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The phylogenetic position of Anomochilidae (Reptilia: Serpentes): first evidence from DNA sequences

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Abstract

Previously, anilioids (Aniliidae, Anomochilidae, Cylindrophiidae and Uropeltidae) were considered the only extant, non-macrostomatan alethinophidian snakes. Although their monophyly and intrarelationships remained poorly established, their fossoriality, small gape, and inferred phylogenetic position have been important evidence in orthodox scenarios about early snake evolution. Recent molecular studies including aniliids, cylindrophiids and uropeltids indicate anilioid polyphyly, with the latter two families comprising a clade nested within Macrostomata. We carried out the first molecular phylogenetic analysis to include the very poorly known and seemingly rare Anomochilidae. Only partial sequences of 12S and 16S rRNA mitochondrial genes could be amplified from tissue collected from a single dead specimen of *Anomochilus leonardi*. Amplification failed for nuclear and other mitochondrial genes, and for all the investigated genes for the holotype and paratype of *A. leonardi*. Analyses recovered a para- or polyphyletic Anilioidea. *Anomochilus* is recovered as most closely related to *Cylindrophis maculatus* (rendering Cylindrophiidae possibly paraphyletic). The relatively small amount of available data produces only moderate levels of support, but the stability of taxa and agreement across different analytical methods and with larger analyses of snake phylogeny support the abandonment of Anilioidea as a natural taxon, and the recognition of a higher category for a clade comprising Asian anilioids (Anomochilidae, Cylindrophiidae and Uropeltidae).

Key words: Anilioidea – *Anomochilus* – Evolution – Phylogeny – Serpentes – Systematics

Introduction

Snake phylogenetics is 'confusing and contentious' (Lawson et al. 2004, p. 285). Although there is consensus that the basal split among living snakes is between Scolecophidia (worm and blind snakes) and Alethinophidia (all other snakes), the configuration of basal splits among alethinophidians is unclear, with notable disagreements between morphological and molecular evidence.

Traditionally, Anilioidea is considered to comprise four major lineages of burrowing snakes (McDiarmid et al. 1999) – South American Aniliidae (one species, *Anilius scytale*), South Asian Uropeltidae (eight genera, *c.* 47 species) Southeast Asian Anomochilidae (two species of *Anomochilus*), and Asian Cylindrophiidae (10 species of *Cylindrophis*). Although their intrarelationships and status as a natural, monophyletic group remained open questions, extant anilioids were until very recently considered to lie outside a clade (Macrostomata) comprising all non-anilioid alethinophidians (e.g. Scanlon and Lee 2000; Tchernov et al. 2000; Lee and Scanlon 2002).

Because they were considered the only non-macrostomatan alethinophidians, the concept and biology of Anilioidea attracted considerable interest. Interpretations of the early history of snake evolution were informed by studies of anilioid anatomy, behaviour and ecology (e.g. Rieppel 1980; Greene 1983; Zaher 1994; Cundall 1995; Lee et al. 1999; Cundall and Greene 2000). In particular, knowledge of anilioid biology has been an important part of the, until very recently consensual, understanding that the most recent common ancestor of alethinophidians was perhaps burrowing and fed on narrow, heavily bodied prey (e.g. Greene 1983; Cundall and Greene 2000; Rieppel and Kearney 2001). This orthodoxy has been challenged recently by several phylogenetic analyses of DNA sequence data that indicate that anilioids do not neatly form the sister group(s) to macrostomatans (Vidal and David 2004).

Thus, rather than representing corroborating evidence for a subterranean phase in early snake evolution, fossoriality might instead explain why at least some anilioids are cryptic, 'regressed macrostomatans' (G. Underwood, personal communication; Vidal and Hedges 2002).

Anomochilus Berg, 1901 comprises two species known from only nine specimens. *A. leonardi* Smith, 1940 is known from five specimens from peninsular Malaysia (Yaakob 2003) and one from Malaysian Borneo (Stuebing and Goh 1993), and *A. weberi* (Lidth de Jeude, 1890) from two specimens from Sumatra and one from Indonesian Borneo (Cundall and Rossman 1993; David and Vogel 1996). The claim that a recent study was the first to include mitochondrial DNA data for representatives of all families of extant alethonophidians (Lawson et al. 2004) depends on Anomochilidae not being considered a family (*contra*, e.g. McDiarmid et al. 1999). Here we report the first DNA sequences for any anomochilid and use them to test the monophyly of Anilioidea and its constituent families that are represented by more than one sampled taxon (Cylindrophiidae and Uropeltidae).

Previous hypotheses

Considerations of morphological data have mostly hypothesized that Anilioidea is monophyletic (Rieppel 1988; Scanlon and Lee 2000; Tchernov et al. 2000; Lee and Scanlon 2002; Rieppel and Zaher 2000) or perhaps paraphyletic (Cundall et al. 1993; Greene 1997; Cundall and Greene 2000), but that they are collectively the proximate outgroup(s) to other (macrostomatan) alethinophidians (e.g. Rieppel 1977). In contrast, all analyses of DNA sequence data have agreed that anilioids are not monophyletic, are possibly polyphyletic, and that they do not lie outside a clade comprising all other alethinophidians (Vidal and Hedges 2002, 2004; Wilcox et al.

2002; Lawson et al. 2004; Vidal and David 2004). Analysis of allozyme and immunological data (Cadle et al. 1990) also concluded that anilioids were probably not monophyletic because of the great distance between *Anilius* and *Cylindrophis maculatus*. To date, *Anomochilus* has been included only in phylogenetic considerations of morphology, which have resulted in several competing hypotheses of its relationships to other snakes (Fig. 1).

Methods

DNA extraction, amplification and sequencing

Small pieces of muscle tissue stored in ethanol were obtained from three specimens of *A. leonardi*: the recently collected sixth specimen deposited in the Forest Research Institute Malaysia, Kepong, FRIM 0026 (Yaakob 2003), and the holotype (BMNH 1946.1.17.4) and paratype (BMNH 1952.1.2.63) from the Natural History Museum, London, UK. The first of these specimens was found on the surface and had been dead for an unknown period (Yaakob 2003), and the latter two were catalogued more than 50 years ago and have an incompletely known preservational history. Attempts to extract genomic DNA were made using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) and standard phenol–chloroform techniques (Sambrook et al. 1989), which had been successful in extracting amplifiable mtDNA from other historic BMNH snake material (> 60 years old, unpublished data). Amplification of DNA by PCR was attempted using various primers for those mitochondrial and nuclear genes recently used to estimate snake phylogeny. Specifically, we attempted all paired combinations of the following primers in amplification and sequencing: L14724 (Meyer and Wilson 1990), cytochrome *b* 1, cytochrome *b* 2 (Kocher et al. 1989), the forward primer of cytochrome *b* 2, CB3-3′ (Palumbi 1996), L14910, and H16064 (Burbrink et al. 2000) for the cytochrome *b* gene; ND4 and LEU (Forstner et al. 1995) for the ND4 gene; 12Sa and 12Sb for the 12S rRNA gene (Kocher et al. 1989); 16Sa and 16Sb for the 16S rRNA gene (Palumbi 1996); L39, HC3 (Vidal and Hedges 2002), and G74 (Saint et al. 1998) for the C-mos gene; L2408, H2920, and H2928 (Vidal and Hedges 2004) for the RAG1 gene. Amplification was unsuccessful for the BMNH holotype and paratype, and for FRIM 0026 was achieved only for 12S and 16S rRNA.

Taxon and character sampling

Other than *A. leonardi*, our analyses included 21 species representing 18 genera and most major lineages of extant snakes, including
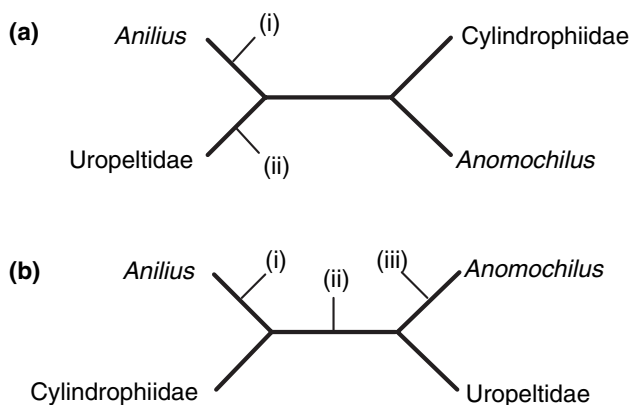

Fig. 1. Two of the three possible unrooted trees depicting interpretations of the relationships among anilioid snakes based on considerations of morphology. Numbers in parentheses indicate variable positions of the root: (a) (i) McDowell (1975, 1987); (ii) Underwood (1967) and Rieppel (1977). (b) (i) Tchernov et al. (2000) and Lee and Scanlon (2002); (ii) Scanlon and Lee (2000); (iii) Cundall et al. (1993) and Cundall and Greene (2000)

representatives of all anilioid families (Appendix). Xenophiidae was not included because only cytochrome *b* data are available, and this taxon seems not to be of immediate relevance to the interrelationships of anilioids (Lawson et al. 2004). There is a wealth of morphological (e.g. Underwood 1967; Lee and Scanlon 2002) and molecular (e.g. Vidal and Hedges 2004) evidence that the basal split among extant snakes is between Scolecophidia and Alethinophidia, and we rooted trees with the scolecophidian *Ramphotyphlops braminus*.

Data analysis

Sequence length differences were small (range of 771–781 base pairs for concatenated 12S and 16S), necessitating few gaps in the alignment. Sequence alignment was performed manually with MUST2000 (Philippe 1993). Our manual alignment (EMBL-ALIGN database accession ALIGN_000848) was based on a much larger data set for snakes (N. Vidal, unpublished data). For 12S, alignment was guided by a secondary structure model (Hickson et al. 1996) and it built on a previous alignment (Vidal et al. 2000) that has been independently appraised (Kelly et al. 2003). In our 16S alignment, one obvious and highly variable loop region of up to 31 base pairs was removed (see accessioned alignment). Following this, an additional alignment of the concatenated 12S and 16S data was produced using T-Coffee (Notredame et al. 2000) with default parameters.

The few remaining gaps were treated as missing data. Maximum likelihood (ML) and Bayesian inference (BI) analyses used the general time reversible (GTR) model (Rodríguez et al. 1990), with rate variation across sites modelled with a four category discrete gamma distribution (G) and proportion of invariant sites (I). This was selected as the best model as judged by both available criteria in Modeltest (v3.06, Posada and Crandall 1998). Empirical base frequencies were used in implementing the GTR model in tree estimation. Maximum parsimony (MP) and ML analyses were performed with PAUP* 4.0b10 (Swofford 1998), using heuristic searches comprising 100 and 10 random addition sequences, respectively, and TBR branch swapping. Maximum likelihood distance (MLD, using PAUP) analysis used the minimum evolution objective function. BI was carried out using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001) with the same model used for ML analysis. The Metropolis coupled, Markov chain Monte Carlo analysis was run for 1 500 000 generations (first 1000 discarded as 'burn-in'). Trees were sampled every 1000 generations.

Support was evaluated with non-parametric bootstrap proportions (BPs – 1000 pseudoreplicates), decay indices (DIs) and Bayesian posterior probabilities (BPPs). The phylogenetic stability of each taxon was quantified using leaf stabilities based on the bootstrap difference measure (Thorley and Wilkinson 1999), determined using RadCon (Thorley and Page 2000) from sets of bootstrap trees. Suboptimal trees conforming to several *a priori* hypotheses were recovered from searches enforcing user-defined topological constraints.

Differences between optimal and suboptimal trees were assessed using a non-parametric test (Templeton 1983) for MP analyses, and Kishino–Hasegawa (Kishino and Hasegawa 1989) and Shimodaira–Hasegawa (Shimodaira and Hasegawa 1999) tests for ML analyses. The more conservative two-tailed versions of these tests were used. Interpretation of the results of these tests is problematic (e.g. Goldman et al. 2000; San Mauro et al. 2004), so that rejection of the null hypothesis is interpreted cautiously (see Wilkinson et al. 2003).

Results

Successful PCR amplifications yielded products of expected size containing negligible site ambiguity upon sequencing both strands. There is no obvious reason to suspect that the new data derive from nuclear copies of mitochondrial sequences. Our manual alignment comprises 794 sites, of which 472 are constant, 101 variable but parsimony uninformative, and 221 parsimony informative. The automated alignment produced by T-Coffee yielded an alignment of 796 sites, 222 of which are parsimony informative. The two alignments are very similar and yielded the same two MPTs, and thus we continued all

subsequent analyses with our manual alignment (EMBL-ALIGN_000848). There is no significant variation in base composition across the manual alignment as a whole (tests for homogeneity, p = 1.0, d.f. = 63) or across the variable (p = 0.477) or parsimony informative (p = 0.198) sites only.

Relationships among major lineages of snakes inferred by all methods of analysis (Fig. 2) are generally in agreement with recent DNA analyses. Thus, anilioids are not monophyletic, *Cylindrophis* and Uropeltidae are more closely related to each other than to *Anilius*, and *Anilius* + tropidophiids lie outside a clade comprising other 'macrostomatans' + cylindrophiids + uropeltids (Vidal and Hedges 2002, 2004; Wilcox et al. 2002; Lawson et al. 2004; Vidal and David 2004). In all analyses, *Anomochilus* forms a clade with *C. maculatus*, and beyond that with cylindrophiids and uropeltids, supporting Vidal and Hedges' (2002) expectation that Asian anilioids are monophyletic, and the conclusions of some evaluations of morphology (e.g. Underwood 1967; McDowell 1975, 1987) that *Cylindrophis* and *Anomochilus* are especially closely related.

Indications of support are given by BPs, BPPs, DIs, leaf stabilities, and agreement across different methods (Fig. 1). Apart from the (in our view) preposterously high BPPs (see Suzuki et al. 2002; Cummings et al. 2003 for discussion of this phenomenon), support is generally uncompelling but, in addition to *A. leonardi* + *C. maculatus*, several clades are recovered in all analyses, including all anilioids other than *Anilius* (Asian anilioids); Uropeltidae; Sri Lankan uropeltids; and Sri Lankan uropeltids + *Brachyophidium*. Tests comparing optimal and suboptimal trees indicate that anilioid monophyly is relatively implausible, but hypotheses we are less able to dismiss as implausible include anilioid paraphyly, cylindrophiid monophyly, and *Anomochilus* + Uropeltidae (Table 1). All anilioids and tropidophiids are the only taxa with above average leaf stability in MP and MLD analyses. *Anomochilus* is among the seven most stable taxa. The selected root is a little below average stability, but its exclusion from analyses does not change the relationships within the ingroup. No taxa are particularly unstable and the mean value is quite high such that, on average, bootstrap support for all quartets in parsimony pseudoreplicates is 83%.

Discussion

The following discussion carries the caveat that we managed to generate only a relatively small quantity of mitochondrial

Fig. 2. Estimates of snake relationships based on mitochondrial 12S and 16S rRNA sequences from analyses using (a) Bayesian inference, showing bootstrap proportions and decay indices from parsimony analysis; (b) maximum likelihood, showing bootstrap proportions. For the Bayesian tree, posterior probabilities for all nodes are maximal (100). The relationships in the strict consensus of the two MPTs (not shown) are the same as in the Bayesian tree except that *Cylindrophis* + *Anomochilus* is monophyletic; boas, pythons and Caenophidia together are para- rather than monophyletic; the *Uropeltis* and *Rhinophis* species form a trichotomy. Maximum likelihood and Bayesian analyses used a GTR + I + G model with substitution rates set at 39.3282 (A–C), 67.2233 (A–G), 24.7268 (A–T), 0 (C–G), 214.004 (C–T), 1 (G–T); empirical base frequencies of 0.384 (A), 0.2353 (C), 0.1784 (G), 0.2023 (T); a four category discrete approximation of a gamma distribution (α = 369); and the proportion of invariant sites set at 0.2867. Anilioid taxa are indicated in bold
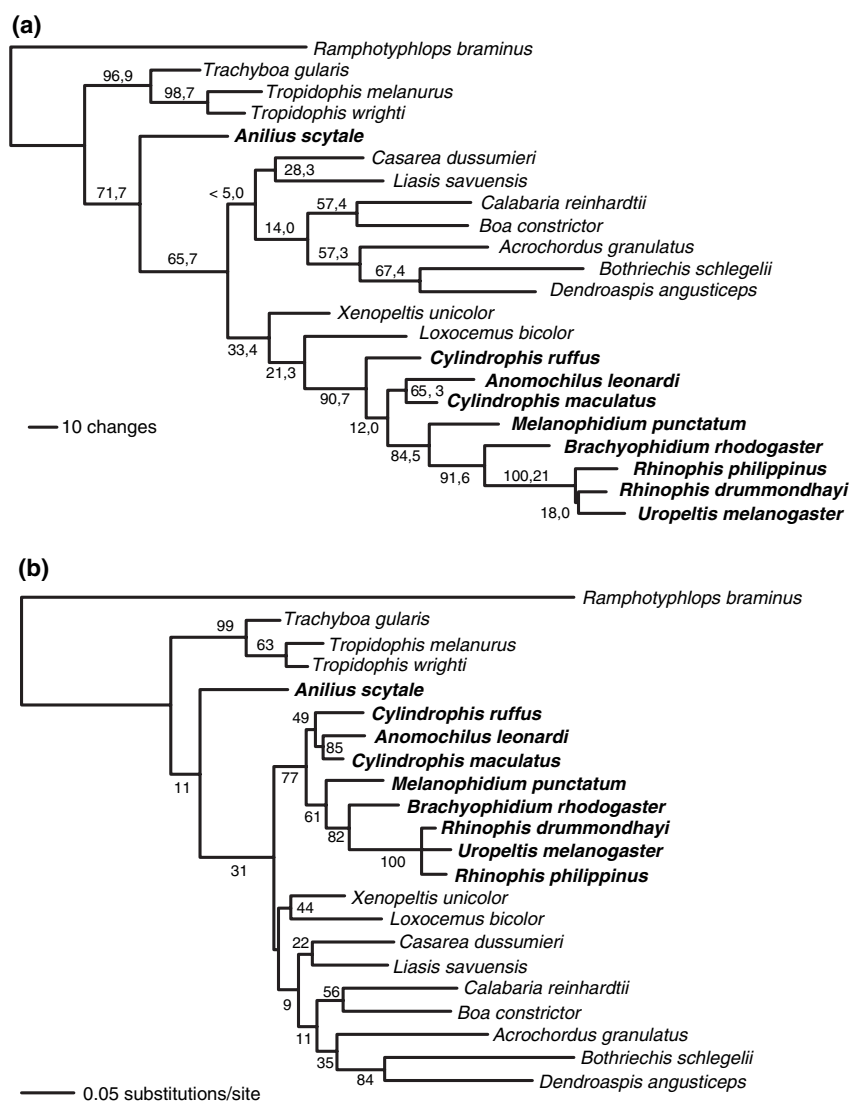
Table 1. Results of tests of differences between optimal parsimony and likelihood trees and the best suboptimal trees including particular hypotheses of the relationships of anilioid snakes

	Parsimony			Maximum likelihood		
	Length	n	Templeton test (p-value)	Likelihood	KH test (p-value)	SH test (p-value)
Optimal	1038	2	NA	5376.56019	NA	NA
(C, U)	1043	2	0.398–0.442	5379.6973	0.513	0.254
Not (U)	1043	1	0.1317–0.166	5383.9391	0.194	0.114
(C)	1043	2	0.09–0.166	5377.9676	0.666	0.320
(Al, U)	1045	2	0.052–0.09	5379.9584	0.438	0.206
Not (C, Al, U)	1045	1	0.052–0.09	5385.7917	0.089	0.071
As (Al, C, U, M)	1047	2	0.139–0.18	Optimal	NA	NA
(As, Al, C, U)	1055	4	0.036–0.052	5396.2796	0.038	0.029

Results are listed in order of decreasingly plausible suboptimal hypotheses, as ranked by parsimony tree length.
Abbreviations for the major anilioid groups are: As, *Anilius*; Al, *Anomochilus*; C, *Cylindrophis*; U, Uropeltidae. Non-tropidophiid macrostomatans are indicated by M. For parsimony, the number of optimal trees (n) is indicated. Abbreviations for tests are: KH, Kishino–Hasegawa; SH, Shimodaira–Hasegawa.

sequence data. Although quantitative support for most nodes is uncompelling, all methods of analysis agree in recovering *Anomochilus leonardi* + *Cylindrophis maculatus*. Templeton, Kishino–Hasegawa and Shimodaira–Hasegawa test results (Table 1) do not allow us to rule out a monophyletic Cylindrophiidae or the hypothesis that *Anomochilus* is more closely related to uropeltids than to cylindophiids (although neither relationship is recovered in any of our analyses). The same tests do not force us to attribute suboptimality of a monophyletic Anilioidea to random sampling error (Templeton test results borderline at p = 0.05 level for some comparisons), but the same is not true of anilioid paraphyly (with tropidophiids outside a clade comprising anilioids and all other alethinophidians). Given the relatively small amount of data, reasonable taxon stability, and agreement across different analyses in our study, and the general agreement with other analyses of larger DNA alignments for snakes where taxon sets overlap with ours, we accept that our data support non-monophyly of Anilioidea and tentatively accept the hypothesis that, within a clade comprising Asian anilioids, *Anomochilus* is most closely related to cylindrophiids.

Our optimal trees agree with Fig. 1a, while all explicit numerical analyses of large morphological data sets (Cundall et al. 1993; Scanlon and Lee 2000; Tchernov et al. 2000; Lee and Scanlon 2002) have agreed on Fig. 1b. None of the explicit morphological analyses of anilioid phylogeny has hypothesized that *Anomochilus* forms a clade with *Cylindrophis*. Underwood (1967) and McDowell (1975, 1987) classified the two genera in a group to the exclusion of Uropeltidae, but this seems to have largely been based on the distinctiveness of the latter family. One morphological character possibly uniting *Anomochilus* and *Cylindrophis* is the shallow fovea dentis on the occipital condyle (Cundall and Rossman 1993; Lee and Scanlon 2002). Previous morphological studies of cylindrophiids have focussed on *C. ruffus* and, to a lesser extent, *C. maculatus*, and they have found differences between these species (e.g. Williams 1959; Rieppel 1979, 1980; McDowell 1987), but ours is the first study to have explicitly tested (and tentatively rejected) *Cylindrophis* monophyly. Future studies might profitably carry out taxonomically broader investigations to conduct more stringent tests of cylindrophiid monophyly. The biogeographic relationships of India, Sri Lanka and Southeast Asia are of great interest (e.g. Gower et al. 2002;

Bossuyt et al. 2004), and the generality of patterns could be further tested with a well resolved and supported phylogeny for Asian anilioids.

Unsurprisingly, our analyses retrieved the same uropeltid intrarelationships as Bossuyt et al. (2004; Fig. 2c) using a similar data set. That *Melanophidium* joins other uropeltids at a basal split was previously proposed based on morphological evidence (Rieppel 1977, 1980; McDowell 1987; Rieppel and Zaher 2002). Other molecular analyses have recovered *Anilius* + Tropidophiidae as monophyletic (Vidal and Hedges 2002, 2004) or paraphyletic, with *Anilius* being sister to tropidophiids + other alethinophidians (Wilcox et al. 2002; Lawson et al. 2004). This study detected signal for the converse paraphyletic arrangement.

Anilioid relationships have important implications for understanding evolution of feeding mechanisms and trends of fossoriality in early snake evolution. Analyses have yet to agree on whether para- or polyphyly is most likely, but expanded DNA sequence data sets perhaps offer the best chance of resolving this robustly, and thus for establishing a framework for improved understanding of early snake evolution. Our analyses indicate that *Anomochilus* is embedded within Asian anilioids, so that future studies of the interrelationships of major snake lineages might not be unduly affected by a lack of additional sequence data for this genus, especially if cylindrophiids and uropeltids can be more densely sampled. Anilioidea might have to be abandoned as a natural higher taxon, but non-aniliid anilioids would seem to constitute a natural group. Because of previous disagreements over the name and rank of higher taxa within Anilioidea, and until more robust estimates of phylogeny are established, we suggest that 'Asian anilioids' is a useful term for this group.

Acknowledgements

Zussammenfassung

Phylogenetische Stellung der Anomochilidae (Reptilia: Serpentes): erste Evidenz basierend auf DNA Sequenzen

Anilioide Schlangen (Aniliidae, Anomochilidae, Cylindrophiidae, Uropeltidae) wurden bisher als einzige rezente, nicht macrostomate alethinophide Schlangen angesehen. Obwohl die Monophylie sowie die Verwandtschaftsbeziehungen innerhalb der anilioiden Schlangen wenig etabliert sind, haben ihre Fossorialität, Öffnungsweite des Maules und vermutete Stellung im phylogenetischen System wichtige Evidenzen für orthodoxe Evolutionsszenarien früher Schlangen geliefert. Jüngste molekulare Analysen unter Einbeziehung von aniliiden, cylindrophiiden und uropeltiden Schlangen weisen auf eine Polyphylie der anilioiden Schlangen, wobei die letzteren beiden Familien ein Monophylum innerhalb der Macrostomata bilden. Die vorliegende Arbeit ist die erste molekulare phylogenetische Analyse unter Einbeziehung der weitgehend unbekannten und anscheinend seltenen Anomochilidae. Von Gewebeproben eines einzigen, tot aufgefundenen Exemplars von *Anomochilus leonardi* konnten lediglich partielle Sequenzen der mitochondrialen 12S und 16 S rRNA Gene amplifiziert werden. Die Amplifikation von Kern- und anderen mitochondrialen Genen, sowie aller untersuchten Gene von Holo- und Paratypus von *A. leonardi* schlug fehl. Die Analysen ergaben para- oder polyphyletische Anilioidea. *Anomochilus* is nächst verwandt zu *Cylindrophis maculatus* (Cylindrophiidae somit möglicherweise paraphyletisch). Die relativ geringe verfügbare Datenmenge führt zu lediglich moderat unterstützenden Ergebnissen. Die Stabilität der untersuchten Taxa sowie deren Übereinstimmung unabhängig von verwendeten Analyseverfahren und im Rahmen umfassender phylogenetischer Analysen legen die Abschaffung der Anilioidea sowie die Annerkennung eines höherrangigen Monophylums asiatischer anilioider Schlangen nahe.

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Appendix

Taxa included in analysis. Format is: Family, *Genus species* Authority (GenBank accession numbers for 12S, and 16S rRNA).

Typhlopidae, *Ramphotyphlops braminus* (Daudin, 1803) (AF544751, AF544823); Aniliidae, *Anilius scytale* (Linnaeus, 1758) (AF544753, AF544826); Cylindrophiidae, *Cylindrophis ruffus* (Laurenti, 1768) (AF544744, AF544817); Cylindrophiidae, *Cylindrophis maculatus* (Linnaeus, 1758) (AY700991, AY701022); Anomochilidae, *Anomochilus leonardi* Smith, 1940 (AY953430, AY953431); Uropeltidae, *Melanophidium punctatum* Beddome, 1871 (AY700993, AY701024); Uropeltidae, *Brachyophidium rhodogaster* Wall, 1921 (AY700992, AY701023); Uropeltidae, *Rhinophis drummondhayi* Wall, 1921 (Z46447, Z46477); Uropeltidae, *Rhinophis philippinus* (Cuvier, 1829) (AF512740, AF512740); Uropeltidae, *Uropeltis melanogaster* (Gray, 1858) (AF512739, AF512739); Xenopeltidae, *Xenopeltis unicolor* Reinwardt, 1827 (AF544752, AF544825); Loxocemidae, *Loxocemus bicolor* Cope, 1861 (AF544755, AF544828); Bolyeridae, *Casarea dussumieri* (Schlegel, 1837) (AF544754, AF544827); Tropidophiidae, *Tropidophis melanurus* (Schlegel, 1837) (AF544757, AF544830); Tropidophiidae, *Tropidophis wrighti* Stull, 1928 (Z46445, Z46476); Tropidophiidae, *Trachyboa gularis* Peters, 1860 (AF544756, AF544829); Pythonidae, *Liasis savuensis* Brongersma, 1956 (AF544748, AF544820); Boidae, *Boa constrictor* Linnaeus, 1758 (Z46470, Z46495); Calabariidae, *Calabaria reinhardtii* (Schlegel, 1848) (Z46464, Z46494); Acrochordidae, *Acrochordus granulatus* (Schneider, 1799) (AF544738, AF544786); Viperidae, *Bothriechis schlegelii* (Berthold, 1846) (AF057213, AF038888); Elapidae, *Dendroaspis angusticeps* (Smith, 1849) (AF544764, AF544792).