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Molecular Phylogeny of the Ranid Frogs from Southwest India Based on the Mitochondrial Ribosomal RNA Gene Sequences

Atsushi Kurabayashi[1], Mitsuru Kuramoto[2], Hareesh Joshy[3] and Masayuki Sumida[1]*

[1]*Institute for Amphibian Biology, Graduate School of Science, Hiroshima University,*
Higashihiroshima, Hiroshima 739-8526, Japan
[2]*3-6-15, Hikarigaoka, Munakata, Fukuoka 811-3403, Japan*
[3]*Rondano Bio-diversity Research Laboratory, St. Aloysius*
College, Mangalore 575-003, India

ABSTRACT—The Western Ghats of Southwestern India are known as one of the world's "hotspots" of biodiversity. We collected frog specimens from the family Ranidae and investigated the phylogenetic relationships among ranid species, particularly among the *Fejervarya,* a genus whose morphological diagnostic characteristics and phylogenetic features remain little known. We analyzed partial sequences of the mitochondrial 12S (428 bp) and 16S rDNAs (549 bp). Results showed that the members of *Fejervarya* form a monophyletic group with the genera *Hoplobatrachus* and *Euphlyctis* among ranid genera. This confirms the recent allocation of *Fejervarya,* including it within the subfamily Dicroglossinae. The mitochondrial rDNA data in our study also appeared to be useful as a marker to distinguish *Fejervarya* species without morphological differences. The phylogenetic tree based on the 16S rDNA sequence showed a correlation between *Fejervarya* phylogenies and their geographic distributions. Lastly, our results suggested the recent occurrence of a radiation event of *Fejervarya* species in the Indian-Sri Lankan region.

Key words: India, molecular phylogeny, Ranidae, Dicroglossinae, *Fejervarya*

INTRODUCTION

The first comprehensive list of Indian amphibians, provided by Inger and Dutta (1986), included 181 species. Through a series of additions, omissions, and nomenclature changes by Chanda and Ghosh (1988), Das and Whitaker (1990), Dutta (1992), Deuti (1996) and Dutta (1997), the compilation of Indian amphibians has now increased to 216 species. Indian anuran species are most abundant in the Western Ghats of the West Peninsular region, one of the "hottest hotspots" of biodiversity in the world (Myers *et al*., 2000; Meegaskumbura *et al*., 2002). Inger and Dutta (1986) listed a total of 17 genera and 99 species of anurans belonging to 6 families in this region. Daniels (1998) listed 103 anuran species, most of them endemic to the Western Ghats, a region where new species have been described year after year (Das and Ravichandran, 1998; Vasudevan and Dutta, 2000; Biju and Bossuyt, 2003).

In spite of the numerous morphological and molecular studies recently employed to reexamine the relationships of anurans (e.g., Laurent, 1986; Ford and Cannatella, 1993; Hedges and Maxon, 1993; Hills *et al*., 1993; Hay *et al*., 1995; Emerson *et al*., 2000), the phylogenetic relationships still remain unclear for some taxa. One such problematic anuran group is the genus *Fejervarya* widely distributed from Pakistan to Japan and Indonesia. A number of species from this genus have been collectively recognized as a single species, *Rana limnocharis* (Dubois and Ohler, 2000), mainly due to their indistinct morphologies. However, more recent detailed analyses have demonstrated that *Rana limnocharis* (as previously used) actually constitutes many distinct species. Based on a detailed study of mating calls and morphology, Dubois (1975) showed that no fewer than four distinct species had been distributed and confusedly classified together under the name *Rana limnocharis* in Nepal alone. In studies published in later decades, Dubois (1984, 1987, 1992) also proposed that the whole group was composed of at least 15 species, and probably many more. Furthermore, Veith *et al*. (2001) detected two sympatric species in Java using allozyme and mitochondrial 16S rDNA data. Finally, Iskandar (1998) and Dubois and Ohler (2000) proposed that the frogs usually known as the "*Rana limnocharis* group" should be regarded as a distinct genus, *Fejervarya,* within the subfamily Dicroglossinae. Recent authors seem to accept this system, and Frost (2004) listed 31 *Fejervarya*

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* Corresponding author. Phone: +81-82-424-7482;
Fax : +81-82-424-0739;
E-mail: msumida@hiroshima-u.ac.jp

species in his comprehensive checklist. Nonetheless, the revision of the genus is still lacking (Dubois, 1984), and the phylogenetic relationship within the genus is poorly known.

From May to June 2003, we collected 28 frog specimens from the Western Ghats Mountain area. The frog specimens contained eight genera of the family Ranidae, including 10 individuals from the genus *Fejervarya.* The samples were deemed to support an examination of the aforementioned problems of this genus. In the present study we sequenced mitochondrial 12S and 16S rRNA genes (rDNAs) and compared the sequence divergences of these genes between *Fejervarya* and other ranid taxa. We also explored the genetic and phylogenetic relationships among the *Fejervarya* specimens from the Western Ghats and other available *Fejervarya* species.

MATERIALS AND METHODS

Frogs examined

Twenty-eight frog individuals were collected from Mangalore, Kudremukh, and Madikeri. All three of these localities are located in the southern parts of Karnataka (Table 1), and the latter two are located in the Western Ghats. Immediately after collection, a clipped toe was dissected from each frog, fixed in 600 ul of a fixing solution (RNAlater, Qiagen), and stored for later use in total DNA extraction. Twenty of the 28 frogs were preserved in 10% formalin, and the fixed samples were deposited in the Rondano Biodiversity Research Laboratory of St. Aloysius College (Table 1). The other eight frogs were identified easily and released.

Species identification was based primarily on the external morphology of each specimen. We followed Dubois's (1992) taxonomical allocation. The 19 specimens identified among the 28 specimens collected comprised 13 species of nine genera in two families (Table 1). One specimen, a frog of an apparently novel species from the genus *Philautus,* was described as the *Philautus luteolus* species reported by Kuramoto and Joshy (2003).

The remaining nine specimens were all members of the genus *Fejervarya,* but we could not identify these specimens exactly. Ten species of the genus *Fejervarya* are known to occur in our sampling areas of the Western Ghats (Frost, 2004), and we carefully examined our specimens in reference to the original descriptions, monographs, and specimens in the Bombay Natural History Society (BNHS). The morphology of one female *Fejervarya* specimen (cat. No. 030607-01 in Table 1) was quite similar to that of *F. brevipalmata*, but there were several remarkable differences. The interorbital space of our specimen was smaller than those of *F. brevipalmata* specimens of BNHS, for example, and the upper eyelid was wider. Thus, in this study, we treated this specimen as *F.* cf. *brevipalmata*. The morphologies of two male *Fejervarya* specimens (cat. Nos. 030610-07, 08) were similar to those of *F. nilagirica* specimens measured by Boulenger (1920) in length ratios of some body parts [e.g., leg-length / snout-vent length (SVL) and tibia-length / SVL]. However, we treated the two male *Fejervarya* specimens as *F.* cf. *nilagirica* based on a considerable difference found in the head sizes. The remaining six *Fejervarya* specimens had small body sizes: average SVL=32.5 mm for female (N=4, cat. Nos. 030607-03, 04, 030526-02, and 030526-04) and 24.1 mm for male (N=2, cat. Nos. 030523-02, 04). These specimens resembled the "*F. limnocharis*" specimens deposited in BNHS and also matched the original description of *F. syhadrensis* (Annandale, 1919) fairly well. Given that the name of *F. limnocharis* is no longer applicable to Indian frogs (Sumida et al., unpublished data), and noting several differences between our specimens and *F. syhadrensis* (e.g., ratios

Table 1. Specimens used in this study.

Family Subfamily	Species	Locality**	Specimen		Accession Nos.	
			Nos. of frogs	Cat. Nos. ***	12S	16S
Bufonidae	***Bufo melanostictus*** Schneider, 1799	Mangalore	1	Release	AB167899	AB167927
Ranidae						
Rhacophorinae	***Philautus luteolus*** Kuramoto & Joshy, 2003	Kudremukh	1	030610-01	AB167904	AB167932
	Philautus wynaadensis (Jerdon, 1854)	Mangalore	2	030523-01,05	AB167901, 02	AB167929, 30
	Philautus wynaadensis (Jerdon, 1854)	Kudremukh	1	030610-02	AB167903	AB167931
	Polypedates maculatus (Glay, 1833)	Mangalore	2	Release	AB167905, 06	AB167933, 34
	Rhacophorus malabaricus Jerdon, 1870	Madikeri	1	Release	AB167900	AB167928
Nyctibatrachinae	***Nyctibatrachus major*** Boulenger, 1882	Kudremukh	1	Release	AB167907	AB167935
Raninae	***Rana aurantiaca*** Boulenger, 1904	Kudremukh	1	030610-04	AB167909	AB167937
	Rana malabarica Tschudi, 1838	Mangalore	1	Release	AB167908	AB167936
Dicroglossinae	***Euphlyctis cyanophlyctis*** (Schneider, 1799)	Madikeri	1	030607-02	AB167910	AB167938
	Euphlyctis hexadactylus (Lesson, 1834) (hpEA)*	Mangalore	3	030525-01,02, 030606-02	AB167911, 12, 14	AB167939, 40, 42
	Euphlyctis hexadactylus (Lesson, 1834) (hpEB)*	Mangalore	1	030606-01	AB167913	AB167941
	Hoplobatrachus tigerinus (Daudin, 1802)	Mangalore	2	Release	AB167915, 16	AB167943, 44
	Fejervarya rufescens (Jerdon, 1854)	Mangalore	1	030526-03	AB167917	AB167945
	Fejervarya* cf. *brevipalmata	Madikeri	1	030607-01	AB167918	AB167946
	Fejervarya* cf. *nilagirica	Kudremukh	2	030610-07, 08	AB167921,22	AB167949,50
	***Fejervarya* sp.** (hpA)*	Madikeri	2	030607-03, 04	AB167919,20	AB167947,48
	***Fejervarya* sp.** (hpB)*	Mangalore	4	030523-02,04, 030526-02,04	AB167923-26	AB167951-54

*Names of haplotype groups found in *F.* sp. and *E. hexadactylus* specimens. **Geographic distances: 56 km between Mangalore and Kudremukh and 107 km between Mangalore and Madikeri. ***In the Rondano Biodiversity Research Laboratory of St. Aloysius College (RBRL), India.

Table 2. Fejervarya species used in this study.

Species	Haplotype*	Origin of sample	Distribution***	Accession Nos.	References
Fejervarya **sp.**	hpA	Western Ghats (India)	---	AB167947,48	this study
Fejervarya **sp**	hpB	Western Ghats (India)	---	AB167951-54	this study
Fejervarya **cf. *brevipalmata***	---	Western Ghats (India)	---	AB167946	this study
Fejervarya **cf. *nilagirica***	---	Western Ghats (India)	---	AB167949,50	this study
Fejervarya rufescens (Jerdon, 1854)	---	Western Ghats (India)	South India	AB167945	this study
Fejervarya syhadrensis (Annandale, 1919)	---	Sri Lanka	Eastern and western India, Sri Lanka, Nepal, Pakistan	AY141843	Meegaskumbura *et al.* (2002)
Fejervarya greenii (Boulenger, 1905)	---	Sri Lanka	Sri Lanka (and possibly south India)	AY014378	Kosuch *et al.* (2001)
Fejervarya kirtisinghei (Manamendra-Arachchi and Gabadage, 1996)	---	Sri Lanka	Sri Lanka	AY014380	Kosuch *et al.* (2001)
Fejervarya limnocharis (Gravenhorst, 1829)	S5-A	Sumatra (Indonesia)	Wide range from western to southern and eastern Asia (i.e., India, Indonesia, Thailand, Laos, Pakistan, Nepal, China, Philippines, Japan)	AJ292021	Veith *et al.* (2001)
	---	Laos		AF161024	Marmayou *et al.* (2000)
	Hiro	Hiroshima (Japan)		AB070732	Sumida *et al.* (2002)
Fejervarya iskandari Veith, Kosuch, Ohler, and Dubois, 2001	J2-B	Java (Indonesia)	Java (Indonesia)	AJ292017	Veith *et al.* (2001)
Fejervarya vittigera (Wiegmann, 1834)	---	Philippines	Philippines	AY313683	Evans *et al.* (unpublished)
Fejervarya cancrivora (Gravenhorst, 1829)	Cancri(T/P)**	Philippines/Thailand	Wide range from southern to eastern Asia	AB070738	Sumida *et al.* (2002)

*Haplotype names used in each reference are indicated. **Philippines and Thailand specimens have the same 16S sequence. ***According to Frost (2004) and Dubois & Ohler (2000).

head-length / head-width and eye-diameter / tympanum-diameter), we noted those six unidentified specimens as *F.* sp. Our present materials probably involve several cryptic species.

PCR and DNA sequencing

Partial fragments of mitochondrial 12S and 16S rDNAs were PCR amplified from total DNA extracted from the fixed toe of each frog using a DNA extraction kit (DNAeasy, Qiagen). Two sets of primers, FSO1 - R16 and F51 – R51 (Sumida *et al.*, 2002), were used to amplify the 5' portion of 12S rDNA (corresponding to position 1 - 422 in *Xenopus laevis* 12S; Dunon-Bluteau and Brun, 1986) and the 3' portion of 16S rDNA (corresponding to position 884 – 1,448 in *Xenopus* 16S; Roe *et al.*, 1985), respectively. PCR mixtures were prepared with an Ex-Taq kit (Takara) according to the manufacturer's protocol. The 12S and 16S rDNAs were amplified by 35 cycles consisting of 10 sec at 98°C, 30 sec at 50°C, and 1 min 30 sec at 72°C. The resultant PCR fragments were purified with a purification kit (MinElute, Qiagen) and directly sequenced with the above primers using a cycle sequencing kit (DYEnamic ET dye terminator, Amersham) and automated sequencer (ABI 373A, Applied Bio System). The resultant sequences were deposited in the EMBL/GenBank/DDBJ database (Table1).

Data analyses

Alignment data. The resultant 12S and 16S rDNA sequences from 28 frog specimens and those of an out-group *Xenopus laevis* were aligned using the ClustalW program (Thompson *et al.*, 1994), with ambiguous sites manually eliminated. Initially, we made the 12S and 16S rDNA alignment data individually. The alignment data of 12S and 16S contained 428 and 549 sites. After conducting the permutation homology test (Farris *et al.*, 1995) to check whether the 12S and 16S sequences were suitable for combination, we combined the 12S rDNA and 16S rDNA data (total 977 sites) in phylogenetic analyses for ranid taxa. We also prepared a set of alignment data constituting 396 sites of 16S rDNA sequences from 14 *Fejervarya* taxa (including nine *Fejervarya* species available in DNA database and five examined in this study, see Table 2) and from an outgroup *Hoplobatrachus tigerinus* to assess relationships within the genus *Fejervarya.* The fragment of 16S rDNA has been applied as a guide to distinct cryptic *Fejervarya* species (Veith *et al.*, 2001).

In the following analyses, gap sites in the alignments were treated as missing data. The alignment data used in this study are available at: http://home.hiroshima-u.ac.jp/~amphibia/sumida/kura/India_align.html.

Phylogenetic analyses. Based on the alignment data, phylogenetic analyses were performed by the Maximum-likelihood (ML), Neighbor-joining (NJ) and Maximum-parsimony (MP) methods using PAUP* 4.10b (Swofford, 2001). For ML and NJ analyses, best-fit substitution models were selected by MODELTEST ver. 3.06 (Posada and Crandall, 1998) with AIC consideration. The robustness of the resultant trees was evaluated with bootstrap (BP) proportions. For the ranid families, topologies of resulting trees were compared using the multi-scale bootstrap techniques, the Kishino-Hasegawa (KH) test (Kishino and Hasegawa, 1989) and the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) using PAUP (see Table 3).

RESULTS

Nucleotide sequences

Nucleotide sequences were determined in a 5' portion of 12S rDNA and a 3' segment of 16S rDNA from 28 frog specimens. After adding an outgroup, *Xenopus laevis* (Roe *et al.*, 1985; Dunon-Bluteau and Brun, 1986), there were 428 and 549 sites in alignment matrices used for further analyses. The 12S rDNA alignment contained 250 variable sites of which 207 were parsimoniously informative, and the 16S rDNA alignment provided 258 variable sites of which 205 were informative.

Fig. 1 shows the mean sequence divergences of 12S

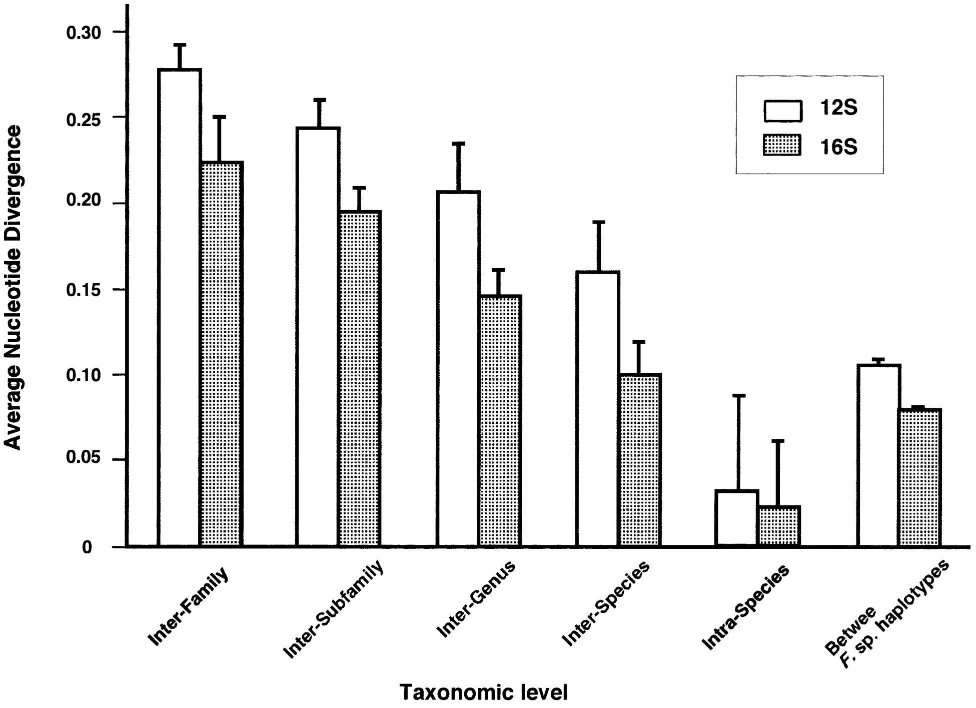

Fig. 1. Mean sequence divergences at various taxonomic levels. The pairwise divergences (uncorrected *p* value) of both 12S and 16S rDNA sequences for all taxa were calculated from the alignment data and the mean values (±SD) at each taxonomic level represented here. The mean divergence values at all taxonomic levels compared here were significantly different (P<0.01, calculated by Tukey's method). Gap sites in the alignments were treated as missing data in the calculations. The values of *F.* sp. were excluded from the calculations at intra-species levels.

and 16S rDNAs at each taxonomic level. The magnitudes of the divergences were related to the taxonomic level applied here: inter-family comparison showed the largest value (27.68 and 22.28% for 12S and 16S, respectively) and intra-species comparison showed the lowest (3.17 and 2.21% for 12S and 16S, respectively).

In the specimens of *Fejervarya* sp., we found 2 distinct haplotype groups, that is, hpA and hpB, and this grouping was consistent with the proximity of their sampling localities (see Table 1). Within each haplotype group, only 2 and 1 substitutions were observed in the 12S and 16S rDNAs, respectively. Among these groups, however, there were 43 and 41 nucleotide changes in the 12S and 16S rDNAs. The average divergences among these haplotypes (10.52 and 7.93% for 12S and 16S, respectively) were much higher than those obtained at intra-specific comparison, yet lower than those of the inter-species (Fig. 1). We also found two distinct haplotype groups, hpEA and hpEB, in *Euphlyctis hexadactylus*. The 12S and 16S rDNA divergence between these two haplotypes were 12.02 and 8.72%, respectively. The 16S rDNA sequences of both hpEA and hpEB also diverged from that of Sri Lankan *E. hexadactylus* (AF215389; Vences *et al.*, 2000) by 5.95 and 8.75%, respectively. The sequence divergences among these haplotype groups were considerably higher than those observed in the intra-species comparison (Fig. 1).

The divergences of the 12S rDNA were larger than those of the 16S rDNA at all taxonomic levels compared

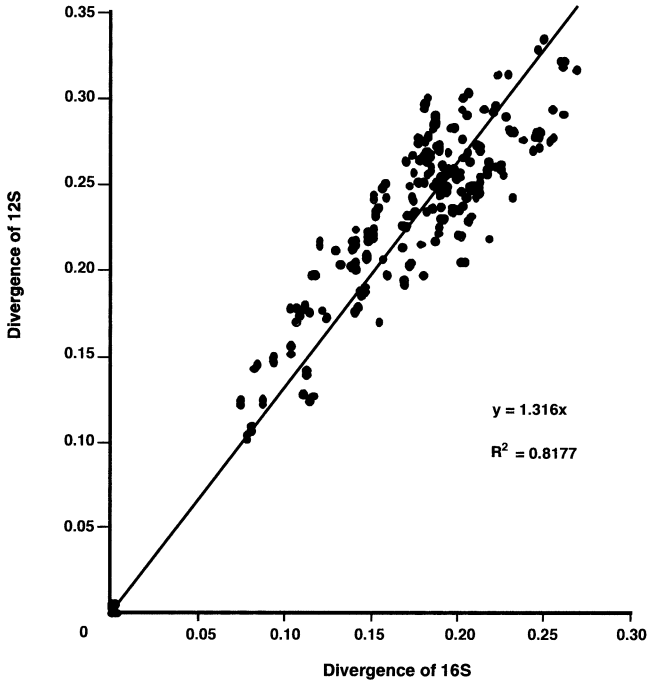

Fig. 2. Dependence plots of sequence divergences of the 12S rDNA on the 16S rDNA. The fitted regression line was calculated from the plots and shown. The inclination of the line indicates the difference of the substitution rate between the genes.

(Fig. 1). The present results showed that the nucleotide substitution of the 12S rDNA occurred at a rate 1.32 times higher than that of the 16S rDNA (Fig. 2). Even with this difference, however, the partition homogeneity test (Farris *et al.*, 1995) revealed that our 12S and 16S rDNA data did not possess significantly conflicting phylogenetic signals (homogeneity between 12S and 16S rDNAs was not rejected by *P*=0.48). We thus combined the 12S and 16S rDNA data and used the combined data for our subsequent phylogenetic analyses for ranid taxa. In our selection of the substi-

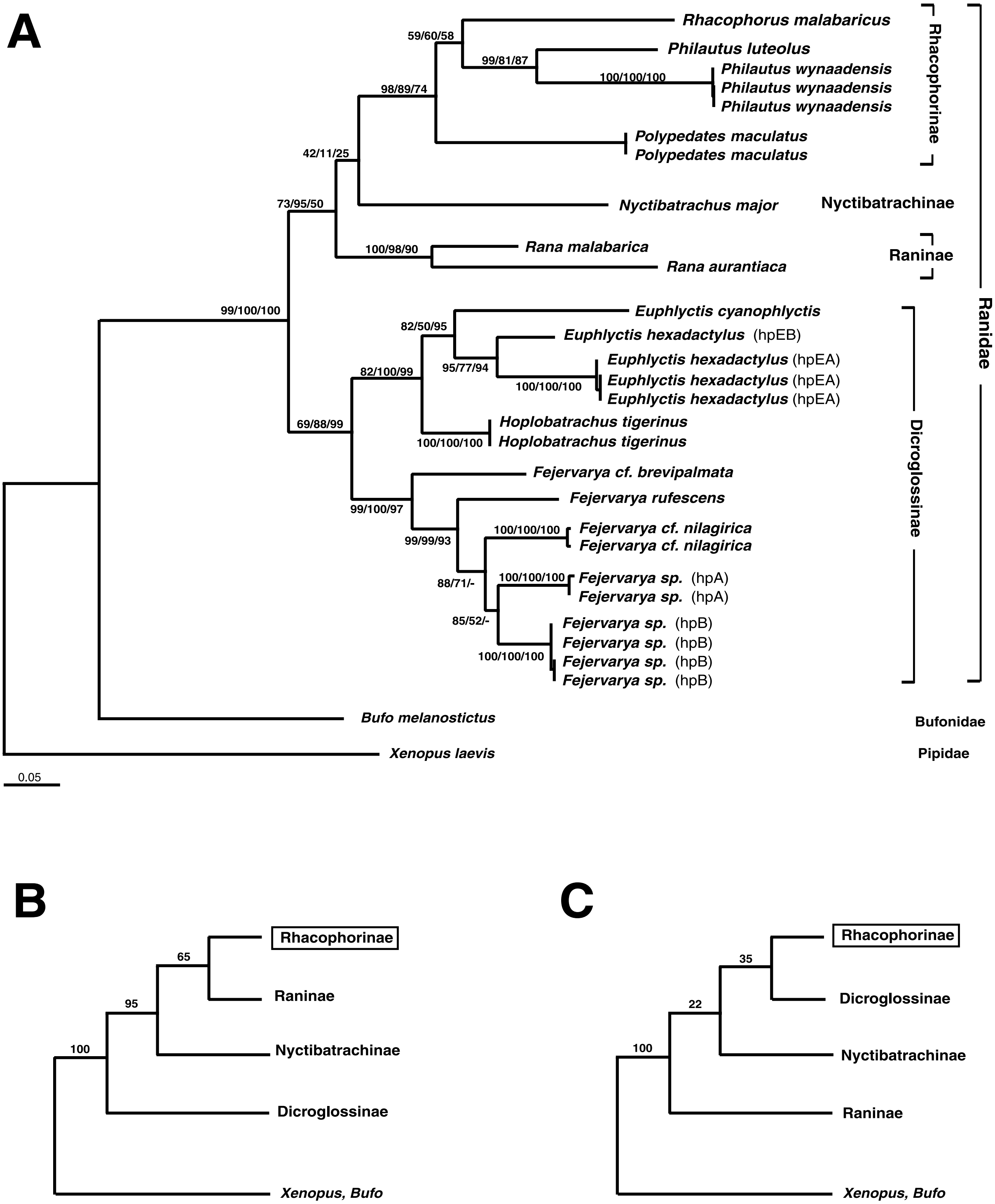

Fig. 3. Phylogenetic trees for ranid taxa based on 12S and 16S rDNA sequences. A: ML tree. The numbers on each node indicate BP values calculated by ML/NJ /MP. BP values were calculated based on 2,000 replicates in the case of NJ and MP and by 500 in the case of ML. The scale bar shows genetic distance based on the ML setting (see text). B: The tree topology and the BP values generated by NJ. C: The strict consensus tree of 12 shortest MP trees.

tution model for the combined data set, ModelTest (Posada and Crandall, 1998) suggested a general time-reversible model (GTR+G+I) as best fitting, with a proportion of invariable sites (I=0.27) and a gamma distribution (α shape parameter=0.64). Thus, we used the substitution model to carry out ML and NJ analyses. For the ML analysis, empirical base frequencies (A: 0.34, C: 0.24, G: 0.18, T: 0.24) and substitution rates ([A–C]=4.52, [A–G]=11.71, [A–T]=7.15, [C–G]=0.58, [C–T]=29.52, [G–T]=1.00) were also adopted.

Phylogeny of ranid taxa and position of the genus *Fejervarya*

Fig. 3A shows the resultant ML tree (-ln *L*=8148.73) and BP values of ML/NJ/MP trees. Figs. 3B and 3C indicate alternative tree topologies generated by NJ and MP, respectively. The monophyly of the Ranidae in our trees was supported with sufficient statistical values (BP=99/100/100). This monophyletic group was recognized to include four subgroups corresponding to the ranid subfamilies, i.e., Raninae, Rhacophorinae, Nyctibatrachinae, and Dicroglossinae (see Fig. 3). Most of these groupings were supported by high BP values (Fig. 3A). However, the relationships among these 4 subfamilial clades were not resolved completely. The rhacophorine clade seemed to have close affinity with *Nyctibatrachus* and *Rana*. The BP values in ML and NJ well supported the monophyly of Rhacophorinae, Nyctibatrachinae and Raninae (BP=73/95). The BP value in MP supported this relationship more weakly (BP=50), and the consensus tree of 12 MP trees (Fig. 3C) showed monophyly of the Rhacophorinae and the Dicroglossinae instead of the former and Raninae and/or Nyctibatrachinae. The alternative tree topologies generated by ML, MP and NJ analyses were tested by Kishino-Hasegawa and Shimodaira-Hasegawa tests. The results indicated that there were no statistically significant differences among their log-likelihood values (Table 3).

Fejervarya taxa analyzed here made a clear monophyletic group, and this clade was included in the dicroglossine clade with the genera *Euphlyctis* and *Hoplobatrachus* (Fig. 3). The latter two formed a single clade and the *Fejervarya* clade was their sister group. The *Fejervarya* taxa split into 5 clades, i.e., *F.* cf. *brevipalmata, F. rufescens, F.* cf. *nilagirica*, and two others corresponding to the two groups haplotype (hpA and B) found in *F.* sp. specimens.

Genetic and phylogenetic relationships of *Fejervarya* taxa

Partial sequences of 12S and 16S rDNAs have been reported in studies on several *Fejervarya* species. Among them, the 3’ sequence of 16S rDNA has served as a useful guide to distinguish cryptic *Fejervarya* species (Veith *et al.*, 2001). We also used the 16S rDNA portion to assess genetic variations and phylogenetic relationships among the *Fejervarya* taxa studied here and reported elsewhere (Table 2). We compiled an alignment data set including 396 sites of 16S rDNA sequences from 13 *Fejervarya* taxa and an outgroup *Hoplobatrachus tigerinus*. As there was no nucleotide change in the 16S rDNA portion of each haplotype group found in the *F.* sp. specimens, we used only one sequence from each haplotype group.

Table 4 shows sequence divergences and nucleotide substitutions of the 16S rDNA data among *Fejervarya* taxa. Among the inter-species, the largest divergence value (19.58%) was observed between *F.* cf *nilagirica* and *F. iskandari* and the smallest divergence value (4.69%) was observed between *F. greenii* and *F. kirtisinghei*. The mean value of the inter-specific divergences was 14.62% (±SD=3.50). Within *F. limnocharis*, the values ranged from 0.52% (Sumatra vs. Laos) to 3.64% (Sumatra vs. Japan) and the mean value was 2.42% (±SD=1.67). Between two haplotype groups found in the *F.* sp. specimens, the divergence value was 10.24%. This value was much larger than the largest value of the intra-species (3.64%) and 2.2 times higher than the minimum value of inter-specific comparisons (4.69%) of other *Fejervarya* species. Our results suggested that the divergence value between the two haplotype groups corresponds to those among distinct species. The 16S rDNA sequence of hpA was almost identical with that of *F. syhadrensis*. There were only 3 nucleotide substitutions between them, and the divergence value (0.77%), fell within the intra-specific range of *F. limnocharis*.

Based on the 16S rDNA data set, we reconstructed phylogenetic trees with ML, NJ and MP methods. The GTR model with I (=0.4839) + G (α shape parameter=0.7831) proposed by MODELTEST was used for ML and NJ analyses, and empirical base frequencies (A: 0.29, C: 0.25, G: 0.20, T: 0.26) and substitution rates ([A–C]=24.30, [A–G]=43.97, [A–T]=40.20, [C–G]=0.70, [C–T]=131.18, [G–T]=1.00) were additionally applied in the ML reconstruction.

Fig. 4 shows the ML tree (-ln *L*=2009.89) and BP values

Table 3. Comparison of log-likelihood scores among the alternative tree topologies using KH and SH tests.

Tree Topology	Method*	-ln *L*	-ln *L* difference	*P* value**	
				KH	SH
(Dicroglossinae, (Raninae, (Nyctibatrachinae, Rhacophorinae)))	ML	8148.73147	best	–	–
(Dicroglossinae, (Nyctibatrachinae, (Raninae, Rhacophorinae)))	NJ	8152.70878	3.97731	0.1369	0.3691
(Raninae, (Nyctibatrachinae, (Dicroglossinae, Rhacophorinae)))	MP	8151.91078	3.17931	0.2955	0.4227

*Tree reconstruction methods that supported each topology (see Fig. 3 and text). ** *P* values were calculated by RELL method from 10,000 replications. The values were not significant (> 0.05) among any of the compared topologies.

Table 4. Pairwise sequence divergences and nucleotide differences of *Fejervarya* 16SrDNA. Pairwise sequence divergences (uncollected *p* value) and numbers of nucleotide differences (transitions/transversions) are shown in lower-left and upper-right diagrams, respectively.

	sp. hpA	**sp. hpB**	**cf. *nilagirica***	***syhadrensis***	***greenii***	***kirtisinghei***	***rufescens***	**cf. *brevipalmata***	***lim.*(Sumatra)**	***lim.*(Laos)**	***lim.*(Japan)**	***iskandari***	***vittigera***	***cancrivora***
***F.* sp. hpA**	**sp. hpA**	26/13	25/14	2/1	33/11	30/12	29/21	28/19	40/28	40/28	41/30	39/35	37/29	39/31
***F.* sp. hpB**	0.1024	**sp. hpB**	21/12	24/13	24/8	23/8	25/16	27/20	36/29	35/29	38/27	36/30	35/24	36/26
F.* cf. *nilagirica	0.1030	0.0873	**cf. *nilagirica***	25/15	30/13	26/14	28/19	27/24	40/26	38/26	39/28	41/33	35/27	38/30
F. syhadrensis	0.0077	0.0975	0.1056	***syhadrensis***	31/12	28/13	26/22	30/18	41/27	41/27	42/29	40/33	38/29	39/32
F. greenii	0.1150	0.0838	0.1131	0.1126	***greenii***	13/5	24/16	27/20	37/27	37/27	39/25	40/32	48/22	40/26
F. kirtisinghei	0.1095	0.0809	0.1048	0.1071	0.0469	***kirtisinghei***	25/19	30/19	28/28	28/28	30/26	36/35	42/23	37/25
F. rufescens	0.1303	0.1073	0.1229	0.1251	0.1042	0.1143	***rufescens***	28/24	34/27	32/27	33/27	32/36	41/26	38/32
F.* cf. *brevipalmata	0.1228	0.1227	0.1335	0.1257	0.1223	0.1273	0.1351	**cf. *brevipalmata***	31/29	29/29	32/31	29/30	37/28	28/28
***F. limnocharis* (Sumatra)**	0.1780	0.1707	0.1740	0.1785	0.1678	0.1464	0.1593	0.1566	***lim.*(Sumatra)**	2/0	10/4	25/22	30/24	34/30
***F. limnocharis* (Laos)**	0.1780	0.1680	0.1687	0.1785	0.1678	0.1464	0.1541	0.1514	0.0052	***lim.*(Laos)**	8/4	25/22	28/24	32/30
***F. limnocharis* (Japan)**	0.1859	0.1706	0.1766	0.1864	0.1678	0.1464	0.1568	0.1646	0.0364	0.0312	***lim.*(Japan)**	24/22	27/20	33/26
F. iskandari	0.1945	0.1742	0.1958	0.1925	0.1895	0.1863	0.1784	0.1547	0.1227	0.1227	0.1201	***iskandari***	26/24	30/30
F. vittigera	0.1728	0.1551	0.1635	0.1760	0.1834	0.1698	0.1750	0.1696	0.1403	0.1351	0.1221	0.1303	***vittigera***	29/14
F. cancrivora	0.1832	0.1629	0.1793	0.1862	0.1728	0.1619	0.1829	0.1462	0.1662	0.1610	0.1533	0.1566	0.1117	***cancrivora***

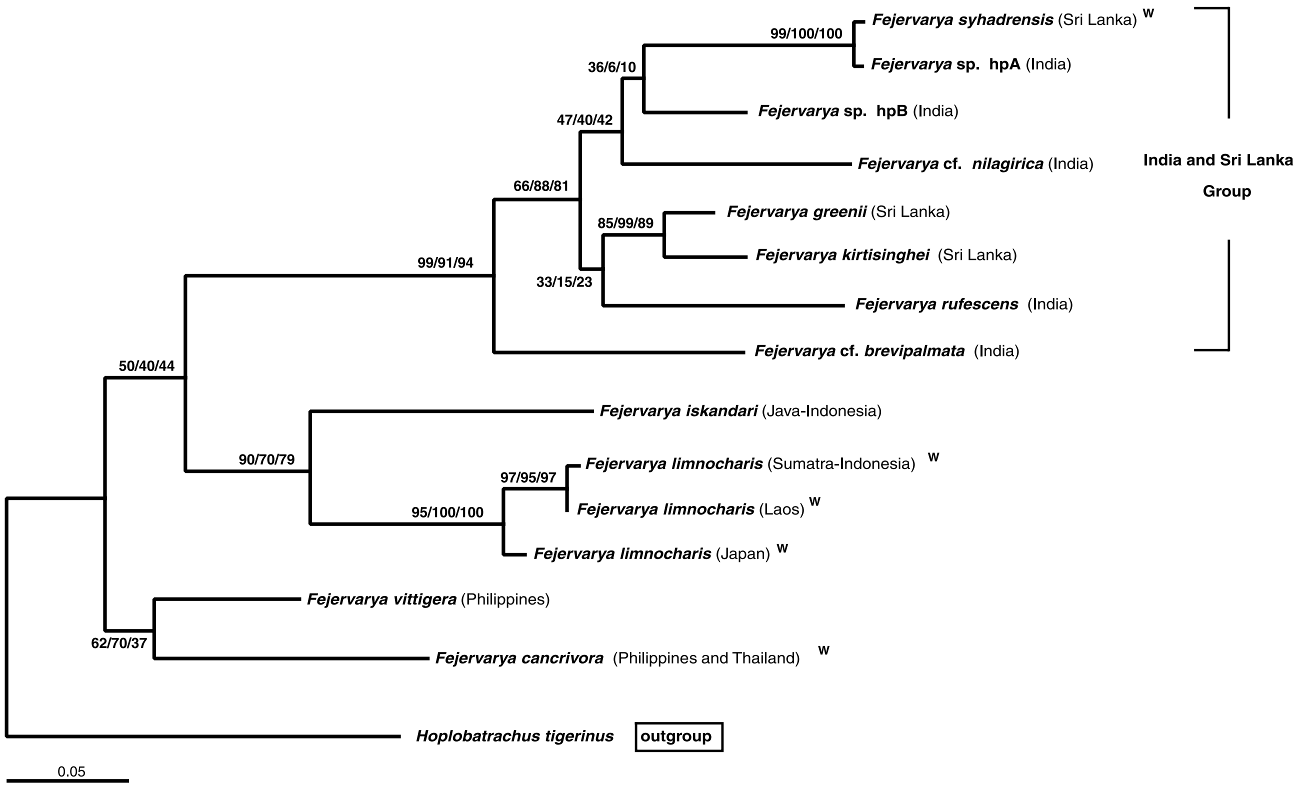

Fig. 4. *Fejervarya* phylogeny based on the 16S rDNA sequence. The ML tree is shown here. The origins of the samples are indicated in parentheses after the taxon names. ^{W} indicates species with wide distribution. The BP values are designated in the same manner as in Fig. 3.

of the ML/NJ/MP trees. The *Fejervarya* taxa were divided into three clades in the ML tree. The first clade consisted of *F. vittigera* and *F. cancrivora*, being sister group of the other taxa. The second clade consisted of *F. iskandari* and three populations of *F. limnocharis*. The third clade consisted of *F. greenii*, *F. kirtisinghei*, *F. syhadrensis,* and all of the Indian *Fejervarya* taxa covered in this study. The two haplotypes of the *F.* sp. specimens in the third clade were weakly grouped (BP =36/6/10). *F. syhadrensis* was nested in the group and became a clade with hpA (supported by sufficient BP values=99/100/100).

DISCUSSION

The position of the genus *Fejervarya* among ranid taxa

The species of the three genera used here, *Fejervarya,* *Euphlyctis,* and *Hoplobatrachus* were formerly regarded as members of the genus *Rana*. Dubois (1987, 1992) challenged this notion by suggesting that the genus *Rana* (as traditionally understood) was a heterogeneous group, and he removed many species from *Rana* to other taxa (including the three genera mentioned above). The *Fejervarya* was first recognized as a subgenus of the genus *Limnonectes,* but more recently it has been considered a distinct genus (see Dubois and Ohler, 2000). Furthermore, Dubois (1992) allocated these genera to the subfamily Dicroglossinae in the family Ranidae. Dubois's system seems to be accepted by recent authors, and its validity has been (partly) buttressed by several molecular studies (Bossuyt and Milinkovitch, 2000, Kosuch *et al*., 2001, Roelants *et al*., 2004). In our trees, (Fig. 3) the members of the Dicroglossinae recognized by Dubois (1992) formed a clear clade and split from the typical members of the genus *Rana*. Our results therefore confirm the Dubois scheme for the allocation of the genus *Fejervarya*, even though we did not analyze other dicroglossine genera (*Ceratobatrachus*, *Conraua*, *Paa,* etc.; Dubois, 2003).

Genetic and phylogenetic relationships within the genus *Fejervarya*

Many members of the genus *Fejervarya* have been often regarded as a single species, *Rana limnocharis* (see Dubois and Ohler, 2000). However, several detailed analyses demonstrated that *Rana limnocharis* (as traditionally used) is comprised of many distinct species, and these species have now been reassigned to the genus *Fejervarya*. Dubois and Ohler (2000) reported that at least 14 *Fejervarya* species were taxonomically valid, and Frost (2004) listed 31 species in this genus. Despite this accumulation of knowledge, the accurate identification of some *Fejervarya* species remains difficult due to poorly differentiated morphologies. Biochemical and molecular analyses have proven to be very useful for this identification. Based on allozyme markers, Toda *et al*. (1997, 1998a, b) discovered sympatric cryptic species in the *Rana limnocharis* (=*F. limnocharis*) group without morphological differences. Veith *et al*. (2001) also detected *F. iskandari* from an Indonesian population of *F. limnocharis* using allozyme and 16S rDNA sequence data and gave a morphology checklist. Our specimens in the present study included six unidentified *Fejervarya* individuals without remarkable morphological differences, and two different haplotype groups were found among them. The divergences between these haplotype groups corresponded to inter-specific divergences of other *Fejervarya* species, suggesting that the specimens were members of two distinct species. Furthermore, one haplotype, hpA, showed an extreme similarity to the haplotype of *F. syhadrensis*. According to Frost (2004), 17 *Fejervarya* species (including *F. syhadrensis*) are distributed in India and Sri Lanka. Our unidentified *Fejervarya* specimens might correspond to any of the species in his list. While a lack of critical morphological evidence still prevents us from annotating the *F.* spp. (see Materials and Methods; Kuramoto and Joshy are now further examining their morphologies), our results clearly showed the usefulness of 16S rDNA data in distinguishing *Fejervarya* species with poorly differentiated morphologies.

While there has been little phylogenetic information on the *Fejervarya* species, our tree (Fig. 4) based on 16S rDNA data now provides some aspects of their relationships. It is particularly interesting to note that the relationships seem to correlate with geographical distribution patterns. The lineages leading to *F. cancrivora* and *F. limnocharis* appeared to diverge from other taxa in early periods. These deeper branchings were consistent with their very wide distributions (Table 2). In addition, *F. cancrivora* and *F. limnocharis* become monophyly with *F. vittigera* and *F. iskandari*, respectively. *Fejervarya vittigera* and *F. iskandari* showed overlapping distributions with the former species, but they were only reported from restricted regions (Table 2). This phylogenetic and biogeographic information may suggest that these endemic species originated from the ancestral lineages of the widely distributed species in each local area. Finally, all *Fejervarya* taxa distributed in India and Sri Lanka (eight taxa used here) formed a clade located at a derived phylogenetic position. Furthermore, the lengths (= genetic distance) of the ancestral nodes connecting these India-Sri Lanka taxa were shorter than those of *Fejervarya* species in other areas. Such tree topology may suggest the occurrence of a recent and rapid radiation of *Fejervarya* species in the India and/or Sri Lanka areas. It is also noteworthy that *F. syhadrensis,* a species with a relatively wide distribution (Table 2), is allocated to the most derived position within the India-Sri Lanka lineage. This implies that the species spread over a wide geographic area during a relatively short period after the radiation of the India-Sri Lanka group.

Limitations in the available specimens and molecular data have prevented us from covering any more than one-third of the known *Fejervarya* species in this study. Further molecular works and their cross-references with morphological and ecological studies will provide new insight into the phylogeny and the taxonomic system of the genus *Fejervarya*.

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REFERENCES

Annandale N (1919) The fauna of certain small streams in the Bombay presidency. Rec Indian Mus 16: 106–161

Biju SD, Bossuyt F (2003) New frog family from India reveals an ancient biogeographical link with the Seychelles. Nature 425: 711–714

Bossuyt F, Milinkovitch MC (2000) Convergent adaptive radiations in Madagascan and Asian ranid frogs reveal covariation between larval and adult traits. Proc Natl Acad Sci USA 97: 6585–6590

Boulenger GA (1920) A monograph of the South Asian, Papuan, Melanesian and Australian frogs of the genus *Rana*. Rec Indian Mus 20: 1–226

Chanda SK, Ghosh AK (1988) Addenda to the amphibian fauna of India. J Bombay Nat Hist Soc 85: 626–627

Channing A (1989) A re-evaluation of the phylogeny of Old-World treefrogs. S Afr J Zool 24: 116–131

Daniels RJR (1998) A field guide to the frogs and toads of the Western Ghats: India. Private circulation. (Reproduced from Cobra 27: 1–25; 28: 1–24; 29: 1–13)

Das I, Ravichandran MS (1998) A new species of *Polypedates* (Anura: Rhacophoridae) from the Western Ghats, India, allied to the Sri Lankan *P. cruciger* Blyth, 1852. Hamadryad 22: 88–94

Das I, Whitaker R (1990) Herpetological investigations in the Western Ghats, South India. Part-I. The Vanjikadavu and Nadukani forests, Kerala state. Hamadryad 15: 6–9

Deuti K (1996) Amphibians of India: Some more species. Cobra 24: 18–20

Dubois A (1975) Un nouveau complexe d' espèces jumelles distinguées par le chant: les grenouilles du Népal voisines de *Rana limnocharis* Boie (amphibiens, anoures). C R Acad Sci Paris Ser D 281: 1717–1720

Dubois A (1984) Note préliminaire sur le groupe de *Rana limnocharis* Gravenhorst, 1892 (amphibiens, anoures). Alytes 3: 143–159

Dubois A (1987) Miscellanea taxomica batrachologica (I). Alytes 5: 7–95

Dubois A (1992) Notes sur la classification des Ranidae (Amphibiens Anoures). Bull Mens Soc Linn Lyon 61: 305–352

Dubois A, Ohler A (2000) Systematic of *Fejervarya limnocharis* (Gravenhorst, 1829)(Amphibia, Anura, Ranidae) and related species. 1. Nomenclatural status and type-specimens of the nominal species *Rana limnocharis* Gravenhorst, 1829. Alytes 18: 15–50

Dubois A (2003) True frogs (Ranidae). In "Grzimek's Animal Life Encyclopedia, 2nd ed. Vol 6, Amphibians" Ed by M Hutchins, WE Duellman, N Schlager, Gale, Farmington Hills, pp 245–264

Dunon-Bluteau D, Brun G (1986) The secondary structures of the *Xenopus laevis* and human mitochondrial small ribosomal subunit RNA are similar. FEBS Lett 198: 333–338

Dutta SK (1992) Amphibians of India: Updated species list with distribution record. Hamadryad 17: 1–13

Dutta SK (1997) Amphibians of India and Sri Lanka (checklist and bibliography). Odyssey Publishing House, Orissa (India)

Emerson SB, Richards C, Drewes RC, Kjer M (2000) On the relationships among ranoid frogs: a review of the evidence. Herpetologica 56: 209–230

Farris JS, Källersjö M, Kluge AG, Bult C (1995) Testing significance of incongruence. Cladistics 10: 537–553

Ford L, Cannatella D (1993) The major clade of frogs. Herpetological Monographs 7: 94–117

Frost DR (2004) Amphibian species of the world: an online reference, V3.0. Electronic database available at http://research.amnh.org/herpetology/amphibia/index.html

Hay J, Ruvinsky S, Hedges SB, Maxon LR (1995) Phylogenetic relationships of amphibian families inferred from 12S and 16S ribosomal RNA genes. Mol Biol Evol 12: 928–937

Hedges SB, Maxon LR (1993) A molecular perspective on lissamphibian phylogeny. Herpetological Monographs 7: 27–42

Hills DM, Ammerman LK, Dixon MT, de Sa OR (1993) Ribosomal DNA and the phylogeny of frogs. Herpetological Monographs 7: 118–131

Inger RF, Dutta SK (1986) An overview of the amphibian fauna of India. J Bombay Nat Hist Soc 83: 135–146

Iskandar DT (1998) The amphibians of Java and Bali. Research and Development Center for Biology, Lipi, Indonesia

Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J Mol Evol 29: 170–179

Kosuch J, Vences M, Dubois A, Ohler A, Böhme W (2001) Out of Asia: mitochondrial DNA evidence for an oriental origin of tiger frogs, genus *Hoplobatrachus*. Mol Phylogenet Evol 21: 398–407

Kuramoto M, Joshy H (2003) Two new species of the genus *Philautus* (Anura: Rhacophoridae) from the Western Ghats, southwestern India. Curr Herpetol 22: 51–60

Laurent RF (1986) Sous-classe des lissamphibiens. Systematique. In "Traite de zoologie, Tome 14, Batraciens, Fasc 1B" Ed by PP Grasse and M Delsol, Masson, Paris, pp 594–797

Meegaskumbura M, Bossuyt F, Pethiyagoda R, Manamendra-Arachchi K, Bahir M, Milinkovitch MC, Schneider CJ (2002) Sri Lanka: An amphibian hot spot. Science 298: 379

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GA, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403: 853–858

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817–818

Roe BA, Ma DP, Wilson RK, Wong JFH (1985) The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. J Biol Chem 260: 9759–9774

Roelants K, Jiang J, Bossuyt F (2004) Endemic ranid (Amphibia: Anura) genera in southern mountain ranges of the Indian subcontinent represent ancient frog lineages: evidence from molecular data. Mol Phylogenet Evol 31: 730–740

Shimodaira H, Hasegawa M (1999) Multiple comparison of log-likelihoods with applications to phylogenetic inference. Mol Biol Evol 16: 1114–1116

Sumida M, Kondo Y, Kanamori Y, Nishioka M (2002) Inter- and intraspecific evolutionary relationships of the rice frog *Rana limnocharis* and the allied species *R. cancrivora* inferred from crossing experiments and mitochondrial DNA sequences of the 12S and 16S rRNA genes. Mol Phylogenet Evol 25: 293–305

Swofford DL (2001) "PAUP*. Phylogenetic Analysis Using Parsimony (* and other methods). Version 4." Sinauer, Sunderland

Thompson JD, Higgins DG, Gibson T J (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680

Toda M, Nishida M, Matsui M, Wu GF, Ota H (1997) Allozyme variation among east Asian populations of the Indian rice frog, *Rana limnocharis* (Amphibia: Anura). Biochem Syst Ecol 25: 143–159

Toda M, Matsui M, Nishida M, Wu GF, Ota H (1998a) Genetic divergence among Southeast and East Asian populations of *Rana limnocharis* (Amphibia: Anura), with special reference to sympatric cryptic species in Java. Zool Sci 15: 607–613

Toda M, Nishida M, Matsui M, Wu GF, Ota H (1998b) Genetic variation in the Indian rice frog, *Rana limnocharis* (Amphibia: Anura), in Taiwan, as revealed by allozyme data. Herpetologica 54: 73–82

Vasudevan K, Dutta SK (2000) A new species of *Rhacophorus* (Anura: Rhacophoridae) from the Western Ghats, India. Hamadryad 25: 21–28

Veith M, Kosuch J, Ohler A, Dubois A (2001) Systematics of *Fejervarya limnocharis* (Gravenhorst, 1829) (Amphibia, Anura, Ranidae) and related species. 2. Morphological and molecular variation in frogs from the Greater Sunda islands (Sumatra, Java, Borneo) with the definition of two species. Alytes 19: 5–28

Vences M, Wanke S, Odierna G, Kosuch J, Veith M (2000) Molecular and karyological data on the South Asian ranid genera *Indirana*, *Nyctibatrachus* and *Nannophrys* (Anura: Ranidae). Hamadryad 25: 75–82

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