; Brandley et al. (2005)), there is essentially no change in the reconstructed ancestral condition (45–46 vs 46–48 PSV). Thus, regardless of which of these non-Malagasy skinks are actually most closely related to the Malagasy clade, it is very unlikely that any other potential outgroup relationship would drastically change the ancestral parsimonious reconstruction presented in this study. Thus, we are confident that the ancestor of the Malagasy clade was already elongated and had a moderately high number of presacral vertebrae.

The evolutionary implication for our hypothesized ancestral condition of the Malagasy clade is that the lowest exhibited numbers of presacral vertebrae are actually derived through loss or reduction in PSV number. While the loss of presacral vertebrae appears to have occurred independently in multiple ‘Amphiglossus’ lineages, in no cases does the starting ancestral condition in the beginning of a sequence of loss events exceed the hypothesized ancestral condition for the Malagasy clade (i.e., 46–48 PSV). In other words, our parsimonious ancestral reconstructions do not support the reduction (=reversal) of presacral vertebrae numbers in any lineages that possess a derived increased presacral vertebrae condition (i.e., >48 PSV). Within the Malagasy clade, it appears that once a lineage has started increasing the number of presacral vertebrae from the hypothesized ancestral Malagasy condition (which has occurred independently multiple times), there are no subsequent reversals in these groups. The only apparent exception to this ‘rule’ may be in Paracontias. Based on our preferred phylogeny (Fig. 2), the parsimony character reconstruction suggests there has been a slight reduction of presacral vertebrae number in P. hildebrandti (53–54 → 51 PSV). However, the slight apparent difference falls within the range of presacral vertebrae variation exhibited within P. hildebrandti (50–55 PSV; Andreone and Greer, 2002; Brygoo, 1980b). It is interesting that there are two additional Paracontias species (P. rothschildi, 46 PSV and P. milloti, 47 PSV; Andreone and Greer, 2002; tissues lacking) that exhibit presacral vertebrae numbers that are essentially the same
as the hypothesized ancestral Malagasy condition. Future studies that may include these missing species could be very useful for further investigating the pattern of presacral vertebrae evolution leading to and within Paraconchias.

The patterns of evolution of the number of presacral vertebrae within the Malagasy "scincine" clade also have implications for taxonomy. Some past taxonomic decisions within this clade have been largely based on the observed number of presacral vertebrae. Within "Amphiglossus," Brygoo (1984b) described the subgenus "Madascincus" for those small-bodied species with the lowest number of presacral vertebrae (29–30 PSV). The "Madascincus" species included in this study (i.e., "A." melanopleura and "A." mouroundavae) are not each other’s closest relative, but are nested within a clade (Clade C; Fig. 3) containing other "Amphiglossus" species with >30 presacral vertebrae. Another taxon previously recognized based on presacral vertebrae number is Androngongo (PSV > 48; Brygoo, 1987). Unfortunately, we were able to include only one species of Androngongo (i.e., "An. crenni" sensu Brygoo, 1981b, 1987) in our study of Malagasy "scincine" phylogeny. However, our data strongly support the placement of "An. crenni" (54–57 PSV) as closely related to a species of "Amphiglossus" exhibiting a large number of presacral vertebrae (i.e., "A." tanyssoma, 52–53 PSV). In general, given our strong results that the number of presacral vertebrae have independently decreased and increased multiple times within the Malagasy "scincine" clade, we agree with Andreone and Greer (2002) that the number of presacral vertebrae should not be the sole (or major) character used to diagnose groups within the Malagasy "scincine" clade.

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Appendix A

List of all species of “Scincinae” known to occur on Madagascar, with previously supposed subgeneric assignments.

Amphiglossus alluaudi (Brygoo, 1981) (formerly Androngongo)
Amphiglossus (Madascincus) ankodabensis (Angel, 1930)
Amphiglossus andranovahensis (Angel, 1933)
Amphiglossus anosyensis Raxworthy and Nussbaum, 1993
Amphiglossus ardouini (Mocquard, 1897)
Amphiglossus astrolabi Duméril and Bibron, 1839
Amphiglossus crenni (Mocquard, 1906) (formerly Androngongo)
Amphiglossus decaryi (Angel, 1930)
Amphiglossus elongatus (Angel, 1933) (formerly Androngongo)
Amphiglossus frontoparietalis (Boulenger, 1889)
Amphiglossus gastrostictus (O’Shaughnessy, 1879)
Amphiglossus igneocaudatus (Grandidier, 1867)
Amphiglossus intermedius (Boettger, 1913)
Amphiglossus macrocercus (Günther, 1882)
Amphiglossus macrolepis (Boulenger, 1888)
Amphiglossus mandady Andreone and Greer, 2002
Amphiglossus mandokava Raxworthy and Nussbaum, 1993
Amphiglossus (Madascincus) melanopleura (Günther, 1877)
Amphiglossus (Madascincus) mouroundavae (Grandidier, 1872)
Amphiglossus melanurus (Günther, 1877)
Amphiglossus minutus Raxworthy and Nussbaum, 1993
Amphiglossus namus Andreone and Greer, 2002
Amphiglossus ornaticeps (Boulenger, 1896)
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