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MOLECULAR PHYLOGENETICS OF SRI LANKAN *ICHTHYOPHIS* (AMPHIBIA: GYMNOPHIONA: ICHTHYOPHIIDAE), WITH DISCOVERY OF A CRYPTIC SPECIES

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ABSTRACT. **–** Based on previous morphological analyses, the caecilian amphibian (Gymnophiona) fauna of Sri Lanka has been considered to consist of three endemic species of the ichthyophiid genus *Ichthyophis*, two of which have a lateral yellow stripe. We examined the relationships of Sri Lankan caecilians using partial sequences of mitochondrial 12S and 16S rRNA and cytochrome *b* genes for 18 Sri Lankan *Ichthyophis* from 14 localities. Based on the latest keys, these 18 samples represent one striped (*I. glutinosus*) and one unstriped (*I. orthoplicatus*) species. Sequences for these samples were aligned against previously reported sequences for Indian and Southeast Asian *Ichthyophis*, and analysed using parsimony, maximum likelihood, distance and Bayesian methods. Results from all methods are in close agreement. Inferred trees strongly support the hypothesis that Sri Lankan caecilians are monophyletic, though their relationships to other Asian caecilians are unclear. While most of the striped specimens that key out as *I. glutinosus* comprise a clade, a small subset from a single locality are robustly recovered as more closely related to the unstriped *I. orthoplicatus*. These individuals are interpreted as a possibly new, morphologically cryptic species. The *I. glutinosus* clade is the most widespread among our samples, and it contains some weakly supported, but consistently recovered hierarchical structure. Most notably, all specimens from the southwestern corner of Sri Lanka comprise a clade, possibly representing a relatively recent dispersal from the central highlands.

KEY WORDS. **–** caecilians, evolution, mitochondrial DNA, Sri Lanka, systematics, taxonomy.

INTRODUCTION

Caecilian amphibians (Gymnophiona) are represented in Sri Lanka with certainty only by species of the South and Southeast Asian ichthyophiid genus *Ichthyophis* Fitzinger (see Nussbaum & Gans, 1980). The most recent taxonomic revision recognised three endemic species based on a combination of numbers of annuli and colour pattern (Nussbaum & Gans, 1980), the unstriped *Ichthyophis orthoplicatus* Taylor and the longitudinally yellow-striped *I. glutinosus* (Linnaeus) and *I. pseudangularis* Taylor. Gower et al. (2002) included single individuals of *I. glutinosus* and *I. orthoplicatus* in broader molecular phylogenetic analyses of Ichthyophiidae, and showed them to be more closely related to each other than to any striped or unstriped species from elsewhere in Asia, and Bossuyt et al. (2004) corroborated this result with a much wider sampling of Sri Lankan *Ichthyophis*.

Although a seemingly relatively small radiation, the Sri Lankan caecilians are of broader importance. *Ichthyophis glutinosus* is the type species of its genus and family, and one of the few caecilians to have had its mitochondrial genome sequenced (San Mauro et al., 2004). The striped Sri Lankan caecilians are the subject of the most detailed study ever published on the biology of any gymnophionans (Sarasin & Sarasin, 1887–1890), a work that has recently informed debates about the origin of the amniotic egg (Wilkinson et al., 2002). Sri Lankan caecilians are also of interest to biogeographers of South

Table 1. Details of voucher specimens of Sri Lankan *Ichthyophis* included in this study. Index numbers in the left hand column correspond to locations indicated in Fig. 1. *I. orthoplicatus* lacks the lateral yellow stripe present in *I. glutinosus*. The specimens listed as "*I.* sp." are striped and key out as *I. glutinosus*, but phylogenetic results suggest they are a distinct species. Voucher specimens are deposited in the Department of National Museums, Colombo (MW field tags), the Wildlife Heritage Trust of Sri Lanka, Agrapatana (WHT), and the Zoology Department of the Natural History Museum, London (BMNH). * indicates individuals for which 12S and 16S rRNA and cytochrome *b* sequences were previously published by Gower et al. (2002). † specimen incorrectly reported as MW 1733 in Gower et al. (2002). All other sequences were previously published by Bossuyt et al. (2004).

	Taxon	Voucher	Total annuli	Province	District	Locality	Altitude (m)
1	*I. orthoplicatus*	MW 1722*	302	Uva	Badulla	Cannavarella Group, nr. Passara	1100
2	*I. orthoplicatus*	WHT 5151	296	Uva	Badulla	Kandahena Estate, Bibilegama	915
3	*I.* sp	MW 1730	335	Sabaragamuwa	Ratnapura	Welegama, Haldummula	782
4	*I.* sp.	BMNH 2000.348	343	Sabaragamuwa	Ratnapura	Welegama, Haldummula	750
5	*I.* sp.	BMNH 2000.349	327	Sabaragamuwa	Ratnapura	Welegama, Haldummula	750
6	*I. glutinosus*	WHT 5152	336	Uva	Badulla	Kandahena Estate, Bibilegama	915
7	*I. glutinosus*	WHT 5164	336	Central	Matale	Gammaduwa, MB line, route to Mousakanda	743
8	*I. glutinosus*	MW 1749	375	Central	Matale	Kandehena, nr. Rattota	673
9	*I. glutinosus*	MW 1746*†	375	Central	Matale	Kandehena, nr. Rattota	673
10	*I. glutinosus*	MW 1733	360	Central	Kandy	Mawalawatta, nr. Peradeniya	548
11	*I. glutinosus*	WHT 5785	381	Central	Kandy	Pussellawa, between Kandy-Nuwara Eliya	980
12	*I. glutinosus*	MW 1789	368	Sabaragamuwa	Ratnapura	Suudagala	185
13	*I. glutinosus*	WHT 5808	318	Southern	Matara	Paragala, nr. Morawaka	200
14	*I. glutinosus*	MW 1769	340	Western	Kalutara	Tiniyawala, nr. Palawatta	175
15	*I. glutinosus*	MW 1773	335	Southern	Galle	Malgalla, nr. Opata	65
16	*I. glutinosus*	MW 1783	353	Southern	Matara	Hanuford Estate, nr. Deniyaya	500
17	*I. glutinosus*	WHT 5794	369	Southern	Galle	Kottawa, nr. Galle	50
18	*I. glutinosus*	WHT 5792	353	Southern	Galle	Ginidawanawatta, nr. Nakiyadeniya	100

Asia (Gower et al., 2002; Bossuyt et al., 2004). Finally, recent conservation assessments have considered at least some Sri Lankan caecilians to be under threat (IUCN Sri Lanka, 2000; IUCN, 2004).

Here we report on extended molecular analyses of the systematics of Sri Lankan *Ichthyophis* in order to assess lower level variation and relationships, and present evidence for a possibly previously unrecognised, morphologically cryptic species.

MATERIALS AND METHODS

Tissue samples analysed for this study come from two main sources. First, from a joint expedition of the Natural History Museum, London and the Department of National Museums, Colombo in November 2001, and second, from longer-term collecting by the Wildlife Heritage Trust of Sri Lanka. A total of 18 Sri Lankan *Ichthyophis* (Table 1) from 14 localities (Table 1, Fig. 1) were included in molecular analyses. Based on the latest key (Nussbaum & Gans, 1980), these samples represent two species, the striped *I. glutinosus* and unstriped *I. orthoplicatus*. No individuals identifiable as the other nominate, striped species (*I. pseudangularis*) were sampled. One striped specimen (number 13, Table 1) had an annular count that falls below the range previously (Nussbaum & Gans, 1980) reported for the striped *I. glutinosus*, though closer to the lower bound of this range than to the highest value reported for *I. pseudangularis*. We tentatively identified this specimen as *I. glutinosus*. Three specimens (numbers 3–

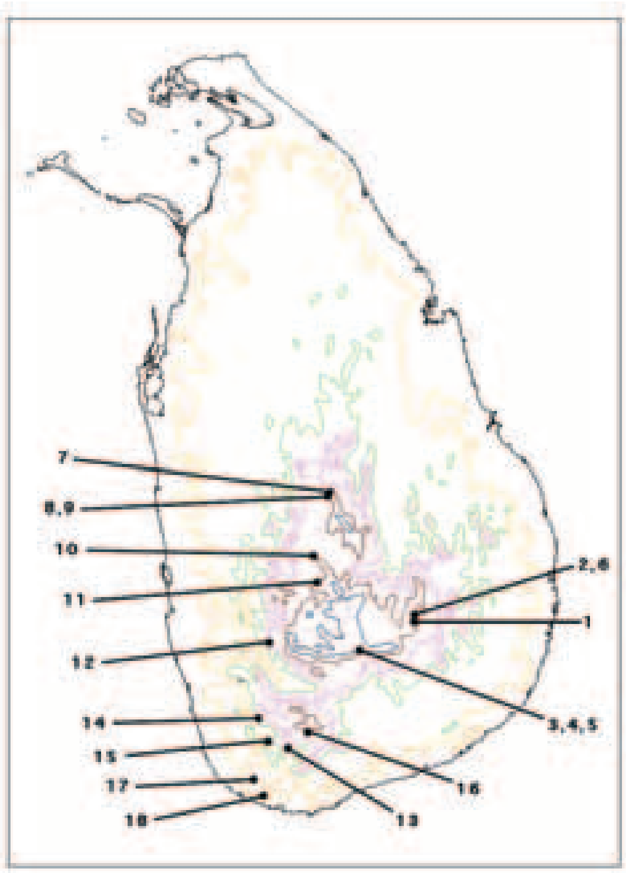

Fig. 1. Map of Sri Lanka showing position of localities from which eighteen samples of *Ichthyophis* were sampled for this study. Contours are indicated for 100, 500, 1000 and 3000 m above sea level. Numbers correspond to specimens listed in Table 1.

5, Table 1) initially identified as *I. glutinosus* are not closely related to that species (see below), and these are referred to as Sri Lankan *I.* sp.

Genomic DNA was extracted from samples of liver and/or muscle/skin stored in absolute ethanol, using a standard phenol/chloroform procedure. Partial sequences of three mitochondrial genes were amplified and sequenced using methods and primers given by Gower et al. (2002). The genes selected, 12S and 16S rRNA and cytochrome *b*, were chosen because of their ability to resolve both shallow and deeper branching within a wider sample of ichthyophiids (Gower et al., 2002), and because some in- and outgroup data were already available. The new sequences (GenBank accession numbers AY700937-990) were first reported and analysed in a different context by Bossuyt et al. (2004).

The Sri Lankan sequences were aligned against a subsample of the *Ichthyophis* sequences previously reported by Gower et al. (2002) from peninsular India (*I.* cf. *malabarensis* Taylor -GenBank accession numbers AY101205, 245 and 245; *I.* cf. *beddomei* Peters, AY101219, 212, 229, 232, 249, 252; and *I.* cf. *tricolor* Annandale, AY101209, 210, 229, 230, 249, 250) and mainland Southeast Asia (*I. bannanicus* Yang,AY101215, 235, 255; and *I.* sp., AY101217, 237, 257). Apart from *I.* cf. *malabarensis*, all these are striped species. This alignment was used to test the hypothesis that the Sri Lankan *Ichthyophis* are monophyletic (Gower et al., 2002; Bossuyt et al., 2004) and a second, more restricted alignment of only the Sri Lankan sequences was produced to reduce the number of sites excluded because of alignment ambiguity. These two data sets are referred to as the "full" and "Sri Lankan" alignments. Sequences were aligned by hand. Length differences were resolved by inserting alignment gaps, positions that could not be aligned unambiguously were excluded, and alignment gaps were treated as missing data.

Parsimony, maximum likelihood (ML), maximum likelihood distance (MLD), and LogDet distance (LDD) analyses were performed with PAUP* 4.0b10 (Swofford, 1998). MLD and LDD used the minimum evolution objective function. Tree searches were heuristic with 100 (parsimony) or 10 (ML) random addition sequences and TBR branch swapping. Bayesian analyses were performed with MrBayes 2.01 (Huelsenbeck & Ronquist, 2001). The Metroplis coupled, Markov chain Monte Carlo analyses were run with four chains for 1,500,000 generations. Trees from the first 1000 generations were discarded as "burn in", but subsequently trees were sampled every 1000 generations. ML, MLD and Bayesian analyses used models of evolution selected using Modeltest (Posada & Crandall, 1998), and the estimated proportion of invariant sites was used in LDD analyses.

Support for clades was measured with parsimony and MLD bootstrap proportions (Felsenstein, 1985) (100 replicates), Bayesian posterior probabilities, and decay indices (Bremer, 1988). Differences between optimal and suboptimal trees were assessed with PAUP* using the Templeton test (Templeton, 1983) and the Kishino-Hasegawa (KH) test (Kishino & Hasegawa, 1989), for parsimony and ML analyses respectively. The more conservative two-tailed versions of these tests were used. KH tests were performed using RELL with 1000 bootstrap replicates. Suboptimal trees for these tests were generated (and decay indices determined) with topologically constrained analyses. 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 was treated with caution (see Wilkinson et al., 2003).

For analyses using the full alignment, trees were rooted with *I.* cf. *malabarensis*, following the results of Gower et al. (2002). Trees recovered from analyses of the Sri Lankan alignment used the rooting implied by analyses of the full alignment.

RESULTS

PCR amplification generally produced single products of expected size, with negligible nucleotide ambiguity. For cytochrome *b* sequences, no gaps or ambiguous alignments were implied and no stop codons were detected in the corresponding amino acid sequences. Thus we have no reason to suspect our data to have come from pseudogenes.

The full and Sri Lankan alignments total 1,555 and 1,597 (284 and 124 informative under parsimony) aligned sites respectively. Details of the three gene data partitions for the two alignments are given in Tables 2a and 2b. Very few length differences exist between the rRNA sites among sequences in the Sri Lankan alignment, and no (12S) or few (16S) sites were removed because of alignment ambiguity.

Considering all characters, there are no significant differences in base composition for either the full (c^2 test for homogeneity, d.f. = 72, $P = 1$) or Sri Lankan (d.f. = 51, $P = 1$) alignment. However, there are significant biases for the subset of informative (under parsimony) characters for the two alignments ($P = 0.023$ and < 0.001 respectively). Examination of partitions of the alignments shows the significant differences to lie in the cytochrome *b* third position sites (full alignment), and cytochrome *b* (for third and all positions) and 16S sites (Sri Lankan alignment). Table 3 shows that, apart from the Indian *I.* cf. *malabarensis*, all taxa with lower than average C+G content in informative sites are Sri Lankan. Furthermore, CG content in these sites is lower for Sri Lankan *I.* sp. and *I. orthoplicatus* than for all *I. glutinosus*.

Analysis of the full alignment recovered 24 most parsimonious trees (MPTs) that differ in the resolution of relationships among *I. glutinosus* individuals, and among the three Sri Lankan *I.* sp. Modeltest recommended TrN + I + G and GTR (Rodriguez et al., 1990) + I + G models based on the hierarchical likelihood ratio tests and Akaike information criterion respectively, and we used the former, simpler model. Analyses with the more complex model did not produce substantially different results (not shown). ML analysis recovered the single tree shown in Fig. 2, which differs from all the MPTs only in the relative positions of the Indian and Southeast Asian species, a result also reported by Gower et al. (2002). MLD, Bayesian and LDD analyses yielded trees (not shown)

Table 2a. Details of character informativeness for the full alignment of 25 South and Southeast Asian *Ichthyophis*.

	cytochrome *b*				12S rRNA	16S rRNA	Total
	All positions	position 1	position 2	position 3			
Constant	449	182	217	50	288	419	1156
Variable - uninformative	54	10	4	40	33	28	115
Parsimony informative	187	38	9	140	42	55	284
Total	690	230	230	230	363	502	1555

Table 2b. Details of character informativeness for the Sri Lankan alignment of 18 *Ichthyophis*.

	cytochrome *b*				12S rRNA	16S rRNA	Total
	All positions	position 1	position 2	position 3			
Constant	601	213	225	163	360	503	1464
Variable - uninformative	5	1	1	3	3	1	9
Parsimony informative	84	16	4	64	14	26	124
Total	690	230	230	230	377	530	1597

very similar to those obtained using ML. All analyses of the full data recovered a monophyletic Sri Lankan *Ichthyophis*, comprising a clade of *I. glutinosus* as sister group to a clade of *I. orthoplicatus* + *I.* sp. The monophyly of Sri Lankan *Ichthyophis* receives maximal support from parsimony and MLD bootstrap proportions, and Bayesian posterior probabilities (Fig. 2). The congruence of results using LDD and other methods suggests that base composition differences in the sequences are not a problem.

All analyses of the Sri Lankan alignment yielded trees that were consistent with the identity and relationships among the primary Sri Lankan lineages recovered in analyses of the full alignment. Analyses recovered eight MPTs. Modeltest again recommended two different models of evolution - TrN + G (hierarchical likelihood ratio tests) and GTR + I (Akaike information criterion), and we used the former, simpler model (although analyses with the more parameter rich model produced congruent results, not shown). Parsimony, ML, MLD, Bayesian and LDD analyses yielded trees that differed only in the relative positions of some *I. glutinosus* individuals.

Measures of quantitative support for relationships in the trees recovered from analyses of the Sri Lankan alignment are shown in Fig. 3. The sister-group relationship between *I. orthoplicatus* and Sri Lankan *I.* sp. is strongly supported by all analyses of both alignments. The monophyly of *I. glutinosus* and of *I.* sp. are also strongly supported, but the monophyly of *I. orthoplicatus* is much more marginal, with little implied molecular evolution having occurred since its split from *I.* sp. Similar patterns of support among Sri Lankan *Ichthyophis* were recovered from analysis of the full alignment (data not shown).

Table 3. Sequences in the full alignment ranked by increasing proportion of C + G in parsimony informative sites. Numbers in parentheses match index numbers of Sri Lankan individuals presented in Table 1.

Taxon	CG proportion
I. sp. (4)	0.36396
I. sp. (5)	0.36396
I. sp. (3)	0.37103
I. orthoplicatus (1)	0.37456
I. orthoplicatus (2)	0.37456
I. glutinosus (12)	0.40141
I. glutinosus (11)	0.40493
I. glutinosus (10)	0.40636
I. glutinosus (18)	0.40989
I. glutinosus (17)	0.40989
I. glutinosus (8)	0.40989
I. glutinosus (14)	0.40989
I. glutinosus (7)	0.41071
I. glutinosus (15)	0.41342
I. glutinosus (16)	0.41342
I. glutinosus (13)	0.41342
I. glutinosus (6)	0.41343
I. cf. *malabarensis* (SW India)	0.41489
I. glutinosus (9)	0.41696
Mean	0.41834
I. sp. (S Thailand)	0.43109
I. cf. *beddomei* (SW India)	0.45936
I. cf. *beddomei* (SW India)	0.46290
I. bannanicus (China)	0.46643
I. cf. *tricolor* (SW India)	0.51409
I. cf. *tricolor* (SW India)	0.52817

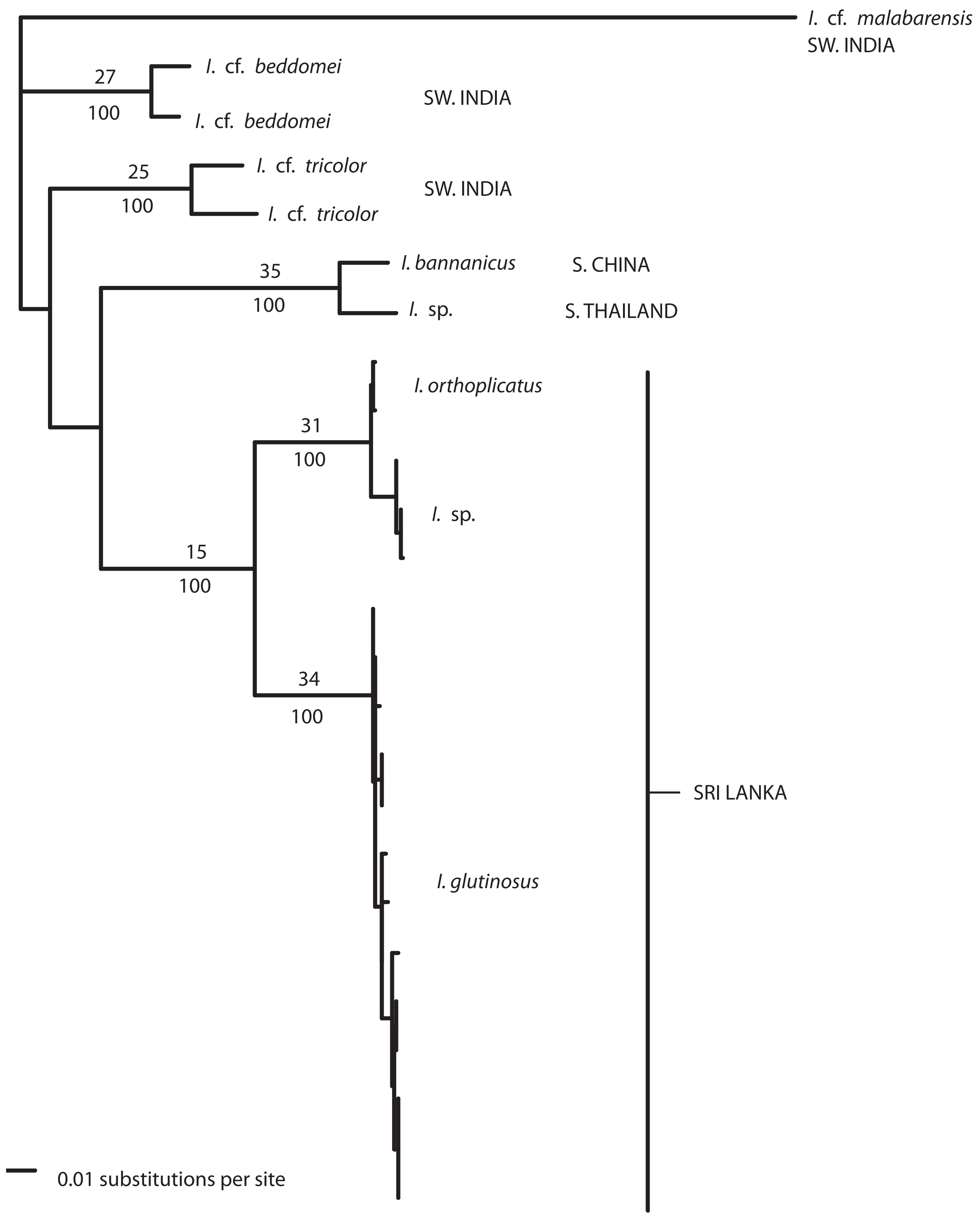

Fig. 2. Single maximum likelihood tree (LnL = 5388.84004) recovered from analysis of the full alignment. The chosen model of evolution (TrN + I + G) employed a symmetric rate matrix with AG and CT substitutions set at 4.6155 and 13.0781 respectively, and all other substitution types set at 1; base frequencies estimated at 0.3453, 0.2538, 0.1458 and 0.2551 for A, C, G and T respectively; a four category discrete approximation of a gamma distribution set at 0.4793, and the proportion of invariant sites set at 0.441. Numbers above branches are decay indices; numbers below branches (all maximal) are bootstrap proportions from parsimony, maximum likelihood distance and LogDet distance analyses, and Bayesian posterior probabilities.

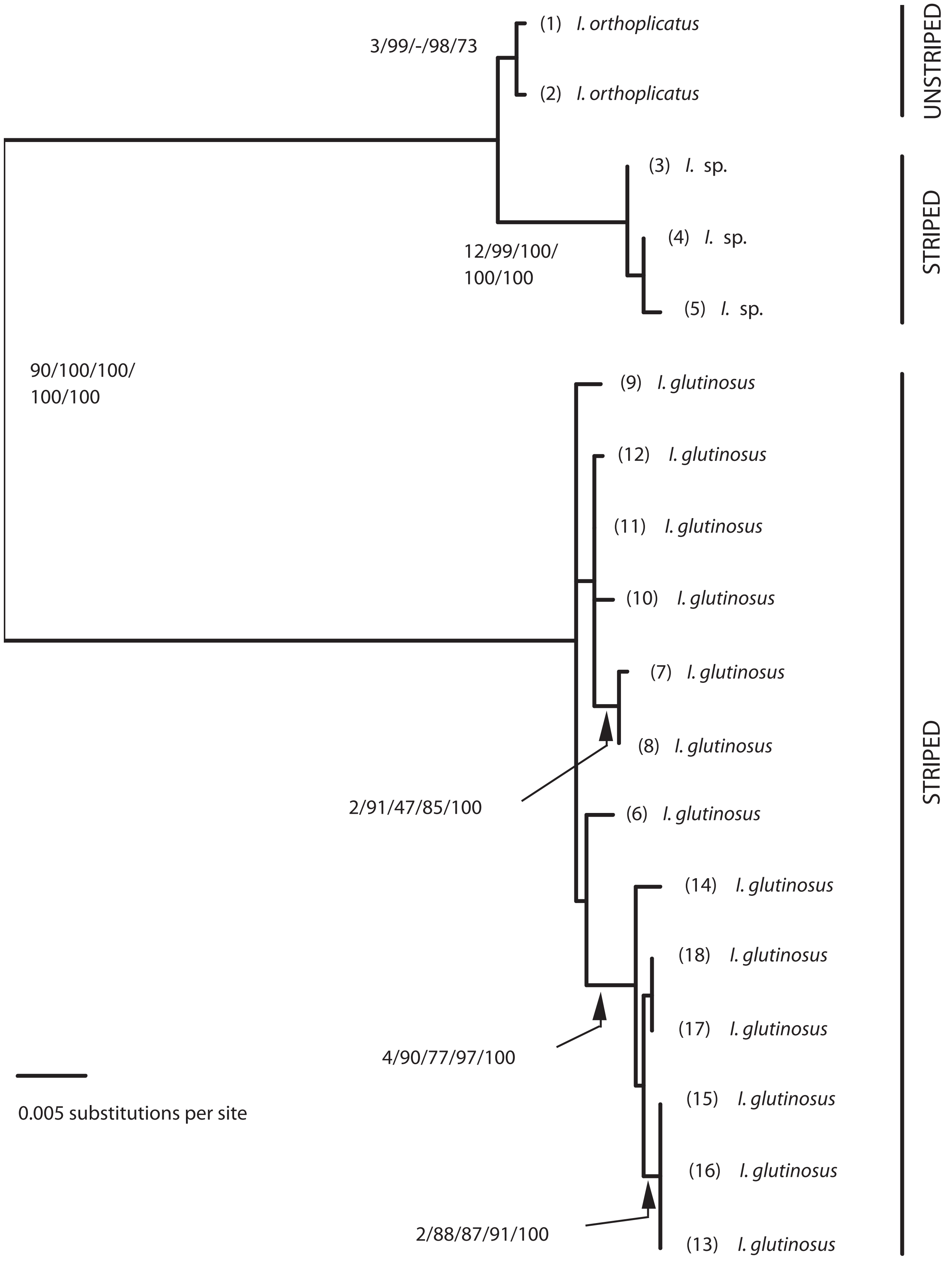

Fig. 3. Single maximum likelihood tree (LnL = 2949.77388) recovered from analysis of the Sri Lankan alignment. The chosen model of evolution (TrN + G) employed a symmetric rate matrix with substitutions set at 3.8572 and 10.3771 for A-G and C-T, and all other substitutions set at 1; base frequencies estimated at 0.3384, 0.2376, 0.1538 and 0.2702 for A, C, G and T respectively; a four category discrete approximation of a gamma distribution set at 0.4793, and the proportion of invariant sites set at zero. Numbers by branches are support values: decay indices/parsimony bootstrap proportions/MLD bootstrap proportions/LDD bootstrap proportions/Bayesian posteror probability. "-" signifies support value < 5.

Table 4. Within (where possible) and between species pairwise % differences for the full alignment of *Ichthyophis* analysed in this study (690 bp of cytochrome b; 363 bp of 12S rRNA; 502 bp of 16S rRNA). Numbers in parentheses indicate number of individuals for each taxon. The pair of *I.* cf. *beddomei* samples represent a divergent subsampling of the three individuals reported by Gower et al. (2002).

	I. cf. *malabarensis*	*I. bannanicus*	*I.* sp. (T)	*I.* cf. *tricolor*	*I.* cf. *beddomei*	*I. orthoplicatus*	*I.* sp. (SL)	*I. glutinosus*
I. cf. *malabarensis* (1)	–							
I. bannanicus (1)	14.7	–						
I. sp. (Thailand) (1)	14.6	3.2	–					
I. cf. *tricolor* (2)	14.4	10.2 –10.3	10.7 – 10.8	3.5				
I. cf. *beddomei* (2)	14.6 –14.7	9.8 – 10	10	8.4 – 8.7	2			
I. orthoplicatus (2)	14.4	11.1	10.9	10.2 –10.3	10.6 –10.7	0.1		
I. sp. (Sri Lanka) (2)	14.4 –14.5	11 –11.1	10.9	10.3 –10.6	10.5 –10.7	1 –1.2	0.1 – 0.3	
I. glutinosus *(13)*	14.3 –14.7	10.3 –10.9	10.5 – 11.1	10.0 –10.4	9.8 –10.6	6.1 –6.5	6.7 – 7.1	0– 1

Table 5. Within and between species pairwise % differences for the alignment of only Sri Lankan *Ichthyophis*.

Pairwise comparison	Number of comparisons	cytochrome *b* 690 base pairs	12S rRNA 377 base pairs	16S rRNA 530 base pairs	Total alignment 1597 base pairs
I. orthoplicatus – I. orthoplicatus	1	0.1	0.3	0	0.1
I. sp. – *I.* sp.	3	0.3 – 0.6	0	0	0.1 – 0.3
I. glutinosus – I. glutinosus	78	0 – 1.6	0 – 0.5	0 – 0.6	0 – 0.9
I. orthoplicatus – I. sp.	6	1.4 – 1.9	0.5 – 0.8	0.9	1.1 – 1.3
I. orthoplicatus – I. glutinosus	26	9.7 – 10.3	2.9 – 3.5	3.6 – 4.2	6.2 – 6.5
I. sp. – *I. glutinosus*	39	10.3 – 10.9	3.5 – 3.7	4.2 – 4.7	6.8 – 7.1

All analyses, irrespective of alignment and method, yielded trees in which three clades were consistently recovered within *I. glutinosus*: (1) a pairing of two of the northernmost *I. glutinosus* samples (7 and 8 in Table 1), (2) a clade of the six most southwestern *I. glutinosus* (13 to 18 in Table 1), and (3) a subset of three of these latter individuals (13, 15, 16). None of these clades has an impressive decay index. The second receives mostly high support from parsimony and LDD bootstrap proportions and Bayesian posterior probabilities (Fig. 3), but is not significantly better supported by the data than alternatives, as judged by Templeton and KH tests ($P >$ 0.1). An *a priori* hypothesis, based on the latest key (Nussbaum & Gans, 1980), is that all the striped Sri Lankan *Ichthyophis* sampled here represent a single species, *I. glutinosus*. Trees consistent with this hypothesis are significantly suboptimal as judged by Templeton and KH tests ($P < 0.0001$, and $P < 0.001$, respectively).

Pairwise differences between individual sequences for each alignment are summarised in Tables 4 and 5. Across the whole of the full alignment, pairwise differences between individuals in the same species range from 0 to 1%, and between individuals from different species from 1 to 14.7%. For the Sri Lankan alignment, these ranges are 0 to 0.9% and 1.1 to 7.1%, respectively. For the Sri Lankan alignment, differences in 12S data are slightly lower than in 16S data, with both these being substantially lower than cytochrome *b* differences (Table 5).

DISCUSSION

The monophyly of all sampled Sri Lankan caecilians is a strongly supported hypothesis, and one that will be further tested if *I. pseudangularis* is included in future molecular and/or morphological analyses. The relationships among the ichthyophiid caecilians of Sri Lanka, India and Southeast Asia are not yet robustly resolved.

Based on mitochondrial DNA sequence data, the three '*I.* sp.'specimens from Welegama represent a striped form that was not recognised in the latest revision of Sri Lankan caecilians (Nussbaum & Gans, 1980). Specimens have apparently not been collected previously from this locality. Nussbaum & Gans' (1980) key based on presence/absence of a stripe and the number of annuli is insufficient to distinguish this form from *I. glutinosus*. Given that this population potentially represents a distinct species, a detailed reappraisal of morphology and taxonomy is required.

Taylor (1968, 1969) had recognised five species of Sri Lankan *Ichthyophis,* but Nussbaum & Gans (1980) synonymised the unstriped *I. taprobanicensis* Taylor and striped *I. forcati* Taylor with *I. orthoplicatus* and *I. glutinosus*, respectively. Unfortunately, none of the type specimens of the three striped Sri Lankan species recognised by Taylor is associated with locality data more precise than 'Sri Lanka'. The possibility that the morphologically cryptic, striped species discovered here through molecular analysis corresponds to Taylor's *I. forcati* needs to be carefully considered. We think it unlikely that the Welegama clade and *I. orthoplicatus* together represent striped and unstriped morphs of a single species. Although the genetic distance between these morphs is not great, it is greater than for *I. glutinosus* from across a wider distribution, the striped specimens are all at (or above) the upper limit for numbers of annuli reported for *I. orthoplicatus* (Nussbaum & Gans, 1980), and preliminary morphological examination suggests there are further differences (e.g. a greater number of tail annuli in the Welegama form). However, our sample sizes are very small and further work will be needed to test our interpretation. It might also be noted that the pairs of Indian individuals labelled *I.* cf. *tricolor* and *I.* cf. *beddomei* (Fig. 2) might also represent more than one species in each case (Gower et al. 2002). Other alternative hypotheses that we believe to be much less likely, are that *I. glutinosus* is not represented in our sample (implying two additional striped species), or that *I. glutinosus* actually corresponds to our *I.* sp. In conclusion, it is most probable either that *I. forcati* will be recovered from synonymy or a new species will be described for the Welegama form. Whatever the outcome, we conclude that the diversity of Sri Lankan *Ichthyophis* is greater than had been thought since 1980, and comprises at least four species.

Interpreted most directly, the trees recovered in this study better fit the hypothesis that the stripe was lost in the evolution of *I. orthoplicatus*, rather than being gained independently in *I. glutinosus* and *I.* sp. However, this is based only on our incomplete sampling of extant lineages, and is only marginally more parsimonious (one loss versus two gains) than the alternative hypothesis. As found by Gower et al. (2002), striped and unstriped ichthyophiids do not constitute monophyletic groups.

Our limited field and phylogenetic results support the conclusions of Nussbaum & Gans (1980) in finding that one Sri Lankan species, *I. glutinosus*, is notably more widespread and variable than the others. Our initial tentative identification of WHT 5808 as *I. glutinosus* is supported by the phylogenetic results, and extends the lower bound in the range in annuli of this species to 318 (previously 329, Nussbaum & Gans, 1980). Of course, ranges in total annuli can be expected to increase with sample size, and only relatively few numbers of individuals of the other Sri Lankan species have been included in morphological and molecular analyses to date.

Local conservation assessments in 1999 considered all three Sri Lankan species of *Ichthyophis* recognised by Nussbaum & Gans (1980) to be threatened (IUCN Sri Lanka, 2000). The latest international assessment (IUCN et al., 2004) categorised them as Least Concern (*I. glutinosus*) or Vulnerable (*I. orthoplicatus* and *I. pseudangularis*), by virtue of small distributions, drought, and pollution. Although our searches were not randomised in any respect, and took place only in agricultural habitats, our findings are consistent with previous reports in finding *I. glutinosus* to be more commonly encountered than *I. orthoplicatus* and *I. pseudangularis*. Indeed, we found no *I. pseudangularis* during our fieldwork, and it is relatively rare in collections. *Ichthyophis glutinosus* seems to survive well in low intensity agriculture often closely associated with human habitation, but there are anecdotal reports that decreases in abundance have occurred in areas of high agrochemical use, such as larger areas of paddy cultivation. The potential fourth (third striped) species of Sri Lankan *Ichthyophis* revealed by this study is probably best considered of data deficient conservation status. However, it was collected adjacent to rice paddy cultivation and a main road, close to housing. The further work required to determine its distribution and abundance will be facilitated by a full morphological characterisation, based on more material, that allows this species to be identified without resort to sequence data.

Conservation assessment depends on accurate taxonomy (e.g. Gower & Wilkinson, 2005). The taxonomy of caecilians is generally poorly understood, and that of ichthyophiids especially confused (e.g. Gower et al., 2002). The findings of this study support Gower et al.'s (2002) view that mitochondrial DNA sequence data can be a valuable tool in remedying this situation. The present work is the most detailed low-level molecular analysis of any group of caecilians yet published. In particular, it indicates that cytochrome *b* sequences are especially informative for examining low-level relationships among closely related caecilian species.

Wilkinson et al. (2003) reported significant differences among CG contents in 12S and 16S rRNA sequences for a global sample of caecilians. Unlike the distribution of differences found by Wilkinson et al. (2003), where sequences for the polyphyletic African caecilians had significantly higher CG contents, the differences found in this study appear to be correlated with phylogeny.

As also reported by Gans (1993: 191), the known distribution of caecilians in Sri Lanka (Fig. 1; Nussbaum & Gans, 1980: fig. 4; Dutta & Manamendra-Arachchi, 1996: figs. 12, 18, 24) essentially appears to match those areas of the island that receive the greatest rainfall (> 2000 mm annually)—the central uplands and the south-western corner (e.g., Dutta & Manamendra-Arachchi, 1996: fig. 6). The subterranean uropeltid snakes of Sri Lanka share a broadly similar geographic distribution to that of the caecilians, although they extend to some drier areas in the North, and generally occupy a higher altitudinal range (e.g. Gans, 1993). Based on phylogenies inferred from immunological and electrophoretic data, Cadle et al. (1990) and Gans (1993) hypothesised that extant lineages of Sri Lankan uropeltids radiated from the south-western quadrant of the island, with dispersals into the central mountains. Although support for the south-western clade within *I. glutinosus* found in this study is not compelling, it is recovered in all analyses and, taken at face value, is more consistent with the hypothesis that extant populations of Sri Lankan caecilians dispersed into the Southwest from the central uplands rather than vice versa. If this is confirmed, it might represent evidence of a relatively recent dispersal into the moist lowlands from an upland refuge, and suggests a different recent biogeographic history to that proposed for uropeltid snakes. Analyses of interrelationships within *I. pseudangularis* would provide a test of the biogeographic hypotheses for Sri Lankan *Ichthyophis* because this species also occurs in both the central uplands and in lower altitude areas in the Southwest (Nussbaum & Gans, 1980).

It remains the case that, as stated by Nussbaum & Gans (1980: 151) "there are still large areas of Sri Lanka in which *Ichthyophis* probably occurs but for which we lack records". Furthermore, there are unconfirmed reports of Sri Lankan records of two other caecilian genera, the ichthyophiid *Caudacaecilia* (Nussbaum & Gans, 1980) and the uraeotyphlid *Uraeotyphlus* (Gans, 1998). Further field and laboratory work is required to gain a fuller understanding of the taxonomy, diversity, evolution, and conservation status of Sri Lankan caecilians.

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