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Endemic ranid (Amphibia: Anura) genera in southern mountain ranges of the Indian subcontinent represent ancient frog lineages: evidence from molecular data

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Abstract

The geological history of the Indian subcontinent is marked by successive episodes of extensive isolation, which have provided ideal settings for the development of a unique floral and faunal diversity. By molecular phylogenetic analysis of a large set of ranid frog taxa from the Oriental realm, we show that four genera, now restricted to torrential habitats in the Western Ghats of India and the central highlands of Sri Lanka, represent remnants of ancient divergences. None of three other biodiversity hotspots in the Oriental mainland were found to harbour an equivalent level of long-term evolutionary history in this frog group. By unceasingly providing favourable humid conditions, the subcontinent's southern mountain ranges have served as refugia for old lineages, and hence constitute a unique reservoir of ancient ranid endemism.

Keywords: Indian subcontinent; Ancient endemism; Ranidae; Metapopulation genetic algorithm; Bayesian divergence time estimates

1. Introduction

Landmasses that have experienced a prolonged period of extensive isolation often hold old endemic lineages of terrestrial and freshwater flora and fauna. Such high-level endemism is apparent on large islands or island groups, such as New Zealand (e.g., Tuataras: Gauthier et al., 1988 and leiopelmatid frogs: Hay et al., 1995), the Seychelles archipelago (e.g., sooglossid frogs: Hay et al., 1995; Ruvinsky and Maxson, 1996) or Madagascar (e.g., lemurs: Sechrest et al., 2002 and mantelline frogs: Bossuyt and Milinkovitch, 2000; Vences et al., 2000a), but also in climatically isolated regions, such as the South African Cape floristic region (e.g., heleophrynid frogs: Hay et al., 1995). When long-term isolation is followed by restoration of contact with other regions, the biotic uniqueness of an area may gradually fade due to floral and faunal interchange. Nevertheless, some previously isolated regions may incidentally retain inconspicuous remnants of a unique ancient biotic composition.

A region potentially harbouring lineages testifying for foregoing periods of isolation is the Indian subcontinent. Indeed, the geological history of the Indian subcontinent has undergone successive episodes during which geological elements may have acted as severe filters of dispersion by allowing only occasional intercontinental exchange of biota. First, the Indian subcontinent detached from Africa ~130 million years ago (Ma) (Krause et al., 1999), as part of the Madagascar–Seychelles–India block. Its long northward drift across the Tethys sea, with disconnection from Madagascar at ~88 Ma (Storey et al., 1995) and the Seychelles at ~65 Ma (Courtillot et al., 1988), ended only in the Palaeogene (Najman et al., 2001), after accretion to the Eurasian block. The first contact between both landmasses momentarily enabled Eurasian animal and plant groups to invade the subcontinent (Briggs, 1989; Prasad and Sahni, 1988), and lineages of Gondwanan origin,

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if they persisted on the drifting subcontinent, to disperse to Eurasia (Bossuyt and Milinkovitch, 2001; Conti et al., 2002; Gower et al., 2002; Wilkinson et al., 2002a). Second, the Cenozoic subsidence of the Indian plate under the Eurasian plate caused the rapid rise of the Himalayan mountain range, creating a barrier to dispersal along the northern limits of the subcontinent. The subsequent uplift of the Tibetan plateau (Chung et al., 1998) strengthened the buffer effect of the Himalayas. Third, although the formation and following erosion of the Himalayas and Indo-Burmese mountain ranges produced extensive sediment deposits in the Bengal basin from the early Tertiary on, a shallow sea, repeatedly extending northward up to the Himalayan foot hills, covered the basin throughout the lower-Tertiary (Alam, 1989). Marine and brackish environments prevailed in this region until as late as the Upper-Miocene (Mannan, 2002), only to be replaced by one of the largest delta complexes on earth. These geological factors may have significantly promoted the long-term isolation of native biota and of lineages that reached the subcontinent by occasional dispersal.

The subcosmopolitan frog family Ranidae is ecologically an extremely diverse amphibian group, represented by approximately 500 species in the Oriental realm (Inger, 1999; Meegaskumbura et al., 2002). A consensus for ranid taxonomy is currently non-existent (Dubois, 1992; Inger, 1996), but recent progress in research on this group has led to the recognition of individual subfamilies for several frog genera endemic to the Indian subcontinent. These taxonomic rearrangements were initially based on the observation of autapomorphic morphological traits in these lineages (Blommers-Schlösser, 1993; Dubois, 1992; Dubois and Ohler, 2001) and are backed in some cases by karyological data (Vences et al., 2000b). Additionally, molecular dating estimates in 14 species of Ranidae (Bossuyt and Milinkovitch, 2001) indicated that several lineages originated on the Indian subcontinent during its trans-Tethys drift. This family therefore constitutes an ideal target group to search for remnants of ancient diversity. We searched for indications of higher-level endemism on the Indian subcontinent through molecular phylogenetic screening of a wide variety of Ranidae from the eight Oriental mainland subfamilies. Our broad taxon sampling, which includes most of the generic diversity in Ranidae from four biodiversity hotspots of mainland Asia (the Indian Western Ghats + Sri Lanka, Indo-Burma, South-Central China, and Sundaland; see Myers et al., 2000), considerably increases the chance to uncover any ancient frog lineage in the Oriental realm.

2. Materials and methods

Our data set is composed of 60 ranoid frog taxa (Table 1), with 55 ranid in-group species, representing 38 genus-group taxa. We mainly followed the taxonomical schemes proposed by Dubois (1992) and Frost (2002). Five species belonging to four other ranoid families served as out-group. The in-group includes 45 Asian species, six Madagascan, one African, two European, and one North American species. When more than two species were available for a particular genus, a preliminary analysis based on one DNA fragment was performed (data not shown) and two or three species representing the largest observed intrageneric divergence were selected. For the Indian genera *Indirana*, *Micrixalus*, and *Nyctibatrachus*, seven, four, and four different species respectively, were preliminarily examined. DNA sequences of 30 species were retrieved from GenBank (Accession Nos. AF249002–AF249064 and AF249098–AF249191). Whole-genomic DNA of other species was extracted from muscle tissue using a standard phenol/chloroform procedure (Sambrook et al., 1989). Two mitochondrial (mt) and three nuclear (nu) DNA fragments were PCR-amplified. The mtDNA fragments are: (i) a ~750 base pair (bp) region covering part of the 12S rRNA gene, the complete $tRNA^{Val}$ gene and part of the 16S rRNA gene and (ii) ~550 bp of the 16S rRNA gene. The nuDNA fragments are: (i) 529–532 bp of exon 1 of the tyrosinase gene, and (ii) 316 bp of exon 1 and (iii) 175 bp of exon 4 of the rhodopsin gene. The primers used for amplification are given elsewhere (Bossuyt and Milinkovitch, 2000). PCR-products were purified following an agarose gel extraction protocol (Qiagen), cycle-sequenced on both strands, and analysed using an ABI 377 automated sequencer (Applied Biosystems). The sequences have been deposited in GenBank under Accession Nos. AY322214–AY322363.

Sequences were aligned using the computer programs SOAP v1.0 (Loytynoja and Milinkovitch, 2000) and ClustalX v1.64 (Thompson et al., 1994) and manually corrected with MacClade v4.0 (Maddison and Maddison, 2000). Plots of transitions (T_i) and transversions (T_v) against uncorrected pairwise distances, and distances corrected according to the general time-reversible (GTR) model of base substitution (Rodríguez et al., 1990), were made to detect saturation in any of the five fragments. Partition homogeneity tests (PHT, Farris et al., 1994) as implemented in the software package PAUP* v4.0b10 (Swofford, 2002), were used to check for significant incongruences between any pair of the five fragments.

Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP*. Heuristic MP searches were executed in 10,000 replicates with all characters unordered and equally weighted, and using tree bisection reconnection (TBR) branch swapping. For likelihood-based phylogeny inference, the Akaike information criterion (AIC, Akaike, 1973) as implemented in the computer program Modeltest v3.06 (Posada and Crandall, 1998) assigned the GTR model, with

Table 1
List of taxa included in this study, with corresponding sequence origins (voucher numbers for species newly sequenced for this study), sampling localities, and GenBank accession numbers

Family	Subfamily	Current genus and species name	Sequence origin/ voucher no.	Locality	Accession nos.
Ranidae	Boophiinae	*Boophis tephraeomystax*	NCBI GenBank	Madagascar	AF249009, AF249039, AF249105, AF249137, AF249168
		Boophis xerophilus	NCBI GenBank	Madagascar	AF249008, AF249038, AF249104, AF249136, AF249167
	Dicroglossinae	*Euphlyctis cyanophlyctis*	NCBI GenBank	India	AF249015, AF249053, AF249111, AF249143, AF249174
		Fejervarya cf. *limnocharis*	NCBI GenBank	India	AF249012, AF249055, AF249108, AF249140, AF249171
		Fejervarya syhadrenis	NCBI GenBank	India	AF249011, AF249040, AF249107, AF249139, AF249170
		Hoplobatrachus chinensis	VUB 0684	Vietnam	AY322221, AY322248, AY322289, AY322307, AY322360
		Hoplobatrachus crassus	NCBI GenBank	Sri Lanka	AF249013, AF249044, AF249109, AF249141, AF249172
		Ingerana tenasserimensis	CAS 205064	Myanmar	AY322236, AY322269, AY322302, AY322308, AY322344
		Limnonectes finchi	VUB 0607	Borneo	AY322230, AY322250, AY322295, AY322306, AY322355
		Limnonectes kuhlii	NCBI GenBank	Vietnam	AF249020, AF249034, AF249116, AF249148, AF249179
		Nannophrys ceylonensis	NCBI GenBank	Sri Lanka	AF249016, AF249047, AF249112, AF249144, AF249175
		Sphaerotheca pluvialis	NCBI GenBank	Sri Lanka	AF249014, AF249042, AF249110, AF249142, AF249173
	Laliostominae	*Aglyptodactylus madagascariensis*	NCBI GenBank	Madagascar	AF249007, AF249036, AF249103, AF249135, AF249166
		Laliostoma labrosa	NCBI GenBank	Madagascar	AF249010, AF249037, AF249106, AF249138, AF249169
	Lankanectinae	*Lankanectes corrugatus*	NCBI GenBank	Sri Lanka	AF249019, AF249043, AF249115, AF249147, AF249178
	Mantellinae	*Mantella madagascariensis*	NCBI GenBank	Madagascar	AF249005, AF249049, AF249101, AF249133, AF249164
		Mantidactylus ulcerosus	NCBI GenBank	Madagascar	AF249006, AF249035, AF249102, AF249134, AF249165
	Micrixalinae	*Micrixalus fuscus*	NCBI GenBank	India	AF249024, AF249056, AF249120, AF249152, AF249183
		Micrixalus kottigeharensis	NCBI GenBank	India	AF249025, AF249041, AF249121, AF249153, AF249184
	Nyctibatrachinae	*Nyctibatrachus aliciae*	NCBI GenBank	India	AF249018, AF249063, AF249114, AF249146, AF249177
		Nyctibatrachus major	NCBI GenBank	India	AF249017, AF249052, AF249113, AF249145, AF249176
		Nyctibatrachus sp. A	VUB 0035	India	AY322224, AY322247, AY322299, AY322315, AY322343
	Occidozyginae	*Occidozyga laevis*	TNHC (DLSUD002)	Philippines	AY322227, AY322262, AY322300, AY322329, AY322342
	Raninae	*Amolops* cf. *ricketti*	VUB 0701	Vietnam	AY322231, AY322261, AY322286, AY322326, AY322352
		Meristogenys kinabaluensis	VUB 0627	Borneo	AY322233, AY322267, AY322292, AY322317, AY322357
		Meristogenys cf. *orphocnemis*	VUB 0630	Borneo	AY322222, AY322254, AY322291, AY322319, AY322358
		Nanorana (Altirana) parkeri	NJNU LS9802	China	AY322219, AY322252, AY322283, AY322333, AY322350
		Nanorana (Nanorana) pleskei	NJNU F97034	China	AY322235, AY322273, AY322282, AY322332, AY322339
		Paa (Gynandropaa) yunnanensis	VUB 0691	Vietnam	AY322229, AY322271, AY322288, AY322309, AY322361
		Paa (Quasipaa) boulengeri	NJNU F96030	China	AY322240, AY322251, AY322280, AY322311, AY322349
		Paa (Quasipaa) cf. *spinosa*	VUB 0713	Vietnam	AY322234, AY322272, AY322284, AY322310, AY322340
		Rana (Aquarana) galamensis	CAS 214840	Kenya	AY322238, AY322270, AY322303, AY322331, AY322337
		Rana (Chalcorana) chalconota	VUB 0610	Borneo	AY322232, AY322268, AY322293, AY322313, AY322341
		Rana (Clinotarsus) curtipes	NCBI GenBank	India	AF249021, AF249058, AF249117, AF249149, AF249180
		Rana (Eburana) livida	VUB 0711	Vietnam	AY322220, AY322258, AY322285, AY322322, AY322353
		Rana (Hylarana) erythraea	VUB 0609	Borneo	AY322228, AY322266, AY322294, AY322323, AY322356
		Rana (Pantherana) sphenocephala	VUB 0558	USA	AY322223, AY322264, AY322297, AY322312, AY322345
		Rana (Pelophylax) lessonae	VUB 0940	Belgium	AY322243, AY322249, AY322276, AY322321, AY322347
		Rana (Pelophylax) nigromaculata	NJNU F97072	China	AY322241, AY322256, AY322278, AY322305, AY322363
		Rana (Pulchrana) signata	VUB 0606	Borneo	AY322237, AY322265, AY322296, AY322316, AY322354
		Rana (Rana) temporaria	NCBI GenBank	Belgium	AF249023, AF249048, AF249119, AF249151, AF249182
		Rana (Rana) zhenhaiensis	NJNU F97004	China	AY322217, AY322253, AY322279, AY322318, AY322346
		Rana (Rugosa) emeljanovi	NJNU 980073	China	AY322218, AY322255, AY322281, AY322320, AY322362

		Rana (Sylvirana) guentheri	VUB 0693	Vietnam	AY322216, AY322259, AY322287, AY322325, AY322351
		Rana (Sylvirana) nigrovittata	VUB 0749	China	AY322242, AY322260, AY322277, AY322324, AY322348
		Rana (Sylvirana) temporalis	NCBI GenBank	India	AF249022, AF249054, AF249118, AF249150, AF249181
		Staurois latopalmatus	VUB 0652	Borneo	AY322239, AY322257, AY322290, AY322327, AY322359
	Ranixalinae	*Indirana sp. A*	NCBI GenBank	India	AF249027, AF249064, AF249123, AF249155, AF249186
		Indirana sp. B	VUB 0319	India	AY322225, AY322298, AY322246, AY322314, AY322338
		Indirana sp. C	NCBI GenBank	India	AF249026, AF249051, AF249122, AF249154, AF249185
	Rhacophorinae	*Philautus charius*	NCBI GenBank	India	AF249032, AF249062, AF249128, AF249160, AF249191
		Philautus microtympanum	NCBI GenBank	Sri Lanka	AF249030, AF249046, AF249126, AF249158, AF249189
		Philautus wynaadenis	NCBI GenBank	India	AF249031, AF249059, AF249127, AF249159, AF249190
		Polypedates cruciger	NCBI GenBank	Sri Lanka	AF249028, AF249045, AF249124, AF249156, AF249187
		Rhacophorus malabaricus	NCBI GenBank	India	AF249029, AF249050, AF249125, AF249157, AF249188
	Microhylidae	Microhyla ornata*	NCBI GenBank	India	AF249003, AF249060, AF249099, AF249131, AF249162
	Hyperoliidae	*Hyperolius sp.**	NCBI GenBank	Kenya	AF249002, AF249033, AF249098, AF249130, AF249161
	Hyperoliidae	*Leptopelis kiwuensis**	CAS 201700	Uganda	AY322214, AY322245, AY322275, AY322328, AY322335
	Arthroleptidae	*Arthroleptis variabilis**	CAS 207822	Equatorial Guinea	AY322226, AY322263, AY322301, AY322330, AY322336
	Astylosternidae	*Trichobatrachus robustus**	ZFMK 66453	Cameroon	AY322215, AY322244, AY322274, AY322304, AY322334

Species indicated by * constitute the out-group. Collection abbreviations: CAS, California Academy of Sciences; NJNU, Nanjing Normal University; TNHC, Texas Natural History Collections; VUB, Vrije Universiteit Brussel; ZFMK, Zoologisches Forschungsinstitut und Museum A. Koenig.

gamma-shape correction for among-site rate heterogeneity (+Γ) and an assumed proportion of invariable sites (+I), as best fitting the observed data. ML searches were performed with substitution rates, gamma-shape parameter (α) and proportion of invariable sites (P_{inv}) estimated from neighbour-joining trees.

Besides conventional ML searches, we applied the recently developed Metapopulation genetic algorithm (MetaGA) (Lemmon and Milinkovitch, 2002a). This heuristic ML search method dramatically reduces the immense computation time associated with conventional ML analyses of large data sets. We conducted 250 independent MetaGA searches using the program MetaPIGA v1.0.2b (Lemmon and Milinkovitch, 2002b), each with strict consensus pruning among four populations. The HKY + Γ + I (Hasegawa et al., 1985) model was applied (the model implemented in MetaPIGA that best approximates GTR + Γ + I), with the T_i/T_v ratio optimized every 200 generations. Searches were started from random trees, and a single best tree per population was kept. The 1000 resulting trees were used to compute a majority-rule consensus tree and calculate posterior branch support values (PBS). Finally, we performed Bayesian analyses using MrBayes v.3.0b4 (Ronquist and Huelsenbeck, 2003). Again, the GTR + Γ + I model was applied, with (default) dirichlet priors for the base frequencies and substitution rate matrix, and uniform priors for α and P_{inv}. Four chains, three heated and one cold (temperature parameter = 0.2), were run simultaneously for 5×10^6 generations, and trees were sampled every 500 cycles. The Bayesian posterior probabilities (BPP) were estimated as the majority-rule consensus of the 8000 last sampled trees. The run was repeated twice, to ascertain convergence towards the same posterior parameter distribution (see Huelsenbeck et al., 2002).

In addition to PBS and BPP, clade confidence was evaluated using decay indices (Bremer, 1994) under MP, and non-parametric bootstrapping (Felsenstein, 1985) under ML (MLBS). To reduce the computation time for the latter, a smaller data set containing 30 taxa was analysed. Taxa were pruned from the data set evenly across lineages in such way that all major lineages supported by our foregoing analyses were still represented in the reduced data set. We ran 100 replicates in PAUP*, with the same parameter settings as for the ML search. We also tested one alternative tree (T_0, with likelihood L_0, see Section 3 for choice of T_0) using parametric bootstrapping (the SOWH-test with partial parameter optimization, see Goldman et al., 2000; Swofford et al., 1996). In order to assess a null distribution for the difference in log likelihood $\delta(\ln L_{\text{ML}} - \ln L_0)$, sequence evolution along T_0 was simulated 100 times, with the program Seq-Gen v.1.2.6 (Rambaut and Grassly, 1997), and according to the GTR + Γ + I model with all parameters estimated from T_0. A significance level of 0.05 is used for rejection of the alternative topology.

In order to evaluate the relative age of divergence of in-group lineages, ultrametric trees were constructed using a Bayesian relaxed molecular clock method developed for multi-gene data sets (Thorne and Kishino, 2002). One internal node served as fixed reference point for comparison of the relative divergence ages. The choice of this node is discussed in Section 3.

3. Results

After removal of 508 nucleotide sites due to ambiguities in the alignments, the total data set consisted of 1895 characters. Of these, 997 were constant and 698 sites were parsimony-informative. The fragment sequences we obtained showed a maximum pairwise divergence of 16.8% (rhodopsin exon 1) to 22.9% (tyrosinase) when uncorrected, and of 19.4% (Rhodopsin exon 1) to 28.3% (tyrosinase) when GTR-corrected. None of the five fragments showed saturation. The PHTs revealed no significant incongruences among any pair of fragments, which justifies their combination in a single data set.

Our MP (Fig. 1), ML (see legend of Fig. 1), MetaGA, and Bayesian searches all corroborate two large clades: one (clade α) comprising the subfamily Occidozyginae, the genus *Ingerana* and all representatives of Dicroglossinae, together with the Paini clade (PBS = 94, BPP = 100), the other (clade β) consisting of Raninae (*excl.* Paini), Rhacophorinae, Mantellinae, Boophiinae, and Laliostominae (PBS = 100, BPP = 98). The bootstrap analysis provided a moderate value for clade β (MLBS = 82) and a marginal value for clade α (MLBS = 54). However, clade α is composed of two subclades, which are both statistically highly supported by all ML analyses (PBS = 99, BPP = 100, MLBS = 93; and PBS = 100, BPP = 100, MLBS = 100, respectively).

The resulting trees are incompatible with several widely accepted taxonomic groupings. The subfamily Raninae is found not to be monophyletic, as a clade containing the genera *Paa* and *Nanorana* (the tribe Paini) is nested well within dicroglossine taxa (PBS = 100, BPP = 100, MLBS = 97), an observation consistent with previous analyses based on 12S rRNA sequences (Jiang and Zhou, 2001). Our analyses also confirm that the Rhacophorinae and a clade composed of three Madagascan subfamilies (Mantellinae, Boophiinae, and Laliostominae) are nested within the Ranidae. This is consistent with previous molecular studies (Bossuyt and Milinkovitch, 2000; Marmayou et al., 2000) but contradicts a recent cladistic analysis based mainly on larval characters (Haas, 2003), in which the Rhacophorinae were suggested to be the sister clade of a Microhylidae–Hyperoliidae assemblage. Both the tree frogs and the Madagascan frogs are still often treated as distinct frog families (Rhacophoridae and Mantellidae, respectively) (Frost, 2002; Haas, 2003; Inger, 1999; Vences and Glaw, 2001) or placed together in a single family (Rhacophoridae) (Richards and Moore, 1998; Richards et al., 2000; Wilkinson et al., 2002b). Additionally, all our analyses corroborated a sister-group relationship between the tree frogs and the Madagascan clade. This relationship was supported by analysis of both the tyrosinase gene and the mitochondrial data independently, and left unresolved when the rhodopsin gene was analysed separately. This result strengthens the findings of previous molecular studies (Bossuyt and Milinkovitch, 2000; Emerson et al., 2000a; Richards et al., 2000) and is consistent with cladistic analyses of morphological data (Blommers-Schlösser, 1993; Channing, 1989).

Finally, all analyses unanimously indicate that four genera endemic to India (*Indirana*, *Micrixalus*, and *Nyctibatrachus*) or Sri Lanka (*Lankanectes*) are not nested within the α- or β-clade. This implies that they diverged prior to the origin of several of the largest recognized subfamilies in Ranidae. All analyses suggest a dual origin for this endemism, although the possibility of more than two origins cannot be eliminated, because the sister-group relationships of *Micrixalus* and *Indirana*, and of *Lankanectes* and *Nyctibatrachus* receive only marginal support. Screening of the 1000 MetaGA trees and the 8000 sampled Bayesian trees, using a constraint filter for monophyly of a group containing the four endemic genera, resulted in the recovery of four trees, and a single tree, respectively, implying a high posterior support (PBS = 99.60, BPP = 99.99) for a multiple origin of these endemics. We verified this outcome by parametric bootstrapping (Fig. 2), which indicated that a single origin for the four endemic taxa implies a significant decrease in likelihood and could be rejected at the 0.05 confidence level ($\delta = 5.492$; $p < 0.03$).

As an alternative approach to evaluate the extent of this endemism, we estimated divergence ages. We therefore constructed ultrametric trees, which allow comparison of the relative ages of divergence lineages. As none of the DNA fragments showed saturation, both mtDNA and nuDNA sequences were included. The highly supported node representing the split between Rhacophorinae and the Madagascan clade served as a fixed reference point. Stratigraphic data for these clades are currently unavailable, but a molecular timescale calibrated with external fossil evidence (Bossuyt and Milinkovitch, 2001) has indicated that this split occurred at 73.1 ± 19.5 Ma. The ultrametric tree based on the Bayesian consensus topology (Fig. 3) shows that each of the endemic genera originated early in ranid evolution. Constraining the Rhacophorinae–Mantellinae split at 73.1 Ma (as shown in Fig. 3) results in Middle- to Upper-Cretaceous time estimates for the individual origins of the four endemic lineages. Even in the most

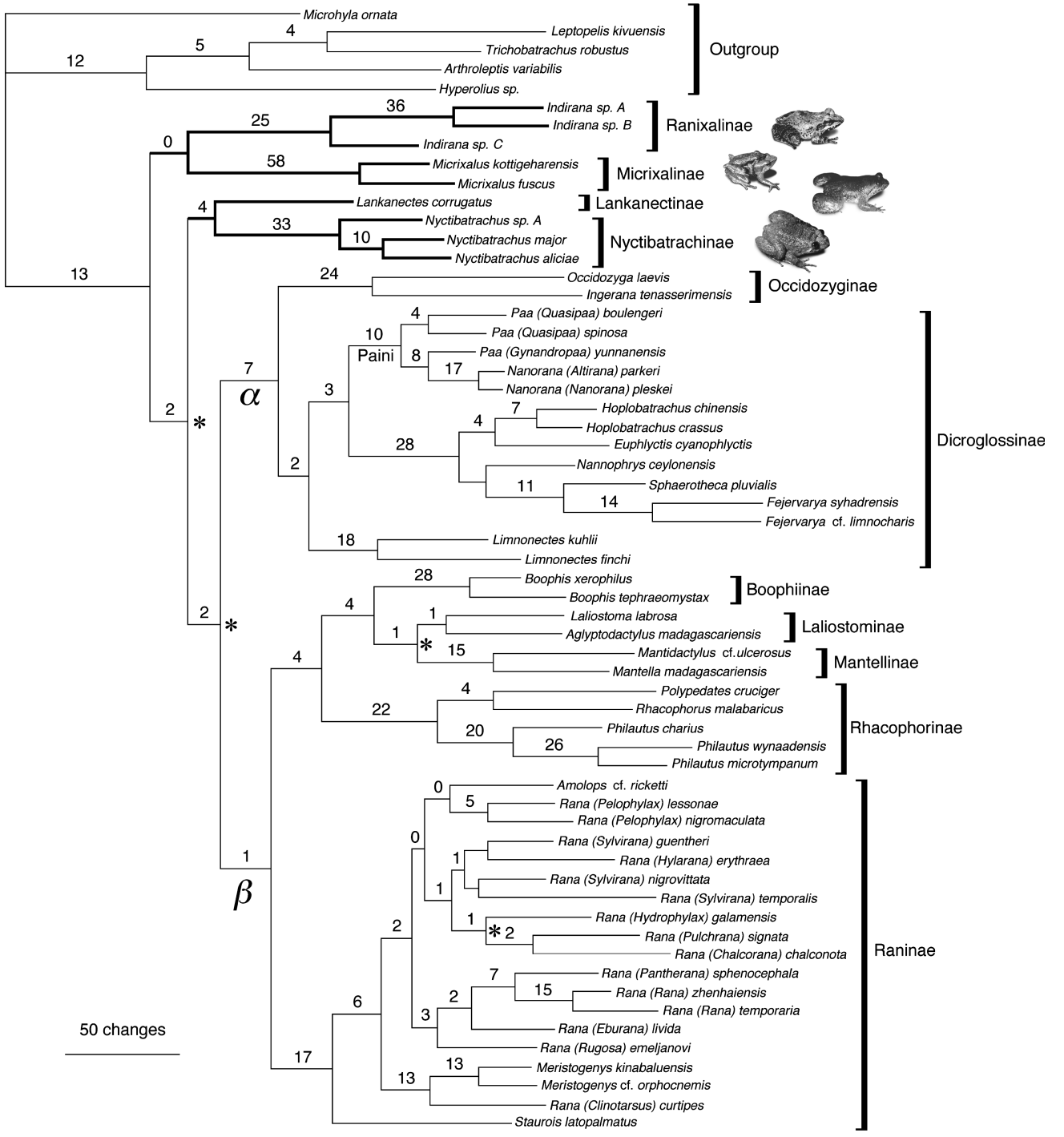

Fig. 1. One of the 24 best trees calculated under maximum parsimony (tree length = 4297). Numbers above internal branches are decay indices. All MP trees show two large clades (α and β) composed of several ranid subfamilies, and basal positions (outside α and β) for subfamilies endemic to the Indian subcontinent (branches indicated in bold, and see pictures). Identical relationships are recovered by our ML search ($-\ln L = 22582.550$), except for four nodes, indicated by an asterisk.

conservative case, when the Rhacophorinae–Mantellinae split is set at its derived lower limit of 53.6 Ma (73.1–19.5 Ma), each endemic genus individually must have originated at least in the Upper-Cretaceous or in the Palaeocene. Additional dating analyses, based on a topology corroborating an independent origin for each of the four endemic subfamilies (not shown), resulted in slightly older relative age estimates. This confirms that, irrespective of their mutual relationships, the origin of each of these endemic lineages is ancient.

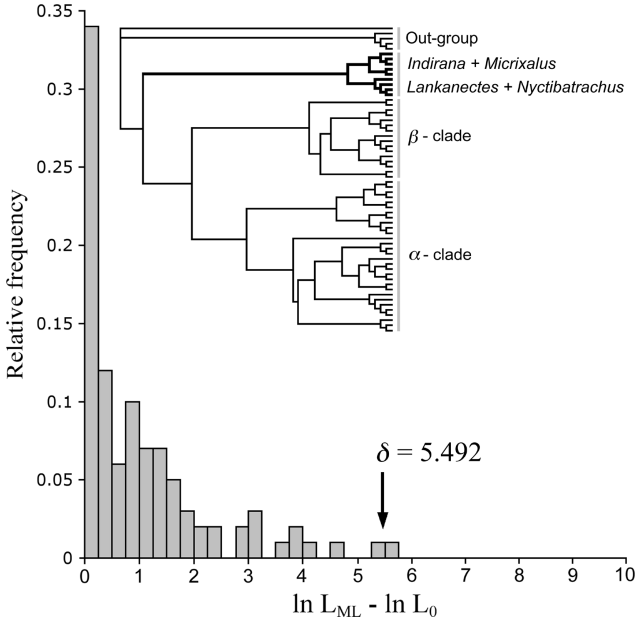

Fig. 2. Result of the parametric bootstrap analysis testing a single origin for the four endemic genera. The null distribution was obtained by plotting $\ln L_{ML} - \ln L_0$ for 100 simulation replicates, T_0 being the ML tree under the constraint of a single origin for the four endemic subfamilies. The observed value for the test statistic δ falls outside the 95% confidence interval, indicating a significantly larger likelihood difference than expected under the null hypothesis, and validating rejection of monophyly of the subcontinent's ancient ranid endemism at the 0.05 significance level.

4. Discussion

Our phylogenetic analyses, combined with the divergence age estimates, indicate that the genera *Indirana*, *Micrixalus*, *Nyctibatrachus*, and *Lankanectes* each represent ancient lineages, their origins predating, or at least being contemporaneous with those of several ranid subfamilies. Given our broad taxonomic sampling in the subcontinent and adjacent regions of the Oriental realm, this observation is a strong indication that the endemic lineages have no close living relatives in any other part of the Asian mainland.

If taxonomy is to be phylogenetically relevant, or even to reflect evolutionary age (see Avise and Johns, 1999), our results will probably influence prevailing opinions concerning the classification of Ranidae, particularly when placed in a broader phylogenetic perspective (e.g., by including all ranid subfamilies). For instance, in consistency with the commonly accepted family rank for the rhacophorine tree frogs and for the Madagascan frog radiation, all lineages that diverged prior to these clades, should be classified into distinct frog families as well. In that case, the family-name Ranidae would remain valid for all taxa currently included in the subfamily Raninae (with exclusion of the genera *Paa* and *Nanorana*). In each of the genera endemic to the subcontinent, remarkably few extant species are described (one in *Lankanectes*, 10 in *Indirana*, and 11 in *Nyctibatrachus* and *Micrixalus*). Furthermore, the high morphological uniformity among species within each lineage contrasts sharply with the extensive morphological and ecological diversifications characterizing other ranid clades such as the Madagascan frog clade (Bossuyt and Milinkovitch, 2000), Rhacophorinae (Meegaskumbura et al., 2002), and Dicroglossinae (Emerson et al., 2000b; Kosuch et al., 2001). The low diversity in species richness and morphology may indicate that living members of each lineage have diverged long after the origin of the branch itself. Interestingly, our divergence age estimates principally corroborate these observations. The ultrametric tree in Fig. 3 reveals extended time gaps between lineage origins of *Indirana*, *Micrixalus*, and *Nyctibatrachus*, and the earliest intrageneric divergences observed (see Section 2). Although it cannot be excluded that these lineages existed as solitary branches without substantially diverging during tens of millions of years, it is likely that multiple offshoot lineages eventually went extinct. In this scenario, the extant frog endemics represent small relict clades that are remnants of a once much more diverse and widespread anuran fauna. Palaeobotanical and geomorphological data indicate that conditions were favourable for a high amphibian diversity on the subcontinent during the Mesozoic and until the mid-Cenozoic, since a tropical wet climate prevailed over the greater part of this landmass until the Miocene (Fawcett et al., 1994; Ramesh, 2001). Yet, given the fact that isolated landmasses are often liable to higher extinction rates, it seems plausible that faunal groups present on the subcontinent have encountered severe bottleneck events. First, the movement of the Indian subcontinent over the Réunion mantle plume at the K-T transition generated the notorious Deccan basalt floods (Courtillot et al., 1988) that afflicted a large part of the subcontinent. Second, the rise of the Himalayas and the Western Ghats set off a dramatic transformation in the Indian subcontinent's climate and vegetation (Gunnell, 2001) during the Upper-Tertiary, eventually leading to aridification and widespread replacement of tropical evergreen vegetation with deciduous savannah vegetation in large parts of the peninsula (Conti et al., 2002; Ramesh, 2001). However, since the uplift of these mountains also induced the onset of a monsoon regime, the forested areas of the Western Ghats and Sri Lankan hills have probably served as Cenozoic refugia by exclusively providing the necessary humid environment and habitat conditions. Without any exception, species of the four endemic genera exhibit, in both larval and adult stages, several traits associated with life in the direct proximity of rocky torrents (Blommers-Schlösser, 1993; Bossuyt and Milinkovitch, 2000). Examples of specialization towards this habitat are the presence of well-developed digital pads in *Indirana*, *Micrixalus*, and *Nyctibatrachus*, and a rare adaptive type of semi-terrestrial tadpole in *Indirana*,

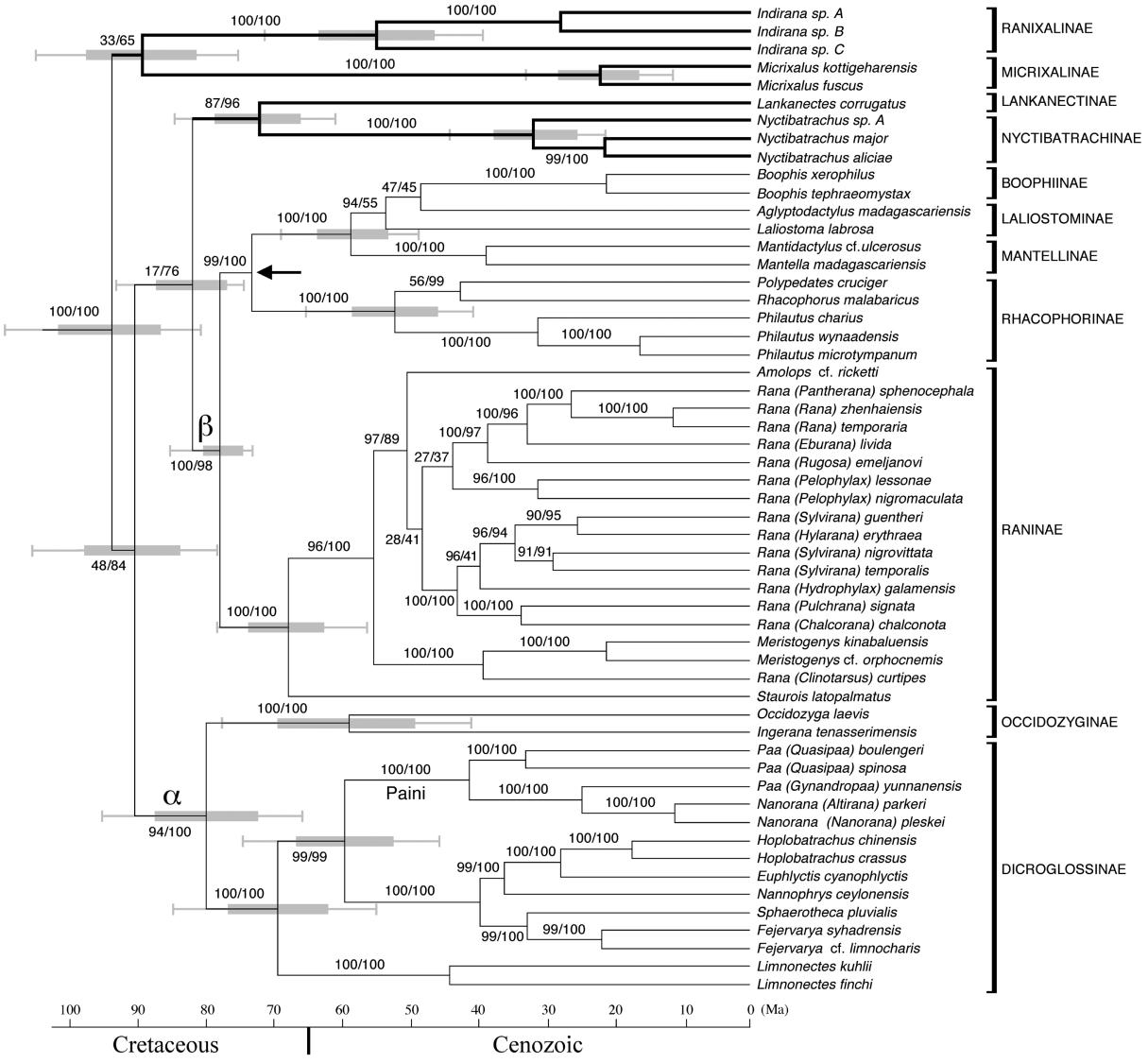

Fig. 3. Bayesian consensus tree topology converted to an ultrametric tree by estimating relative divergence ages for ranid lineages. As a fixed reference point, we used the split of Rhacophorinae and the Madagascan frog radiation (Mantellinae, Boophiinae, and Laliostominae) (indicated by an arrow). The sister relationship of these clades is well-supported in our analyses, and previous molecular dating estimates (Bossuyt and Milinkovitch, 2001) situated their most recent common ancestor at 73.1 ± 19.5 Ma. The timescale below the tree is based on this age estimate. The horizontal bars at internal nodes denote twice the standard deviation value, the thin lines indicate the 95% credibility intervals, for the corresponding divergence age estimate. Numbers at internal branches are MetaGA branch support values (PBS, left) and Bayesian posterior probabilities (BPP, right).

which clings on steep, humid rock faces. Some of the extreme specializations may have restricted the endemics to a narrow range of potential niches, and prevented their subsequent dispersion outside these mountain ranges.

At present, the distribution ranges of the four ancient lineages are confined to two disjunct mountainous regions, jointly classified as one of the 25 global biodiversity hotspots (Myers et al., 2000): *Indirana*, *Micrixalus*, and *Nyctibatrachus* inhabit the Western Ghats mountain chain along the west coast of peninsular India, whereas *Lankanectes* occurs in the central highlands of Sri Lanka. Our results identify these mountain ranges as valuable reservoirs of ranid evolutionary history. Indeed, none of the other three hotspot regions on the Asian mainland was found to harbour an equivalent level of ancient endemic diversity within Ranidae, since all other ancient splits in our ultrametric tree (Fig. 3) merely produce frog clades with large distributions (e.g., Rhacophorinae, Dicroglossinae, and Raninae). The fact that such unparalleled evolutionary history is concentrated in a spatially limited forest area, facing one of the largest demographic pressures of Southeast Asia (Cincotta et al., 2000), stresses the urgent need for revised protection measures for the Western Ghats/Sri Lanka hotspot.

The results presented here are based on the most inclusive molecular phylogenetic study hitherto performed on Asian Ranidae. However, in order to fully comprehend the evolutionary relationships of the subcontinent's endemics with respect to *all* ranid clades, future studies should also incorporate dense taxon sampling in remote landmasses, such as Subsaharan Africa (e.g., Petropedetinae and Pyxicephalinae), the Philippines and islands across the Wallace line (e.g., Platymantinae).

Previous studies in other faunal groups, such as caecilians (Gower et al., 2002; Wilkinson et al., 2002a) and agamid lizards (Macey et al., 2000), revealed long branches for Indian taxa as well. Increased taxon sampling within these groups should clarify whether these long branches indeed represent ancient endemism and hence, whether they are consistent with our findings. Additional phylogenetic surveys, based on broad taxon sampling, will most likely demonstrate more cases of high-level endemism in the southern mountains of the Indian subcontinent. The revelation of similar patterns in other animal and plant groups would result in a major upgrade of the value of these mountain ranges as biodiversity hotspot and as target area for conservation priorities.

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