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Repeated evolution of limblessness and digging heads in worm lizards revealed by DNA from old bones

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The evolutionary relationships of the burrowing amphisbaenians ('worm lizards') have long been controversial for several reasons: the rarity of museum specimens available for study, highly derived morphological conditions that can confound comparative studies and difficulty in obtaining tissues for molecular phylogenetic studies because of their secretive habits in the wild. We present a phylogenetic analysis of two nuclear genes obtained from both fresh tissues and museum specimens of worm lizards. We achieved sufficient taxonomic sampling for analysis by extracting DNA from museum specimens using a modified forensics protocol. Results show the limbless Rhineuridae to be the most basal lineage, whereas the limbed Bipedidae occupy a more derived position as the sister-taxon to a Trogonophidae–Amphisbaenidae clade. This pattern of relationships indicates widespread morphological convergence within the group, including three independent incidences of limb loss. Convergence in skull shape and scalation is also prevalent. Mosaic evolution in the skull versus postcranial skeleton parallels that seen in snake evolution.

Keywords: amphisbaenians; convergence; DNA extraction; limb loss; mosaic evolution; worm lizards

1. INTRODUCTION

Amphisbaenians are fossorial squamate reptiles, nearly all of which are limbless (figure 1). They are a poorly known group with over 150 extant species in 23 genera occurring in the Neotropics, Caribbean Islands, Florida, Baja California, parts of the Mediterranean and Middle East, and sub-Saharan Africa. Many species exhibit dramatic modifications of the cranium related to their highly derived head-first burrowing behaviour in the sandy or friable soils they inhabit. Although limblessness is a hallmark feature of amphisbaenians, the three species in the genus *Bipes* exhibit robust digging forelimbs (figure 1*b*) and a complete pectoral girdle. All other species are limbless externally, but two genera (*Bipes* and *Blanus*) retain internal hindlimb rudiments (Zangerl 1945; Renous *et al.* 1991; Kearney 2002).

(a) *Evolution of cranial versus postcranial skeleton*

Because *Bipes* retains well-developed forelimbs, internal hindlimb rudiments and a relatively unspecialized head, bipedid amphisbaenians have been interpreted as the sister-group to all other amphisbaenians in both traditional taxonomies and more recent morphology-based phylogenetic analyses (Gans 1978; Kearney 2003), with all other amphisbaenians united by the loss of external limbs. However, this interpretation of relationships is in conflict with the known fossil record for the group because, although apparently limbless, some fossil amphisbaenians (family Rhineuridae) display uniquely primitive cranial features that are not present in other amphisbaenians, suggesting that rhineurid amphisbaenians could be the most basal lineage (Berman 1973, 1976). In short, a morphological incongruity exists between extant and fossil amphisbaenians as a result of mosaic evolution of the skull versus the postcranial skeleton: the presence of limbs and the absence of enclosed orbits and certain cranial bones in bipedids contrasts with the absence of limbs and the presence of enclosed orbits and certain cranial bones in rhineurids (Kearney 2003). Because primitive conditions of both the skull and the postcranial skeleton are never found in the same species, a parsimony analysis of morphological characters always reconstructs relationships such that either one or the other is derived, leading to a high degree of homoplasy. This conundrum is one reason why an independent molecular phylogenetic dataset is highly desirable for the group; however, such a dataset has been unavailable owing to the difficulty of obtaining tissues from these elusive reptiles.

(b) *Cranial morphotypes: homologous or convergent?*

Another area of interest concerns the evolution of various cranial morphotypes among worm lizards, all of which are head-first burrowers (Gans 1969, 1974). Amphisbaenians are characterized by highly specialized heads and exhibit four distinct cranial shapes, each associated with a stereotyped burrowing behaviour: a blunt 'round-headed' shape occurs in bipedids and some other groups; a depressed 'shovel-headed' shape occurs in rhineurids and some other groups; a 'spade-headed' shape occurs in trogonophids; and a compressed 'keel-headed' shape occurs in eight genera of amphisbaenids (figure 2). A recent morphology-based phylogenetic analysis resulted in 'shovel-headed' and 'keel-headed' amphisbaenians each forming monophyletic groups (Kearney 2003); however, these conclusions were considered to be tentative given the possibility that supporting characters could be functionally correlated. Again, an independent molecular dataset for testing relationships among these forms was seen as critical.

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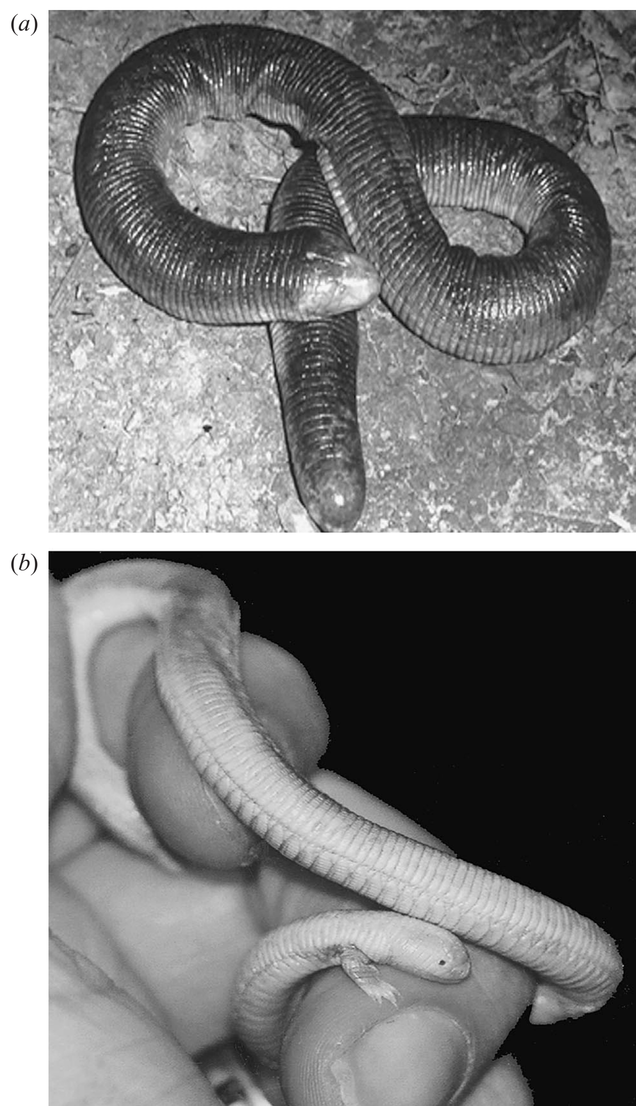

Figure 1. Examples of limbless and limbed amphisbaenian reptiles: (*a*) *Amphisbaena alba* (photograph reproduced, with permission, from Kearney (2003)) and (*b*) *Bipes biporus*.

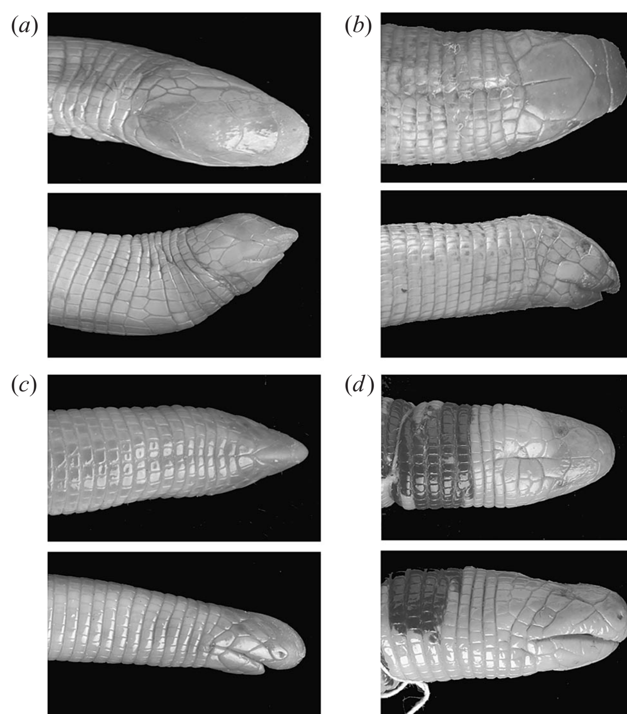

Figure 2. Examples of head shapes of amphisbaenians. Specimens in dorsal (above) and right lateral (below) views. (*a*) A 'shovel-headed' form, *Rhineura floridana*; (*b*) a 'spade-headed' form, *Diplometopon zarudnyi*; (*c*) a 'keel-headed' form, *Anops kingii*; and (*d*) a 'round-headed' form, *Amphisbaena alba*. (Photographs reproduced, with permission, from Kearney (2003).)

(c) *Taxonomically biased fossil record*

An additional confounding factor in studies of amphisbaenian evolution is the taxonomically biased fossil record of the group. Fossil amphisbaenians are known mainly from a number of well-preserved skulls assignable to the Rhineuridae. All of these forms exhibit a 'shovel-headed' cranial morphology in which the snout is dorsoventrally flattened and the skull has a strong craniofacial angle (Berman 1973, 1976). The fossil record of rhineurids extends back to the Upper Palaeocene (Estes 1983) and is exclusively North American. A single surviving relict species, *Rhineura floridana*, occurs in north central Florida and Georgia. No fossil record exists for most other amphisbaenians, which causes some difficulty in interpreting their phylogenetic relationships (Kearney 2003).

(d) *Obtaining DNA for further analysis*

In this study, we surmounted the difficulty of obtaining fresh tissues for many amphisbaenian species by modifying a forensics protocol for extracting DNA from human bones and applying it to skeletonized and fluid-preserved museum specimens, most of which were collected over 40 years ago. Here, we report the results of a phylogenetic analysis based on two nuclear loci (c-*mos* and RAG-1) sampled for 18 amphisbaenian species and six snake and lizard outgroups.

2. METHODS

(a) *DNA amplification, sequencing and alignment*

Total genomic DNA was extracted from either fresh tissue (muscle or liver prepared by the collector for molecular study or removed from a recent ethanol-fixed museum specimen in one case) or museum-specimen bone (consisting of vertebrae or ribs taken from museum skeletal preparations or fluid-preserved specimens). Fresh tissue was extracted using PureGene Animal Tissue DNA Isolation Protocol (Gentra Systems, Inc.). Primers for amplifying and sequencing nuclear DNA were designed from squamate sequences deposited in GenBank or from preliminary amphisbaenian sequences generated in our laboratory (for details on primers, see electronic Appendix A).

Bone samples were extracted using ultraviolet-sterilized supplies inside a Purifier PCR Enclosure (Labconco) in a separate room from that where fresh squamate tissues were extracted. Bone samples were washed three times (at intervals of 2 h, 2 h and 12 h) with 1.5 ml of GTE buffer (100 mM glycine, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to bind excess formalin (Shedlock *et al.* 1997) and for 1 min in 100% ethanol, 5 min in 70% ethanol and 10 min in sterile water. Samples were gently vortexed for 5 s after their placement into a new wash. After washing, the bones were crushed with a mortar and pestle in liquid nitrogen and decalcified by incubating with agitation at room temperature for 48 h in 1.6 ml of 0.5 M EDTA (pH of

8.0). Tubes were centrifuged (8000 r.p.m. for 1 min) to pellet bone fragments, and the EDTA was removed by a pipette and discarded. The pelleted bone fragments were washed twice in 1 ml sterile water and incubated at 56 °C for 3 days in 300 μl of TNES buffer (10 mM Trizma Base, 100 mM NaCl, 10 mM EDTA, 2% sodium lauryl sulphate (SDS, 39 mM) DTT) with daily additions of 300 μg of proteinase-K. The remaining extraction procedure followed the DNeasy Tissue Kit (Qiagen) protocol for animal tissues, with these modifications: 300 μl of AL buffer and 400 μl of 100% ethanol were used rather than 200 μl of each, two spins of 500 μl of the extraction product through the DNeasy mini column were necessary to accommodate the larger extraction volume, a second spin was added for 1 min at full speed after discarding the buffer AW2 flow-through fluid and 60 μl of buffer AE was added to the DNeasy membrane rather than 100–400 μl, after which the membrane was incubated at room temperature for 5 min rather than 1 min before centrifuging.

A 585–588 bp fragment of the oocyte maturation factor Mos (c-*mos*) gene was amplified from fresh tissue by PCR (94 °C for 45 s, 56–60 °C for 30 s and 72 °C for 1 min) for 35 cycles using the light-strand primer L-lizcmos and the heavy-strand primer H-cmosII or H-cmosIII. An 872 bp fragment of the recombination activating protein 1 (RAG-1) gene was amplified from fresh tissue under the same PCR conditions using the primer pair L-RAG1b and H-snRAG1. A 1094 bp fragment that overlapped with the 872 bp fragment was additionally amplified in some taxa using the primer pair L-snRAG1 and H-RAG1b. Amplifying and sequencing DNA extracted from bone samples required the use of primer pairs with 3′ ends positioned a maximum of 224 bp apart. To avoid generating chimeric sequences, primers were designed so that the resulting DNA fragments overlapped by 22–70 bp in variable regions after the primer sequences were trimmed. AmpliTaq Gold (Roche), 4 μl of DNA template and 4 μl of purified 10 mg ml^{-1} bovine serum albumin (BSA; New England BioLabs, Inc.) were used in 25 μl total PCR reactions. A negative control containing all PCR reagents except the DNA template was always included. A total of 357 bp of the c-*mos* gene was amplified from bone samples in two overlapping fragments by PCR (94 °C for 45 s, 54 °C for 30 s and 72 °C for 50 s) for 40 cycles using the primer pairs L-230cmos–H-450cmos and L-420cmos–H-cmosIII. A total of 459 bp of the RAG-1 gene was amplified from bone samples in three overlapping fragments by PCR (94 °C for 45 s, 54 °C for 30 s and 72 °C for 50 s) for 40 cycles using the primer pairs L-140RAG1–H-311RAG1, L-288RAG1–H-430RAG1 or H-455RAG1 or H-470RAG1 and L-385RAG1–H-603RAG1. PCR products were electrophoresed in a 1% low melt agarose gel stained with ethidium bromide and visualized under ultraviolet light. The bands containing DNA were excised and the agarose was digested from bands using GELase (Epicentre Technologies).

PCR products were sequenced in both directions by direct double-strand cycle sequencing using Big Dye v. 3 chemistry (Perkin Elmer). Cycle sequencing products were precipitated with ethanol and 3 M sodium acetate and sequenced with a Prism 3100 Genetic Analyser (ABI). Sequences from bone fragments were compared with all other sequences from squamates generated in our laboratory to verify authenticity. Sequences were edited and aligned with SEQUENCHER v. 4.1 (Genecodes). Three c-*mos* sequences of amphisbaenians were downloaded from GenBank and included in the alignment. All sequences were included in the alignment regardless of length differences. Sequences were translated into amino acids using MACCLADE v. 3.08a (Maddison & Maddison 1992) and the amino acids were used to determine sequence homology in cases of codon insertions or deletions in the alignment.

(b) *Phylogenetic analysis*

Both maximum-parsimony and maximum-likelihood phylogenetic analyses were performed using PAUP* v. 4.0b10 (Swofford 2000). Trees were rooted with the snake taxa *Loxocemus* and *Ramphotyphlops*. Parsimony analyses were performed with equal weighting of transitions and transversions, using a heuristic search algorithm with 1000 random addition replicates of stepwise taxon addition. Branch support was evaluated with 500 pseudoreplicates in a bootstrap analysis. Likelihood analyses were performed using the model of sequence evolution that best described the data as inferred by MODELTEST v. 3.06 (Posada & Crandall 1998). The model selected was HKY + I + G, with a transmission–transversion ratio of 2.3667, proportion of invariable sites of 0.4369, a gamma distribution shape parameter of 3.0181 and base frequencies of A = 0.3038, C = 0.2138, G = 0.2238 and T = 0.2587. Maximum-likelihood analyses were performed with 1000 random addition replicates with stepwise addition of taxa using the heuristic search algorithm and tree bisection–reconnection branch swapping. A Shimodaira–Hasegawa (S–H) test (Shimodaira & Hasegawa 1999), implemented in PAUP* v. 4.0b10, was used to test statistically alternative maximum-likelihood topologies under the model parameters given above.

3. RESULTS

(a) *DNA sequencing results*

Fresh tissues were available for only one-half of the amphisbaenian species analysed here. Testing hypotheses of phylogenetic relationships and morphological character evolution was possible because of the increased taxonomic sampling achieved by obtaining DNA from museum specimens. Sequences of both c-*mos* and RAG-1 were obtained for all taxa in the study, except RAG-1 from the bone sample of *Aulura* (for details on samples used in this study see electronic Appendix B). The c-*mos* alignment contained three insertion–deletion events. First, one codon was missing in all snakes and amphisbaenians except *Rhineura*. Second, seven adjacent codons were missing in amphisbaenians. Third, one codon was missing in *Mabuya* and amphisbaenians. The RAG-1 alignment contained no insertion–deletions.

(b) *Phylogenetic analysis results*

The position of amphisbaenians within other squamates is an area of controversy; because of limited outgroup sampling, this analysis does not address this issue. We focus here on the well-supported relationships found within the Amphisbaenia. The single parsimony and single likelihood trees we obtained differed in only two respects, which do not affect our conclusions: the positions of the outgroup taxa *Gekko* and *Ophisaurus* were reversed, and *Monopeltis* in the likelihood tree is the sister-taxon of *Geocalamus*. Both genes analysed separately under parsimony recover the same topology except that c-*mos* when analysed alone does not resolve trogonophid–amphisbaenid relationships.

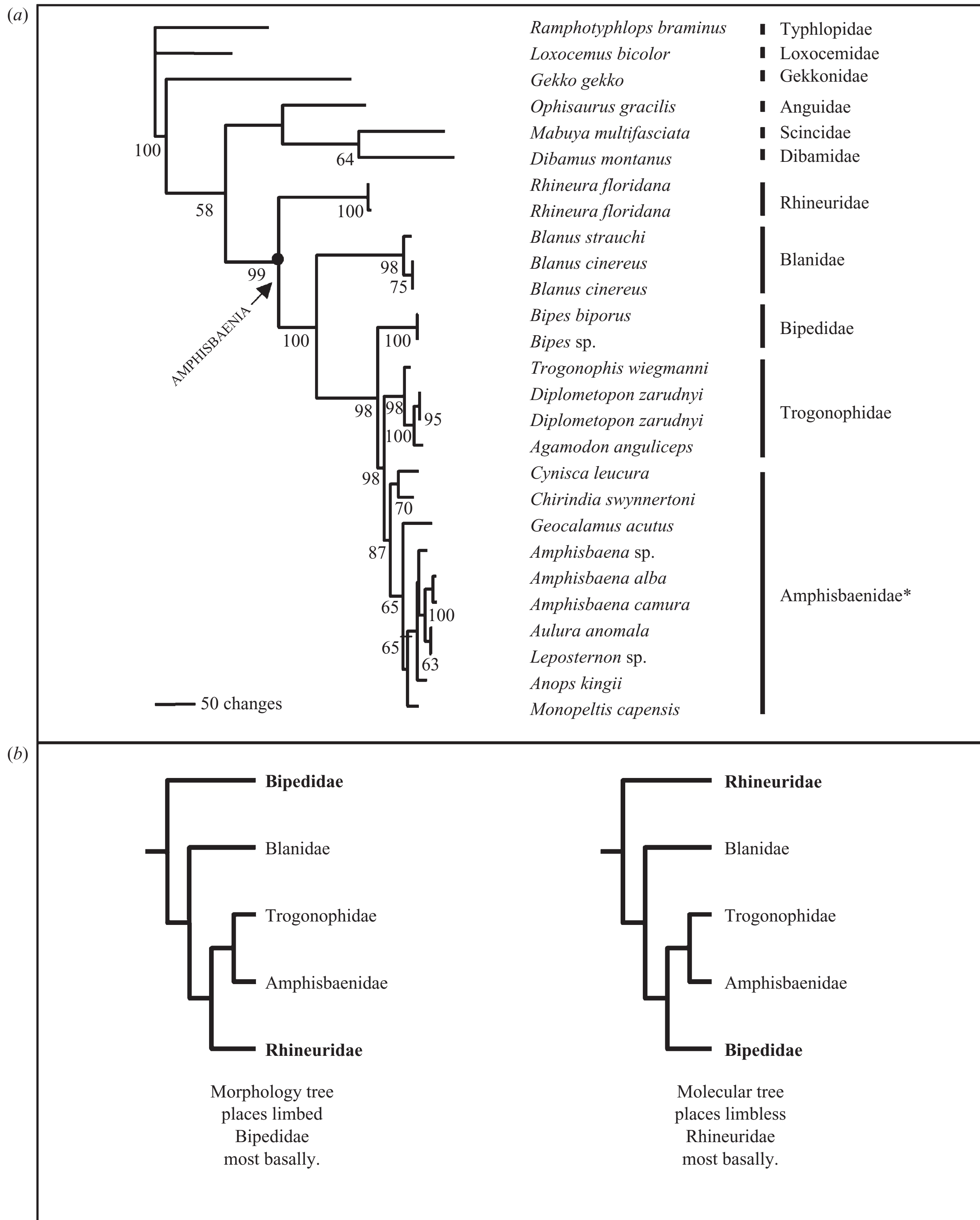

Figure 3. (*a*) The phylogenetic relationships of amphisbaenians based on a parsimony analysis of c-*mos* and RAG-1 nuclear genes. Numbers below nodes are bootstrap values of greater than 50%. *The family Amphisbaenidae here differs slightly in composition from traditional taxonomies (e.g. Gans (1978) included *Blanus* in Amphisbaenidae) and from a recent morphology-based phylogenetic study (e.g. Kearney (2003) removed *Blanus*, *Aulura*, *Leposternon* and *Monopeltis* from Amphisbaenidae). (*b*) Incongruence between morphological (Kearney 2003) and molecular (this analysis) results for family-level amphisbaenian relationships.

The results of our analysis show the limbless Rhineuridae to be the sister-group to all other amphisbaenians, and the limbed Bipedidae to be nested well inside the group as the sister-taxon to a clade composed of Amphisbaenidae and Trogonophidae (figure 3*a*). All four traditionally recognized families (Gans 1978) are recovered except that *Blanus*

is not part of the Amphisbaenidae (see also Kearney 2003). A recent morphology-based analysis (Kearney 2003) and the DNA-based analysis presented here are congruent in supporting the following hypotheses: (i) Amphisbaenia is monophyletic; (ii) Amphisbaenidae is the sister-group to Trogonophidae; and (iii) *Blanus* is not an amphisbaenid, as previously thought (Gans 1978), but a relatively basal lineage within Amphisbaenia (Kearney 2003). The topologies are incongruent in several ways, most notably in that the limbed Bipedidae is most basal in the morphological topology, whereas the limbless Rhineuridae is most basal in the molecular topology (figure 3*b*). The S–H test statistically rejected ($p < 0.05$) an alternative topology constrained with the limbed Bipedidae most basal ($-\ln L = 10\,112.891\,14$) as compared with the optimal topology found here, which places the limbless Rhineuridae most basal ($-\ln L = 9826.696\,77$).

4. DISCUSSION

The phylogenetic result obtained here suggests multiple incidences of limb loss within worm lizards and substantial morphological convergence in other character systems as well (figure 4). Areas of conflict between results from previous morphology-based analyses and the molecular-based study presented here are probably a result of a complex interplay of morphological convergence, incomplete fossil records for some lineages and mosaic evolution of the skull versus the postcranial skeleton, as explained below.

(a) *Evolution of limblessness*

Given the nesting of *Bipes* within amphisbaenians, it is the case either that external limbs were lost independently three times as shown in figure 4, or that they were lost once at the base of the amphisbaenian tree and then regained in *Bipes*. Also, internal hind-limb rudiments present in *Bipes* and *Blanus* were either lost independently in rhineurids and the trogonophid–amphisbaenid clade, or lost at the base of the tree and then regained in *Bipes* and *Blanus*. Although re-evolution of isolated limb elements such as phalanges has been proposed for certain squamate species (Auge 1992; Greer 1992; Whiting *et al.* 2003), to our knowledge no empirically based hypothesis of re-evolution of a complete limb and limb girdle has been proposed. Furthermore, loss or reduction of limbs and limb girdles is prevalent among fossorial and grass-dwelling squamate reptiles. The transition from a quadrupedal lizard-like body form to a limbless (or limb-reduced) elongate snake-like body form has occurred dozens of times in squamate reptiles (Greer 1991; Wiens & Slingluff 2001). Even within genera, some species are fully limbed and some are limbless. Given the incompleteness of the fossil record for most amphisbaenians, the structure of the forelimb and pectoral girdle in *Bipes* (Castañeda & Alvarez 1968; Kearney 2002), the widespread occurrence of multiple losses of limbs among squamates in general (Greer 1991), and the lack of evidence for complete limb re-evolution in any vertebrate clade, we believe that the most plausible inference from this topology is the independent loss of limbs in rhineurids, *Blanus* and the trogonophid–amphisbaenid clade from a tetrapodal ancestor.

(b) *Convergence of cranial morphotypes and other character systems across continents*

Our phylogenetic results also suggest substantial homoplasy in character systems other than limbs (figure 4). For example, all 'shovel-headed' amphisbaenians exhibit numerous similarities including a strong craniofacial angle, enlarged pectoral scales and the complete lack of pectoral girdle elements, similarities that previously led to a hypothesis of monophyly (Kearney 2003). The results obtained here imply that all these features evolved convergently, which is consistent with previous hypotheses that similar skull shapes evolved in parallel among groups occurring on different continents (Gans 1978). In addition, these results suggest that 'keel-headed' and 'round-headed' forms are not monophyletic.

Despite this requirement of substantial convergent evolution in morphological characters, the molecular phylogeny obtained here is more congruent with the geographical distributions of these taxa (Gans 1978) than is the topology obtained from the morphology-based phylogenetic study (Kearney 2003). Specifically, morphological analysis grouped forms occurring on different continents but with similar cranial morphotypes and scalation patterns together, whereas this analysis groups geographically similar species together, requiring numerous convergences in cranial shape and other characters. For example, in the morphology tree, the shovel-headed taxa *Leposternon* (South America), *Rhineura* (North America) and *Monopeltis* (Africa) group together, whereas, in the molecular tree, South American and African taxa each form groups despite exhibiting a wide variety of head shapes.

(c) *Mosaic evolution and its consequences in phylogenetic analysis*

Finally, this analysis underscores mosaic evolution of the skull versus the postcranial skeleton in amphisbaenians, a situation noted previously as a potential problem in reconstructing relationships in the group from morphological data (Kearney 2003). Interestingly, a similar pattern of mosaic evolution occurs among snakes, leading to a similar phylogenetic challenge. The most basal extant snakes are widely believed to be highly modified burrowing forms that are completely limbless externally (Cundall *et al.* 1993; Saint *et al.* 1998; Vidal & Hedges 2004). Recently discovered fossil snakes appear primitive based on postcranial skeletal anatomy, as a result of the retention of hind limbs, but are highly derived with respect to skull anatomy, causing their phylogenetic placement to be controversial: some studies place the limbed fossil snakes as the most primitive snakes (Caldwell & Lee 1997; Lee 1998; Lee & Caldwell 1998; Scanlon *et al.* 1999), whereas others place them with relatively advanced snakes such as pythons and boas (Zaher 1998; Zaher & Rieppel 1999*a,b*; Rieppel & Zaher 2000*a,b*; Tchernov *et al.* 2000). In either case, substantial homoplasy is evident owing to mosaic evolution of the skull versus the postcranial skeleton, as in amphisbaenians. Such a combination of mosaic evolution and incomplete fossil records for some lineages can lead to persistent problems in interpreting relationships through morphological phylogenetic analyses (de Queiroz 1985).

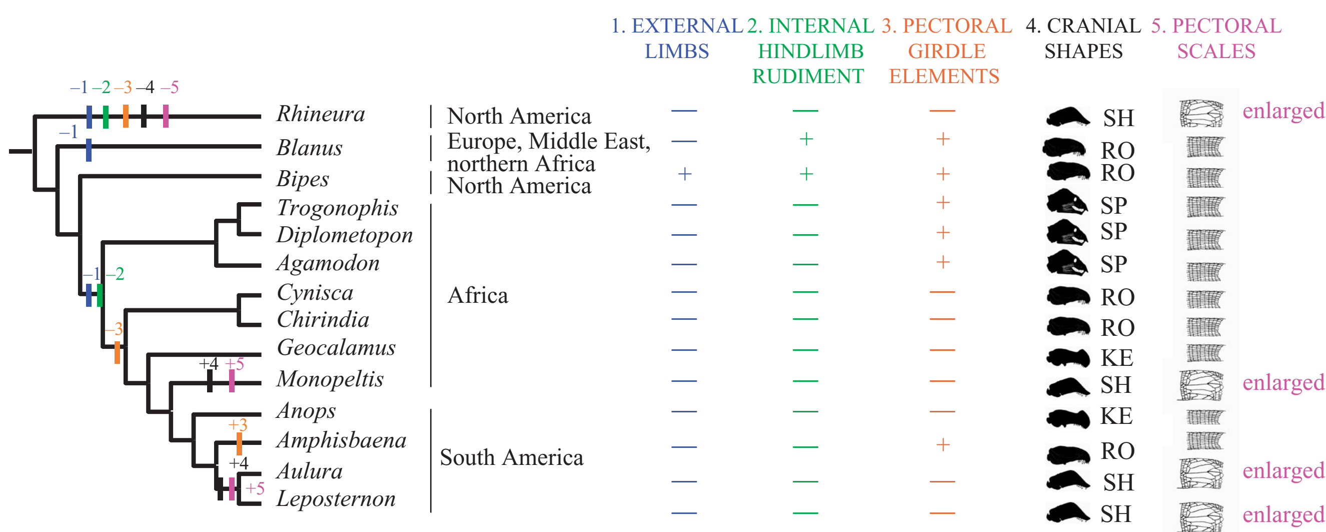

Figure 4. Genus-level tree of amphisbaenian relationships, geographical distributions and optimization of several important morphological features. KE, keel-headed; RO, round-headed; SH, shovel-headed; SP, spade-headed; +, present; −, absent.

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