THE ROYAL SOCIETY

Received 12 March 2002
Accepted 17 April 2002

A molecular phylogeny of ichthyophiid caecilians (Amphibia: Gymnophiona: Ichthyophiidae): out of India or out of South East Asia?

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Recent molecular phylogenetic studies indicate that the rafting Indian plate harboured several isolated vertebrate lineages between *ca.* 130 and 56 Myr ago that dispersed and diversified 'out of India' following accretion with Eurasia. A single family of the amphibian order Gymnophiona, the Ichthyophiidae, presently occurs on the Indian plate and across much of South East Asia. Ichthyophiid phylogeny is investigated in order to test competing out of India and out of South East Asia hypotheses for their distribution. Partial sequences of mitochondrial 12S and 16S rRNA and cytochrome *b* genes for 20 ichthyophiids and proximate outgroups were assembled. Parsimony, maximum-likelihood and distance analyses all recover optimum trees in which uraeotyphlids plus *Ichthyophis* cf. *malabarensis* are the sister taxa to all other *Ichthyophis*, among which the South East Asian taxa are monophyletic. Tree topology and branch lengths indicate that the Indian lineages are more basal and older, and thus are more consistent with the hypothesis that ichthyophiids dispersed from the Indian subcontinent into South East Asia. The estimated relationships also support monophyly of Sri Lankan *Ichthyophis*, and non-monophyly of striped and unstriped *Ichthyophis* species groups. Mitochondrial DNA sequences provide evidence that should assist current problematic areas of caecilian taxonomy.

Keywords: biogeography; evolution; mitochondrial DNA; Uraeotyphlidae; Gondwana

1. INTRODUCTION

Using divergence dates inferred from molecular phylogenies of ranid frogs, Bossuyt & Milinkovitch (2001) argued that multiple lineages of frogs (Order Anura) survived on the drifting Indian subcontinent after it split from Africa–Madagascar (*ca.* 130 Myr ago (Krause *et al.* 1999)) and before accretion with Eurasia (65–56 Myr ago (Beck *et al.* 1995)), and that these lineages subsequently diversified and dispersed 'out of India'. Other evidence consistent with the hypothesis that major lineages originated, and/or were isolated, on a drifting India before colonizing a wider area after accretion with Eurasia comes from considerations of biogeographic patterns of amphibians (e.g. Savage 1973; Duellman & Trueb 1986), molecular phylogenies for acrodont lizards (Macey *et al.* 2000) and ratite birds (Cooper *et al.* 2001), and palynological data for some plants (Morley 1998). This has important implications for interpreting biological history in the Mesozoic and Early Tertiary, and for understanding biogeographic patterns. Here, we use molecular genetic data to test an out of India hypothesis for another of the three orders of amphibians, the Gymnophiona or caecilians.

The mostly fossorial and tropical Gymnophiona are perhaps the least well known tetrapod order, and molecular data have only recently been employed to estimate parts of the group's phylogeny (Hedges *et al.* 1993; Wilkinson *et al.* 2002). Ichthyophiidae is one of the largest of the six caecilian families, with the two constituent genera, *Ichthyophis* and *Caudacaecilia*, including nearly 25% of the *ca.* 160 nominate caecilian species (e.g. Nussbaum & Wilkinson 1989). Ichthyophiidae and its sister taxon Uraeotyphlidae (Wilkinson & Nussbaum 1996; Wilkinson 1997; Wilkinson *et al.* 2002) are the only caecilian families restricted to Asia. Uraeotyphlidae (five nominate species

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DOI 10.1098/rspb.2002.2050

in a single genus; Pillai & Ravichandran 1999) is restricted to the southern part of the Western Ghats of peninsular India. Ichthyophiidae is more widely distributed across south Asia, occurring in the Western and Eastern Ghats of peninsular India, northeast India and Sri Lanka. Furthermore, ichthyophiids are the only caecilians to occur in South East Asia, being distributed on the mainland and on small (e.g. Koh Tao, Thailand) and large (e.g. Sumatra, Borneo) islands west of Wallace's line (e.g. Taylor 1968; Nussbaum & Wilkinson 1989). The distribution of the 34 nominate species of *Ichthyophis* subsumes that of the five species of *Caudacaecilia*.

The origin of South East Asian ichthyophiids is one of the major questions in caecilian biogeography (Hedges *et al.* 1993; Feller & Hedges 1998; Wilkinson *et al.* 2002). Two main alternatives have been proposed to explain their current distribution in Asia. The out of India hypothesis states that ichthyophiids were associated with the drifting Indian plate and that they dispersed into South East Asia following accretion with Eurasia (Duellman & Trueb 1986; Feller & Hedges 1998; Wilkinson *et al.* 2002). Conversely, the out of South East Asia hypothesis is that ichthyophiids dispersed into the Indian subcontinent from South East Asia following accretion (Hedges *et al.* 1993).

Here, we present a test of these competing biogeographic hypotheses. We report the first hypothesis of ichthyophiid and uraeotyphlid interrelationships at the species level, and the first use, to our knowledge, of protein-coding genetic data in estimating phylogenetic relationships within Gymnophiona. We also offer an initial assessment of the evidential potential that mitochondrial DNA (mtDNA) sequence data might have for caecilian taxonomy.

2. METHODS

(a) *Samples*

Ichthyophiids were collected from peninsular India, Sri Lanka, several regions in mainland and peninsular Thailand, southern China and two regions of Vietnam. *Ichthyophis* is known from species groups with and without lateral stripes that Taylor (1968, p. 48) thought 'may actually be generically separable'. Our samples comprise two unstriped (*I. orthoplicatus*; *I.* cf. *malabarensis*) and 13 striped populations of *Ichthyophis*. Sampling is incomplete taxonomically in lacking several species of *Ichthyophis* and *Caudacaecilia*, and geographically in lacking samples from South East Asian islands. Ichthyophiid taxonomy is not adequately established to enable the confident specific identification of many of these samples. Localities for the samples are indicated in figure 1. Details of specimens, vouchers and localities are given in table 1. Three Indian uraeotyphlids and two south American rhinatrematids were employed as proximate outgroups, on the basis of previous findings from morphological (Wilkinson & Nussbaum 1996) and molecular (Hedges *et al.* 1993; Wilkinson *et al.* 2002) analyses.

(b) *DNA extraction, amplification and sequencing*

Genomic DNA was extracted from *ca.* 5 mm^3 of liver preserved in aqueous 95% ethanol, and purified using phenol/chloroform extractions. The primers used in amplification and sequencing were L14724 (Meyer & Wilson 1990), cytochrome *b*1, cytochrome *b*2 (Kocher *et al.* 1989), the forward primer of cytochrome *b*2, and CB3-3′ (Palumbi 1996) for the cytochrome *b* gene, 12Sa and 12Sb for the 12S rRNA gene (Kocher *et al.* 1989), and 16Sa and 16Sb for the 16S rRNA gene (Palumbi 1996). Successful PCR bands were removed and purified. PCR products were sequenced using an ABI 377 automated sequencer (PE Biosystems, Warrington, UK), following the manufacturer's protocols. Each published sequence represents a consensus of both strands. GenBank accession numbers for sequences are given in table 1. Previously published rRNA sequences for *Epicrionops marmoratus* and *Ichthyophis bannanicus* (Hedges *et al.* 1993) and *Ichthyophis* cf. *tricolor* MW 1712 and *Uraeotyphlus* cf. *malabaricus* (Wilkinson *et al.* 2002) were not used because of a lack of exact correspondence with the analysed regions of the new data.

(c) *Phylogenetic analysis*

Sequences were aligned by hand. Length differences were resolved by inserting alignment gaps, and positions that could not be aligned unambiguously were excluded. Parsimony, maximum-likelihood (ML) and distance analyses were performed with PAUP*4b6 (Swofford 1998). LOGDET and maximum-likelihood distance (MLD) analyses used the minimum evolution objective function. ML and MLD analyses used the general time reversible (Rodríguez *et al.* 1990) model (recommended by MODELTEST 3.04; Posada & Crandall 1998), with empirical base frequencies. Rate matrix parameters, gamma-distribution shape parameters and the proportions of invariant sites were estimated iteratively through full likelihood evaluation of optimal trees, beginning with a most parsimonious tree (MPT), until they stabilized. Iterative optimization was also used to estimate the proportion of invariant sites in LOGDET analyses. Alignment gaps were treated as missing data. Tree searches were heuristic with 10 random addition sequences and tree bisection–reconnection branch swapping. Support for clades was measured with bootstrap proportions (Felsenstein 1985) (100 pseudoreplicates) and decay indices determined by enforcing converse topological constraints (Bremer 1988). The significance of length differences between most parsimonious and suboptimal trees found in constrained analyses were assessed using non-parametric tests (Templeton 1983). Significance of log-likelihood differences between optimal and suboptimal ML trees was assessed using the Shimodaira–Hasegawa test (Shimodaira & Hasegawa 1999) employing full optimization and 100 replicates and/or resampling estimated log-likelihood bootstrapping and 1000 replicates, depending on computation time. Trees were rooted with the rhinatrematids *E. marmoratus* and *Rhinatrema bivitattum*, following Wilkinson *et al.* (2002).

3. RESULTS

PCR amplifications generally resulted in single products of expected size, with negligible levels of site ambiguity. For cytochrome *b* sequences, no unambiguous alignments were found and, when translated into amino acid sequences using the vertebrate mitochondrial code, no stop codons were detected, indicating that all sequences are functional. Pairwise comparisons with the four samples for which sequences had been previously published found the *ca.* 850 overlapping base pairs of rRNA sequences to differ by 0 to 0.8%, mostly in the 12S data.

The analysed sequences total 1535 aligned sites. Of these, 940 are invariant and 446 are informative under parsimony. The alignment is available from the senior author upon request. Parsimony analysis of the full data

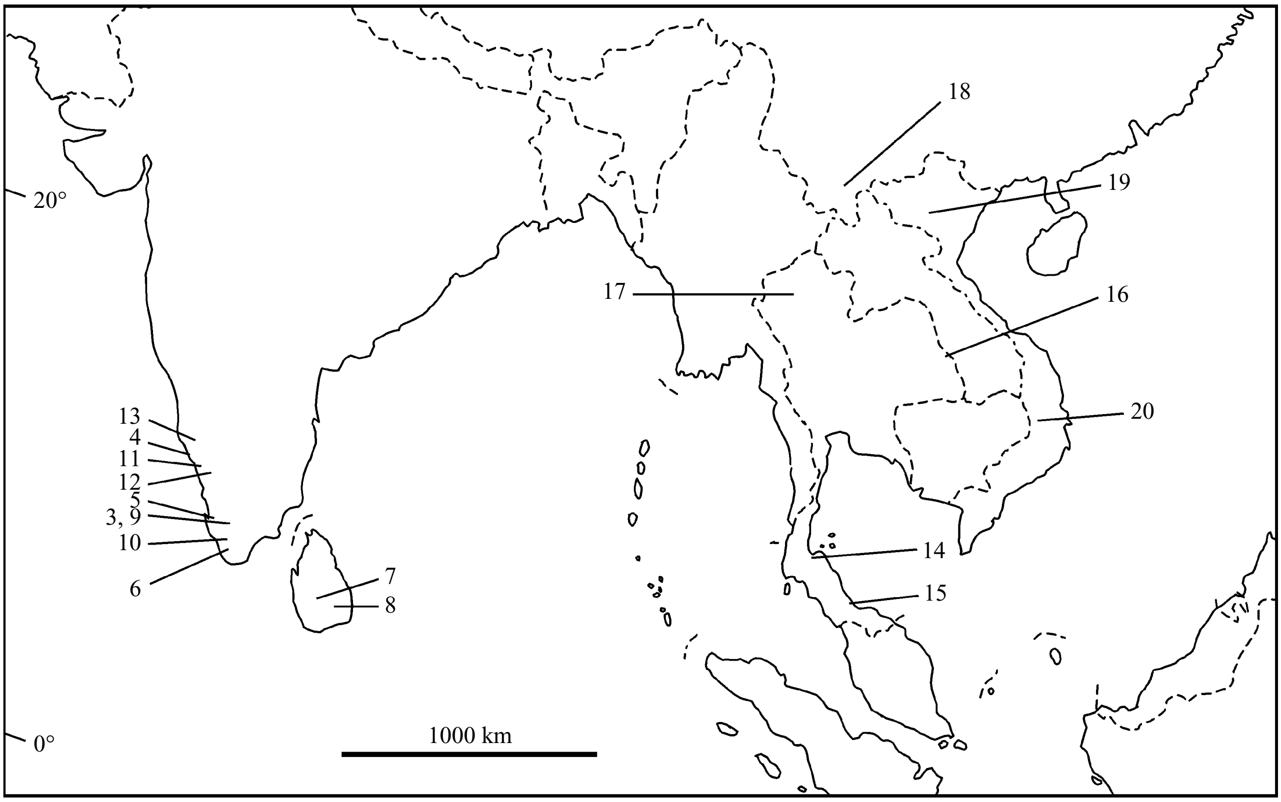

Figure 1. Outline map of the Indian subcontinent and parts of South East Asia, indicating location of numbered samples of uraeotyphlid and ichthyophiid caecilians listed in table 1.

yielded six MPTs varying only in the relative positions of *I. bannanicus* and the three striped *Ichthyophis* sp. specimens from northern Vietnam (taxon 19 in table 1), northeastern (16) and northwestern (17) Thailand. Figure 2*a* shows the strict component consensus of these MPTs. The LogDet distance tree (not shown) differs from this in the Sri Lankan clade being a sister taxon to *I.* cf. *tricolor* plus *I.* cf. *beddomei*, and South East Asian *Ichthyophis*. The ML tree (figure 2*b*) shows a different arrangement of the Sri Lankan, *I.* cf. *tricolor*, and *I.* cf. *beddomei* clades, but otherwise agrees closely with the parsimony and LogDet trees.

Several relationships in the MPTs are well supported as judged by high decay indices, high bootstrap proportions, and Templeton tests in which the best trees have a significantly better fit to the data than alternatives not including specified relationships (figure 2*a*). These well-supported relationships include monophyletic *Uraeotyphlus*, *I.* cf. *tricolor*, *I.* cf. *beddomei*, Sri Lankan *Ichthyophis*, South East Asian *Ichthyophis* and southern Thailand *Ichthyophis*. These relationships, and a *Uraeotyphlus* plus *I.* cf. *malabarensis* clade, are also significantly supported in ML analyses, as judged by Shimodaira–Hasegawa tests ($p < 0.05$). The best suboptimal trees containing monophyletic striped or unstriped *Ichthyophis* fit the data significantly worse under parsimony ($p < 0.013$) and ML ($p \leqslant 0.003$). Relationships absent in the optimal trees but which are relevant to competing biogeographic hypotheses were also tested. Most importantly, seven backbone constraint analyses were run in which at least one of the South East Asian *Ichthyophis* samples was a sister taxon to *Uraeotyphlus* and non-South East Asian *Ichthyophis*. These were thus designed to find the best trees in which the inferred ancestral area for ichthyophiids is either equivocal or favouring South East Asia. The analyses all found the same optimal trees, including a monophyletic South East Asian group, such that the ancestral area is equivocal. These trees are a significantly worse fit to the data under parsimony (18 extra steps; $p < 0.003$), but not ML (−ln greater by 6.3139; $p = 0.17$).

4. DISCUSSION

(a) *Historical biogeography*

Caecilians might be excellent subjects for historical biogeography because their largely subterranean lifestyle perhaps limits their dispersal, already diminished by the inability to cross major salt-water barriers shared with other extant amphibians (e.g. Nussbaum 1984). Thus, their present day distribution might be expected to reflect terrestrial dispersal and continental drift. The caecilian fossil record is very poor (Evans 2001), and to date is non-existent for Asia. Historical biogeography of the group must therefore currently be assessed with data from extant distributions, phylogeny and palaeogeography.

Hedges *et al.* (1993, p. 74) stated that 'A testable prediction of this hypothesis, that the ichthyophiids are of Laurasian origin, is that the South East Asian species should show greater phylogenetic structure (i.e. older lineages and more basal branching) than the ichthyophiids of India.' Rough divergence times estimated by Wilkinson *et al.* (2002) indicate that the uraeotyphlid and ichthyophiid lineages diverged on a drifting India before accretion with Eurasia, more consistent with the out of India hypothesis for Ichthyophiidae. We have not estimated divergence times because of a lack of obvious calibration points. Historical biogeography is instead assessed

Table 1. Data for samples and sequences. For each taxon sampled, locality, voucher and GenBank acquisition numbers are presented.
(BMNH, The Natural History Museum, London; MW, field series of the Zoology Department, University of Kerala, Thiruvananthapuram, India and the Department of National Museums, Colombo, Sri Lanka; UMMZ, University of Michigan Museum, Ann Arbor; ZMB, Zoologisches Museum Berlin, Berlin; ZFMK, Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn; ZSM, Zoologisches Staatssammlung München, München. Vouchers were identified on the basis of keys (Nussbaum & Gans 1980; Pillai & Ravichandran 1999; Taylor 1968), previously published identifications (Wilkinson *et al.* 2002), or comparison with type material and/or proximity of collected material to type localities.)

taxon	locality	voucher	GenBank accession numbers: cyt *b*, 12S rRNA, 16S rRNA
1. *Epicrionops marmoratus*	Ecuador, Cotopaxi, San Francisco de las Pampas	UMMZ 190478	AY101246, AY101206, AY101226
2. *Rhinatrema bivittatum*	French Guiana, near Kaw	BMNH 2002.6	AY101247, AY101207, AY101227
3. *Uraeotyphlus* cf. *malabaricus*	India, Kerala, Idukki District, near Vandiperiyar	MW 1711	AY101244, AY101204, AY101224
4. *Uraeotyphlus* cf. *oxyurus*	India, Kerala, Kannur District, near Payyanur	MW 212	AY101243, AY101203, AY101223
5. *Uraeotyphlus narayani*	India, Kerala, Kottayam District, Kannam	MW 1417	AY101242, AY101202, AY101222
6. *Ichthyophis* cf. *malabarensis*	India, Kerala, Thiruvananthapuram District, Palode	MW 198	AY101245, AY101205, AY101225
7. *Ichthyophis glutinosus*	Sri Lanka, Central Province, near Peradeniya	MW 1733	AY101254, AY101214, AY101234
8. *Ichthyophis orthoplicatus*	Sri Lanka, Province of Uva, near Passara	MW 1722	AY101253, AY101213, AY101233
9. *Ichthyophis* cf. *tricolor*	India, Kerala, Idukki District, near Vandiperiyar	MW 322	AY101248, AY101208, AY101228
10. *Ichthyophis* cf. *tricolor*	India, Kerala, Kollam District, near Punalur	MW 1712	AY101249, AY101209, AY101229
11. *Ichthyophis* cf. *beddomei*	India, Kerala, Wayanad District, near Periya	MW 283	AY101250, AY101210, AY101230
12. *Ichthyophis* cf. *beddomei*	India, Kerala, Wayanad District, near Sulthan Bathery	MW 222	AY101251, AY101211, AY101231
13. *Ichthyophis* cf. *beddomei*	India, Karnataka, Hassan District, higher elevations of Subramanya	MW 460	AY101252, AY101212, AY101232
14. *Ichthyophis* sp.	Thailand, Surat Thani Province, Ban Tung Tao	ZSM 1009/2001	AY101257, AY101217, AY101237
15. *Ichthyophis* sp.	Thailand, Songkhla Province, Hat Yai	ZSM 1012/2001	AY101256, AY101216, AY101236
16. *Ichthyophis* sp.	Thailand, Ubon Ratchathani Province, Ban Na Sabaeng	ZSM 1212/2001	AY101260, AY101220, AY101240
17. *Ichthyophis* sp.	Thailand, Chiang Mai Province, Mae Saivalley	ZMB 63600	AY101261, AY101221, AY101241
18. *Ichthyophis bannanicus*	China, Yunnan, Longling	UMMZ 189122	AY101255, AY101215, AY101235
19. *Ichthyophis* sp.	Vietnam, Tam Dao	ZFMK 72351	AY101259, AY101219, AY101239
20. *Ichthyophis* sp.	Vietnam, Mang Xang	ZSM 1013/2001	AY101258, AY101218, AY101238

from tree topology (and its implications for ancestral areas) and branch lengths.

The optimal trees from all analyses contain a basal split into uraeotyphlid (plus *Ichthyophis* cf. *malabarensis*) and ichthyophiid lineages, with the latter containing a South East Asian clade. The relationships of the Sri Lankan and Indian ichthyophiids (excluding *I.* cf. *malabarensis*) are less well resolved; forming either a mono- or paraphyletic outgroup to the South East Asian clade. Considering ancestral areas, the optimal phylogenies are more consistent with the hypothesis that ichthyophiids dispersed from India into South East Asia than *vice versa*. Under parsimony, these phylogenetic hypotheses are significantly better supported than explanations implying a South East Asian or equivocal ancestral area. Under ML, the suboptimal hypothesis of a basal dichotomy into South East Asian ichthyophiids and Indian ichthyophiids plus uraeotyphlids (the optimum backbone constraint topology) is not significantly worse, although this alternative only makes the reconstructed ancestral area for the ingroup equivocal. The Indian (and Sri Lankan) uraeotyphlids and ichthyophiids join the ML tree (figure 2*b*) with longer branches than the South East Asian ichthyophiids. This supports the hypothesis that ichthyophiids dispersed into South East Asia from India, assuming that branch lengths are positively correlated with divergence times.

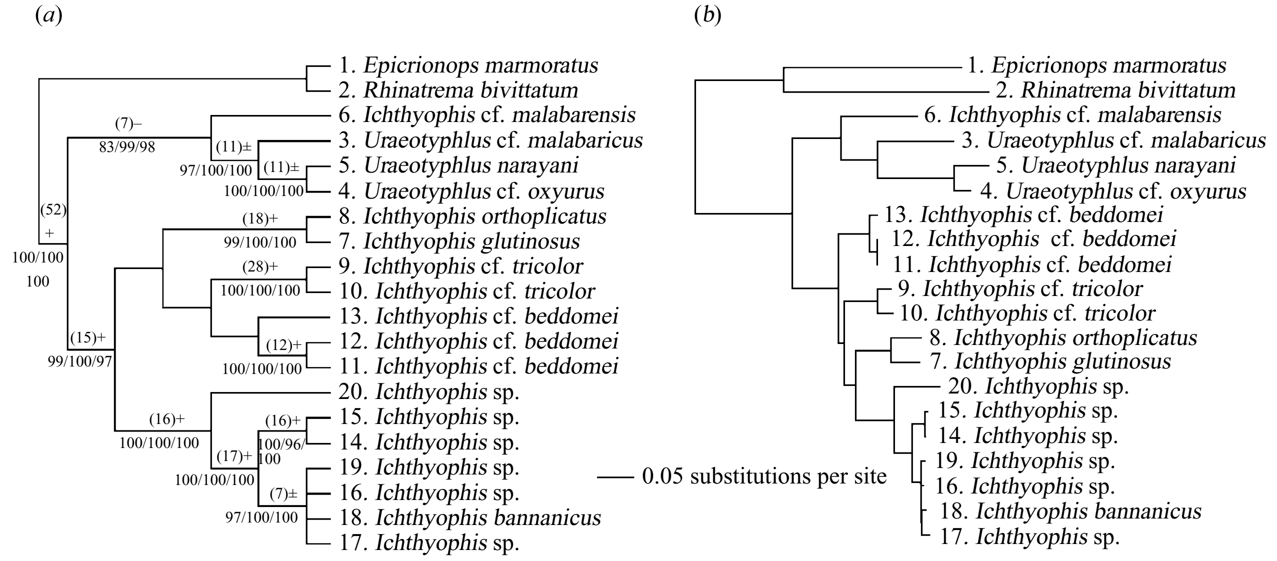

Figure 2. (*a*) Strict component consensus of six most parsimonious trees. Descriptive tree statistics are $L = 1488/1308$, $CI = 0.571/0.512$ and $RI = 0.642/0.642$ with all characters/without uninformative characters. Numbers (in parentheses) above internal branches are the decay index. The symbols following the decay index show the results of Templeton tests of clade support: significant support at $p \leqslant 0.05$ (+) or $p \leqslant 0.1$ (±) cut-offs, or no support at either cut-off (−). Numbers below branches are bootstrap values from parsimony/MLD/LogDet analyses. (*b*) Maximum-likelihood tree, −ln likelihood 8442.10612, showing branch lengths.

Hedges *et al.* (1993) largely viewed the out of South East Asia hypothesis as an out of Laurasia hypothesis, although they also recognized an alternative in which ichthyophiids were instead isolated on non-Indian Gondwanan fragments that are now part of South East Asia, and subsequently dispersed into India. Presently, South East Asia comprises several terraines derived from the northern margin of Gondwanaland in episodes around 350 (South, North and Indochina), 270 (the Cimmerian continental sliver), and 200–140 (West Burma, Lhasa) Myr ago (Metcalfe 1998, 2001). Thus, these terraines broke away from Gondwanaland before India did, and at least the most recent of these to break away may potentially have offered a route for Gondwanan caecilians into Asia. Our phylogenetic hypotheses are most readily interpreted as supporting the simple out of India, Gondwanan origin for ichthyophiids, and Wilkinson *et al.*'s (2002) interval estimate of 123–72 Myr ago for the uraeotyphlid–ichthyophiid divergence supports this by corresponding to the time that India was drifting. However, more complex hypotheses explaining the presence of ichthyophiids in India and South East Asia, such as multiple dispersal events from Gondwana, will be more stringently tested with further sampling and more robust divergence dates.

The out of India hypothesis can be challenged by (i) inferred ancestral areas that are unequivocally non-Indian, (ii) nodes within South East Asian ichthyophiids predating India–Eurasia accretion (65–56 Myr ago), and (iii) fossil South East Asian caecilians older than 56 Myr ago. The discovery of extinct or extant South East Asian uraeotyphlids, or the polyphyly of South East Asian caecilians might not be consistent with the out of India hypothesis in its simplest form. Assuming monophyly of South East Asian ichthyophiids is upheld, divergence dates will ultimately be required to choose between competing out of Gondwana biogeographic hypotheses.

(b) *Phylogeny and taxonomy*

Most areas of caecilian systematics are inadequately understood. Morphological caecilian systematics, especially at low levels, has been dogged by a paucity of obvious external characters and a lack of understanding of their variation that is frequently compounded by small samples, inadequate locality data and lack of detailed study. Ichthyophiid systematics can be considered to be especially confused, probably because of their being rather uniform externally, and in having their external annuli decoupled from underlying body segments (Naylor & Nussbaum 1980). Thus, high intraspecific variability in the number of annuli, one of the most commonly used taxonomic characters, may preclude its systematic value in many cases. The investigation by Nussbaum & Gans (1980) of the external morphology of Sri Lankan caecilians has been, to our knowledge, the only detailed study of ichthyophiid systematics published to date. DNA sequences offer a potential tool, but thus far they have mostly been applied to higher level caecilian phylogeny (Hedges *et al.* 1993; Wilkinson *et al.* 2002) and they remain largely unexplored.

An unexpected finding of this study is the non-monophyly of Ichthyophiidae and *Ichthyophis*. Although this might be remedied by transferring *I.* cf. *malabarensis* to *Uraeotyphlus*, any formal taxonomic revision will require rediagnoses of taxa that must await more thorough knowledge of morphology and relationships within the uraeotyphlid–ichthyophiid clade. This study also demonstrates the improbability that either striped or unstriped species of *Ichthyophis* constitute monophyla, though the direction of evolution and the number of evolutionary events is not known. Taylor's (1968) suggested division of *Ichthyophis* into two genera diagnosed by the presence or absence of stripes is inadvisable. We expect additional molecular data to provide a strong test of Nussbaum & Gans' (1980) hypothesis that Sri Lankan *Ichthyophis* have closer affinities with Indo–Malayan species than with those from peninsular India.

Genetic distances also appear to constitute useful evidence. The *I.* cf. *beddomei* samples MW 222 and MW 283 are from localities 53 km apart and have an uncorrected distance of zero. By contrast, the *I.* cf. *tricolor* samples (MW 322 and 1712) are separated by an uncorrected distance of 3.4%, approaching half the value between these *I.* cf. *beddomei* and *I.* cf. *tricolor* samples (8.3%), but their localities are separated by a similar geographical distance of about 63 km. Whether this represents previously undetected diversity at the specific or subspecific level remains to be fully investigated, but mtDNA data might be useful in directing the focus of time consuming morphological analyses. Interestingly, preliminary unpublished morphometric data are congruent with the identification of two subgroups within these same *I.* cf. *tricolor* samples (Presswell & Oommen 2001). The relatively large genetic distance between the *I.* cf. *tricolor* and *I.* cf. *beddomei* samples, and the possibility that these two groups are not each other's closest relative, are evidence against Nussbaum & Gans' (1980, p. 152) proposal that *I. tricolor* and *I. beddomei* 'may be only geographical races or colour morphs of a single species'. As indicated by ML branch lengths (figure 2*b*), genetic distances among the samples from northern Thailand and Vietnam and southern China (samples 16–19, table 1) are relatively small. Preliminary observations of soft anatomy indicate that at least the northeastern Thailand population is specifically distinct from *I. bannanicus*, so that this part of the ML tree can be taken to indicate relatively recent species level divergence.

The ML tree branch lengths (figure 2*b*) indicate that uraeotyphlids are a relatively old lineage with substantial divergence. The sister-group relationship between *Uraeotyphlus* and *I.* cf. *malabarensis* indicates that the last common ancestor of uraeotyphlids and ichthyophiids probably bore a close resemblance to modern ichthyophiids. All optimum trees provide strong support for the hypothesis that *U.* cf. *oxyurus* and *U.* cf. *narayani* share a more recent common ancestor with each other than either does with *U.* cf. *malabaricus*.

5. CONCLUDING REMARKS

With caveats concerning incomplete sampling, the topology and relative branch lengths of phylogenies estimated from mtDNA sequences clearly support the hypothesis that Asian caecilians are a primarily Gondwanan group. Ichthyophiids and uraeotyphlids were probably isolated (and perhaps diverged) on the drifting Indian subcontinent. Ichthyophiids apparently dispersed into South East Asia after plate accretion in the early Tertiary. There is no evidence that uraeotyphlids dispersed out of India. The data and analyses presented here should form a basis for taxonomic revision and for future investigations into the evolutionary biology of this neglected group.

In addition to many helpful local people and authorities in French Guiana, India, Sri Lanka, Thailand, Vietnam and China, the authors thank J.-C. and E. Baloup, M. Blanc, Jochen Bulian, M. Dewyter, G. Dubost, P. Gaucher, Jon Gower, Wolfgang Grossmann, V. Hequet, R. Janardhanan, K. G. Lalith, K. Kariyawasam, Savitha N. Krishna, Peter Lenk, S. Lochon, Yasantha Mapatuna, J.-C. de Massary, Smita Mathew, John Measey, Jarujin Nabhitabatha, Dinarzarde Raheem, S. R. M. Swarnapali Samaradiwakara, Biju Thomas and family, S. Vishwambaran, Gernot Vogel, K. A. S. Ravindra Wickramanaike, Winai Sae-chew, the staff of the HYDRECO laboratory and two anonymous reviewers. D.J.G. is very grateful to Salvi Carranza for assistance with the molecular research, and to Julia Bartley and Claire Griffin for their work in the Natural History Museum sequencing facility. This work was supported in part by NERC GST/02/832 to M.W., an MRF grant to D.J.G. and M.W. and an EU SYSRESOURCE award to A.K.

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