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Genetic divergences of South and Southeast Asian frogs: a case study of several taxa based on 16S ribosomal RNA gene data with notes on the generic name *Fejervarya*

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Genetic divergences of South and Southeast Asian frogs: a case study of several taxa based on 16S ribosomal RNA gene data with notes on the generic name *Fejervarya*

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Abstract: To elucidate the genetic divergences of several Asian frog taxa, the mitochondrial 16S rRNA gene (16S) sequences of 81 populations across 6 Asian countries were analyzed. In total, 109 haplotypes were found, and the concept of a 3% difference in 16S sequence corresponding to species threshold was applied to define candidate amphibian species, for which corroborating evidence, such as morphology, ecological characteristics, and/or nuclear gene data, is required. *Polypedates leucomystax*, *Hylarana chalconota*, and *Hylarana* sp. from Chantaburi, Maelippet Siberut, and Langkawi Island, respectively, correspond to 3 candidate species and *H. erythraea* from Malaysia/Thailand represents a possible candidate species. *Hylarana* cf. *nicobariensis* from Muara Siberut, *Amolops larutensis* from Gombak, and *Microhyla okinavensis* from Ishigaki Island showed divergences from the topotypic specimen, all suggesting a relevant candidate species. *Microhyla heymonsi* from Malaysia and *M. ornata* from 2 regions (Mudigere and Talapu) in India did not fit any congeneric species based on available 16S data, suggesting 3 possible candidate species in total. Two lineages of *Duttaphrynus melanostictus* from Malaysia denote 2 possible candidate species. Consequently, this study indicates the occurrence of 6 candidate and 6 possible candidate species, and argues that the generic allocation of the *Fejervarya*-*Minervarya*-*Zakerana* complex needs to be studied in detail.

Key words: Amphibia, Mitochondrial 16S ribosomal RNA gene, Genetic divergence, Candidate species, South and Southeast Asia

1. Introduction

A species is a fundamental unit of biology (Mayr, 1982; Ereshefsky, 1992; Claridge et al., 1997). Contemporary concepts of species differ between researchers and also between objective taxa; thus, the concept of what constitutes a species continues to be a vexing problem (Dobzhansky, 1976; de Queiroz, 1998). Although Mayden (1997, 1999) listed up to 24 named species concepts, the phylogenetic species concept and the biological species concept are the most commonly advocated (Futuyma, 2005). The phylogenetic species concept states “a species is an irreducible (basal) cluster of organisms that is diagnosably distinct from other such clusters, and

within which there is a parental pattern of ancestry and descent” (Cracraft, 1989) and “a species is the smallest monophyletic group of common ancestry” (de Queiroz and Donoghue, 1990). For multicellular animals, however, the biological species concept is recommended (Wright, 1940; Mayr, 1942; Dobzhansky, 1950) and has been widely accepted by researchers. In amphibians, morphological, ecological, and/or bioacoustics characteristics have been used to define species. However, investigations of these characteristics are often impractical due to difficulties in observing live animals and/or collecting multiple samples over wide geographic areas. Although it is not sufficient to define a biological species on the basis of molecular

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data alone (Ferguson, 2002), the utility of molecular data in taxonomic studies (e.g., Tautz et al., 2003), especially in searching for cryptic species (i.e. 2 or more species sharing similar external morphology) (e.g., Bickford et al., 2007 [review]; Hasan et al., 2012a; Mila et al., 2012), has been demonstrated. One such procedure is called the “candidate species approach” (Vences et al., 2005a; Fouquet et al., 2007; Vieites et al., 2009). In the present study, the term “candidate species” is used to denote lineages that are generally distinct, for instance on the basis of molecular data (generally mitochondrial [mt]DNA genes), but for which corroborating evidence, such as morphology, ecological characteristics, and/or nuclear gene data, is required.

In many animal taxa, the mt *cox1* gene is often used as a marker for species identification (i.e. DNA barcoding; Herbert et al., 2003, 2004). In amphibians, however, the mt *16S* rRNA gene (*16S*) is considered a useful marker for determining taxonomic affiliation (Vences et al., 2005b).

Although amphibians (number of amphibian populations) are in worldwide decline (Stuart et al., 2004), the number of discovered amphibian species is steadily increasing (Hanken, 1999; Lannoo, 2005; Pyron and Wiens, 2011). For instance, the number of known amphibian species has increased dramatically from 4013 in 1985 to 7164 in 2013 (AmphibiaWeb, August 19, 2013). Numerous researchers have proposed abundant diversity in unrecognized anuran species (in Southeast Asia: Inger, 1999; Emerson et al., 2000; Brown and Guttman, 2002; Bain et al., 2003; Stuart et al., 2006; Chan and Grismer, 2010; Matsui et al., 2010; in Madagascar: Vieites et al., 2009; Glaw et al., 2010; in South America: Jansen et al., 2011; Funk et al., 2012). Inger (1999) proposed that potentially unknown amphibian biodiversity needs to be explored by additional sampling in South Asia as it might disclose detailed information at the species level. In fact, many undescribed and/or cryptic species have been found in complexes previously thought to be single species in South to East Asian countries through morphological, allozyme, and molecular analyses, as well as crossing experiments (*Hoplobatrachus* and *Euphlyctis*: Alam et al., 2008; Hasan et al., 2012b; *Fejervarya*: Sumida et al., 2007; Islam et al., 2008a, 2008b; Kotaki et al., 2010; Kurniawan et al., 2010). In recent years, particularly on the Indian subcontinent, eye-catching discoveries of new species, genera, and/or families have been made (Biju and Bossuyt, 2003; Biju et al., 2011).

Despite these new findings, the diversity of amphibian species in South and Southeast Asia remains underestimated, mainly because of the homoplasy in amphibian morphology (Stuart et al., 2006). Therefore, the discovery of new frog species is an ongoing activity (Ohler et al., 2009). In this study, we sequenced mt *16S* data from frog specimens collected from 20 regions/

localities across 6 Asian countries in an attempt to reveal candidate species. This data will be helpful for revealing hidden anuran biodiversity and will be useful in future taxonomic studies of frogs in South and Southeast Asia for delineating species boundaries using combinations of multiple datasets (i.e. integrative taxonomy and/or the candidate species approach [Dayrat, 2005; Padil et al., 2010]). We also discuss the taxonomic status of several frog taxa of South and Southeast Asia and present notes on the generic name *Fejervarya* on the basis of sequence divergence and our resultant *16S* maximum likelihood (ML) tree.

2. Materials and methods

2.1. Specimens

A total of 159 specimens from 81 populations were collected from 20 localities/regions across 6 Asian countries (Figure 1). Species identification was based mainly on external morphological characteristics (Boulenger, 1920; Dutta and Manamendra-Arachchi, 1996; Iskandar, 1998; Chanda, 2002; Das, 2002; Daniels, 2005; Inger and Stuebing, 2005). We adopted the species naming system used by Frost (2013) with the exception of the species within the genus *Zakerana*, which are treated here as members of *Fejervarya* because the morphological separation of *Zakerana* and *Fejervarya* proposed by Howlader (2011) is based on inadequate comparisons.

All animals (frogs) used in this study were treated in accordance with the ethical regulations for animal experimentation as defined by the Ethics Committee of Hiroshima University, Japan.

2.2. DNA extraction

Total genomic DNA was extracted from the clipped toe of each frog specimen using a DNA extraction kit (DNeasy Tissue Kit, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The extracted DNA solutions were used as polymerase chain reaction (PCR) templates for amplifying a partial *16S* region corresponding to position 3099–4462 of the *16S* gene of *Xenopus laevis* (Accession no. M10217, Roe et al., 1985).

2.3. PCR and sequencing

PCR amplification was performed using a primer set of 12S_3'end_Fow1 and 12S_3'end_Fow2 (Hasan et al., 2012a). The primers were also used for DNA sequencing. We used the following additional primers for sequencing when necessary: F51 and R51 (Sumida et al., 2002), 16S_R530 and 16S_R723 (Hasan et al., 2012a), R1051 (5'-ATGTTTTTGGTAAACAGGCGGG-3'), FR60 (5'-AAGCAAGTCGTAACATGGTA-3'), 16S_Rev1_SMA (5'-GGTTATTGTTAAGCTTTAACGC-3'), and 16S_Rev2_CANCR1 (5'-GAGAAATTAAGCTTTAACGC-3'). The length of the resultant *16S* fragments varied from 1287 to 1354 bp between taxa. PCR mixtures were prepared

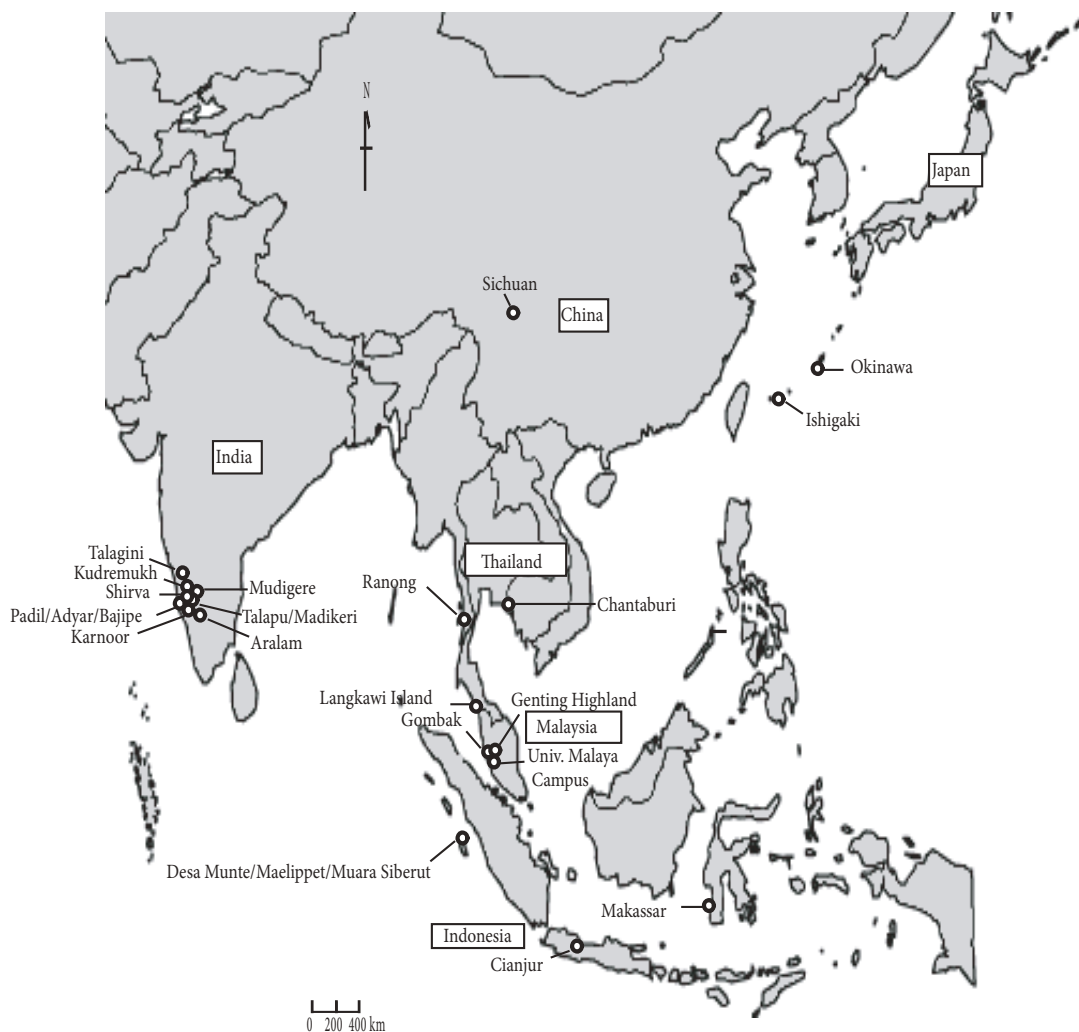


Figure 1. Map showing collection sites of South and Southeast Asian frogs used in the present study.

with the TaKaRa Ex Taq Kit (Takara Bio, Inc., Shiga, Japan), according to the manufacturer's protocol. DNA amplification and sequencing strategies were followed according to the procedure by Hasan et al. (2012a), and PCR products were purified using MicroSpin S-300 HR columns (GE Healthcare, Buckinghamshire, UK). The amplified *16S* segments were directly sequenced on both strands using the BigDye Terminator Cycle Sequencing Kit (ABI) with an automated DNA sequencer (3100-Avant; ABI, Brooklyn, NY, USA). The obtained new sequences that were found in this study were deposited in the DNA Data Bank of Japan (DDBJ) database under the accession numbers AB530548 to AB530656. The specimens used and haplotypes of *16S* data found in this study are shown in Table 1.

2.4. Alignment data and identified haplotypes

The *16S* sequences from the 159 specimens and *X. laevis* were aligned using the ClustalW program (Thompson et al.,

1994). The initial alignment consisted of 1487 nucleotide positions and showed 109 distinct haplotypes. Indel and ambiguous alignment sites were removed from the initial alignment dataset using Gblocks Ver. 0.91b (Castresana, 2000) with default parameters, resulting in 829 well-aligned sites. After deletion of indel and ambiguous sites, several haplotypes had identical sequences, and the initial 109 haplotypes were reduced to 88 haplotypes, which were used for a phylogenetic tree reconstruction (Figure 2; see below). The sequence divergences (uncorrected *p* values) were calculated for the 88 haplotypes using MEGA Ver. 4.0 (Tamura et al., 2007) with the pairwise-deletion option and with indel sites excluded.

Next, we separately prepared alignment datasets for the families of Rhacophoridae, Ranidae, Microhylidae, and Bufonidae using the *16S* data of our specimens and related species from public DNA databases (GenBank, DDBJ, and European Molecular Biology Laboratory [EMBL])

Table 1. Specimens used and haplotypes of 16S rRNA gene found in the present study.

BNHS: Bombay Natural History Society. IABHU: Institute for Amphibian Biology, Hiroshima University.					
RBRL: Rondano Biodiversity Research Laboratory, St. Aloysius College. NA: Not available.					
Species	Collection station		No. of	Voucher/Tissue Ref.	No. of haplotype
	Country	Locality	frogs used		(Accession number)
<i>Euphlyctis cyanophlyctis</i>	India	Madikeri	1	RBRL 030607-02	1(AB530596)
<i>Euphlyctis aloysii</i>		Adyar	1	BNHS 5124	1(AB530594)
<i>Euphlyctis cf. aloysii</i>		Mudigere	1	RBRL 050709-39	1(AB530595)
<i>Euphlyctis mudigere</i>		Mudigere	1	BNHS 5128	1(AB530599)
<i>Euphlyctis hexadactylus</i>		Adyar	2	RBRL 050719-01, 02	2(AB53059-98)
<i>Hoplobatrachus tigerinus</i>		Padil	1	Release	1(AB530600)
<i>Hoplobatrachus rugulosus</i>	Thailand	Ranong Province	1	NA	1(AB530609)
<i>Fejervarya limnocharis</i>	Malaysia	University Malaya Campus	1	IABHU 21015	1(AB530625)
<i>Fejervarya rufescens</i>	India	Bajipe	1	RBRL 030526-03	1(AB530601)
		Padil	1	RBRL 040716-1	1(AB530602)
<i>Fejervarya kudremukhensis</i>		Kudremukh	1	BNHS 4654	1(AB530603)
<i>Minervarya sahyadris</i>		Aralam	2	RBRL 050714-01, 02	2(AB530604-05)
<i>Fejervarya caperata</i>		Mudigere	1	BNHS 5060	1(AB530606)
<i>Fejervarya mudduraja</i>		Madikeri	1	BNHS 4646	1(AB530607)
<i>Fejervarya multistriata</i>	China	Sichuan Province	4	Alive, IABHU 3789	2(AB530611-12)
<i>Fejervarya iskandari</i>	Indonesia	Cianjur, Java	2	IABHU 3816, alive	1(AB530613)
<i>Sphaerotheca dobsonii</i>	India	Bajipe	3	RBRL 040706-13, 14, 060720-01	1(AB530608)
<i>Occidozyga cf. martensii</i>	Thailand	Ranong Province	1	Alive	1(AB530610)
<i>Occidozyga cf. lima</i>	Indonesia	Java	1	Alive	1(AB530619)
<i>Limnonectes shompenorum</i>		Muara Siberut	4	IABHU 20716-20719	1(AB530614)
<i>Limnonectes kuhlii</i>		Desa Munte	7	IABHU 20727- 20732	4(AB530615- 18)
<i>Limnonectes blythii</i>	Malaysia	Langkawi Island	4	IABHU 21101, 21103, 21108, 21118	3(AB530620-22)
<i>Limnonectes laticeps</i>		Gombak	1	IABHU 21020	1(AB530623)
<i>Limnonectes plicatellus</i>		Langkawi Island	1	IABHU 21120	1(1(AB530624))
<i>Polypedates leucomystax</i>	Thailand	Chantaburi	1	NA	1(AB530566)
	Japan	Okinawa	2	Alive	1(AB530567)
	Malaysia	University Malaya Campus	2	IABHU 21024, 21030	2(AB530568-69)
		Langkawi Island	1	IABHU 21119	1(AB530570)
<i>Polypedates pseudocruciger</i>	India	Bajipe	9	RBRL 070721-26, Release	3(AB530550-52)
<i>Rhacophorus lateralis</i>	India	Mudigere	3	RBRL 050709-35, 36, 37	1(AB530548)
<i>Rhacophorus malabaricus</i>		Madikeri	1	Release	1(AB530549)
<i>Raorchestes luteolus</i>		Madikeri	2	RBRL 060709-24, 25	1(AB530553)
		Kudremukh	1	BNHS 4192	1(AB530554)
<i>Raorchestes cf. glandulosus</i>		Talapu	2	RBRL 060709-26, 27	2(AB530555-56)
<i>Raorchestes sp.</i>		Talapu	1	RBRL 060709-28	1(AB530557)
<i>Raorchestes cf. bobingeri</i>		Aralam	1	RBRL 050714-09	1(AB530558)
<i>Pseudophilautus wynaadensis</i>		Padil	4	BNHS 5111-5112, Release	2(AB530559- 60)
		Kudremukh	1	BNHS 5113	1(AB530561)
		Bajipe	1	BNHS 5118	1(AB530562)
		Bajipe	1	BNHS 5120	1(AB530565)

Table 1. (Continued).

		Karnoor	1	BNHS 5115	1(AB530563)
		Aralam	1	RBRL 050714-10	1(AB530564)
<i>Indirana cf. semipalmata</i>	India	Shirva	1	RBRL 060715-02	1(AB530593)
<i>Hylarana aurantiaca</i>		Adyar	2	RBRL 070728-04, 05	1(AB530574)
		Kudremukh	2	BNHS 5106, Release	1(AB530575)
		Talapu, Madikeri	3	BNHS 5108-5109, RBRL 060709-05	2(AB530576-77)
<i>Hylarana temporalis</i>		Talapu, Madikeri	4	RBRL 060709-01, 02, 03, Release	1(AB530578)
<i>Hylarana malabarica</i>		Bajipe	2	RBRL 060724-03, Release	1(AB530579)
<i>Hylarana erythraea</i>	Thailand	Chantaburi	2	NA	1(AB530580)
	Malaysia	Langkawi Island	5	IABHU 21105-21107, NA	3(AB530584-86)
		University Malaya Campus	1	IABHU 21017	1(AB530587)
<i>Hylarana cf. nicobariensis</i>	Indonesia	Muara Siberut	5	IABHU 20707-20711	2(AB530581-82)
<i>Hylarana chalconota</i>		Maelippet Siberut	1	IABHU 20720	1(AB530583)
<i>Hylarana sp.</i>	Malaysia	Langkawi Island	1	IABHU 21104	1(AB530588)
<i>Odorrana hosii</i>		Gombak	3	IABHU 21004-21006	3(AB530589-91)
<i>Amolops larutensis</i>		Gombak	2	IABHU 21007, 21013	1(AB53057892)
<i>Nyctibatrachus major</i>	India	Kudremukh	1	Release	1(AB530571)
		Talapu	2	BNHS 5101, 5103	2(AB530572-73)
<i>Micrixalus saxicola</i>		Madikeri	3	RBRL 040716-37, 38, 39	1(AB530626)
<i>Microhyla heymonsi</i>	Malaysia	University Malaya Campus	2	IABHU 21025-21026	2(AB530636-37)
<i>Microhyla ornata</i>	India	Bajipe	1	Release	1(AB530627)
		Karnoor	2	BNHS 5028-5029	2(AB530628-29)
		Talagini	1	RBRL 040723-04	1(AB530630)
		Talapu, Madikeri	1	RBRL 060709-29	1(AB530631)
		Mudigere	1	BNHS 5036	1(AB530632)
<i>Microhyla berdmorei</i>	Malaysia	Gombak	1	IABHU 21019	1(AB530638)
<i>Microhyla okinavensis</i>	Japan	Okinawa	1	IABHU 5128	1(AB530634)
		Ishigaki	1	IABHU 5263	1(AB530635)
<i>Kaloula pulchra</i>	Thailand	Ranong Province	1	NA	1(AB530633)
	Indonesia	Makassar, Sulawesi	1	NA	1(AB530639)
<i>Duttaphrynus melanostictus</i>	India	Bajipe	2	BNHS 5021, Release	1(AB530640)
		Padil	1	Release	1(AB530641)
		Shirva	1	Release	1(AB530642)
	Thailand	Ranong Province	1	NA	1(AB530645)
	Indonesia	Maelippet Siberut	3	IABHU 20701, 20703-20704	1(AB530646)
	Malaysia	University Malaya Campus	3	IABHU 21021-21022, 21029	2(AB530647-48)
<i>Duttaphrynus scaber</i>	India	Mudigere	3	BNHS 5023-5025	2(AB530643-44)
<i>Ingerophrynus parvus</i>	Malaysia	Gombak	3	IABHU 21009- 21011	3(AB530649-51)
<i>Phrynoidis aspera</i>		Gombak	2	IABHU 21002-21002	1(AB530652)
		Gombak	1	IABHU 21013	1(AB530654)
		Langkawi Island	5	IABHU 21102, 21114-21117	1(AB530653)
<i>Leptobrachium smithi</i>		Langkawi Island	1	IABHU 21112	1(AB530655)
<i>Xenophrys longipes</i>		Genting highland	1	IABHU 21101	1(AB530656)
			159		109

and used in detailed phylogenetic analyses (Figures 3–6). The 16S data from GenBank is listed in Appendix 1. We selected the related taxa and their 16S sequences on the basis of the BLAST search and information from relevant scientific papers. The procedures to calculate sequence divergence (uncorrected *p* values) among the haplotypes for each family were the same as those described above. The 16S sequence lengths of the alignment datasets varied across the 4 families and were shortened from the initial alignment depending on the lengths of 16S sequences obtained from DNA databases. The sequence lengths and total number of operational taxonomic units (OTUs) determined from the alignment data were 404 sites of 75 OTUs for rhacophorids, 397 sites of 57 OTUs for Ranidae, 739 sites of 29 OTUs for microhylids, and 419 sites of 41 OTUs for bufonids.

2.5. Phylogenetic analysis

We first constructed ML and Bayesian inference (BI) trees using the alignment data of the 88 haplotypes of our specimens. Nucleotide substitution models for ML and BI analyses were selected according to the Akaike information criterion and Bayesian information criterion implemented in the Kakusan 3.0 program (Tanabe, 2007). ML analysis was performed using Treefinder (Jobb et al., 2004), and the resultant tree was evaluated by bootstrap analysis with 1000 replicates. BI analysis was performed using MrBayes Ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) with the following settings: number of Markov chain Monte Carlo (MCMC) generations = 30×10^5 and sampling frequency = 100. The burn-in size was determined by checking the convergence of $-\log$ likelihood ($-\ln L$) values using Tracer ver. 1.5 (Rambaut and Drummond, 2007), the first 3×10^5 generations were discarded as burn-in data. The statistical support of the BI tree was evaluated by Bayesian posterior probability.

Further phylogenetic analyses of the families Rhacophoridae, Ranidae, Microhylidae, and Bufonidae were performed by ML and BI. For these analyses, the procedures of ML and BI analyses and the selection

of appropriate substitution models were the same as those described above. Node support of the resultant trees was evaluated by bootstraps (BPs) calculated from 500 replicates for the ML analysis, and BI analysis was performed in the same manner as described above. A summary of alignment data and evolutionary models is shown in Table 2.

3. Results

3.1. Haplotypes and sequence divergences of South to East Asian frogs

The length of the sequenced 16S fragments varied from 1287 to 1354 bp among the 159 specimens examined, and the aligned 16S data set consisted of 1487 nucleotide positions. Of these sites, 480 were variable and 408 were parsimoniously informative. Among the 159 specimens (from 81 populations), a total of 109 haplotypes were identified. A complete list of the haplotypes and their corresponding DDBJ accession numbers is shown in Table 1.

Figure 2 shows the resultant ML tree and BP values for the ML and BI methods and haplotypes of each species form one clade. The average sequence divergence at the individual, population, species, genus, and family taxonomic levels was 0.23%, 2.2%, 7.23%, 11.22%, and 17.35%, respectively (Figure 2). In general, the low 16S diversity in each species clade of dicroglossid frogs indicates the absence of candidate species in the examined dicroglossid specimens. In contrast, the average (and range) of 16S fragment analyzed within each species is as follows: 2.1% (0.1%–3.1%) in *Pseudophilautus wynaadensis*, 3.6% (2.5%–4.5%) in *Polypedates leucomystax*, 4.0% (0.7%–5.8%) in *Hylarana erythraea*, 7.1% in *Microhyla okinavensis*, 8.9% (0.4%–15.7%) in *M. ornata*, and 5.3% (0.3%–7.2%) in *Duttaphrynus melanostictus*. Excluding *Ps. wynaadensis*, all examined taxa showed >3% 16S divergence. Especially, 16S divergence is higher in *P. leucomystax* (between the Okinawa, Univ. Malaya Campus, and Chantaburi populations), *H. erythraea* (between the Langkawi Island/Univ. Malaya Campus and Chantaburi

Table 2. Summary of alignment data and evolutionary models estimated in the Kakusan 3.0 program (Tanabe 2007).

Figure	Total sites	Variable sites	Parsimoniously informative sites	Model	
				ML	BI
Figure 2	829	480	408	GTR + G + I	GTR + G + I
Figure 3	404	164	139	J2 + G + I	GTR + G + I
Figure 4	397	167	141	J2 + G + I	GTR + G + I
Figure 5	739	254	190	GTR + G	GTR + G
Figure 6	419	125	82	J2 + G	GTR + G

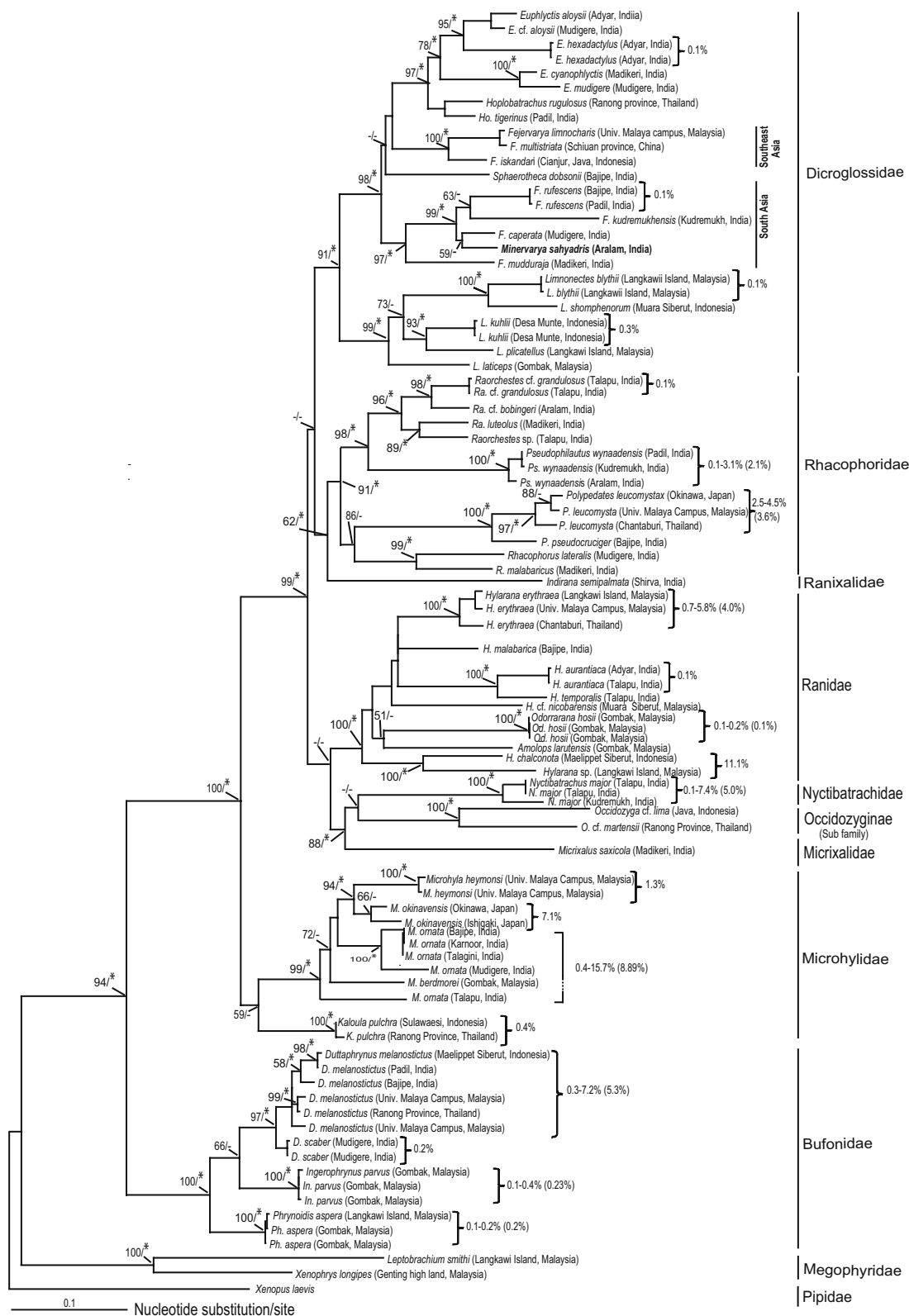


Figure 2. Maximum likelihood (ML) tree based on nucleotide sequences of the mitochondrial 16S rRNA gene from 88 haplotypes of frogs (Table 1), with *Xenopus laevis* as an outgroup. Bootstrap support (>50%) is indicated at nodes in the order of ML (1000) replicates. Asterisks represent Bayesian posterior probability (BPP; * $\geq 95\%$).

populations), *M. okinavensis* (between the Okinawa and Ishigaki populations), *M. ornata* (between the Bajipe, Mudigere, and Talapu populations), and *D. melanostictus* (between the Maelippet Siberut, Padil, Bajipe, Univ. Malaya Campus, and Ranong Province populations). These high *16S* divergence values suggest the presence of many candidate species in our specimens. Haplotypes distribution maps of South and Southeast Asian frogs (candidate and/or possible candidate species) are given in Appendix 2.

3.2. Genetic divergence and phylogenetic positioning of South to East Asian frogs with respect to congener species

In general, there is a low degree of *16S* divergence within each species of the group (Figure 2) in the family Dicroglossidae, indicating the absence of any possible candidate species in our presently examined dicroglossid taxa. Remarkably, *16S* divergence between Southeast and South Asian *Fejervarya* groups is high (11.8% [9.3%–15.1%]). In particular, *Minervarya sahyadris* formed a clade with the South Asian *Fejervarya* group and was remotely related to the Southeast Asian *Fejervarya* group.

To clarify candidate species status, we performed detailed phylogenetic analyses and a *16S* divergence survey for each family using *16S* data from the DNA database data. For the analyses of Rhacophoridae, Ranidae, Microhylidae, and Bufonidae, we incorporated 56, 42, 16, and 26 additional *16S* sequences of related species from the DDBJ, EMBL, and GenBank databases, respectively. The resultant ML trees are shown in Figures 3–6.

3.3. Family Rhacophoridae (Figure 3)

In our ML tree, *Ps. wynaadensis* specimens from India (Padil, Karnoor, and Bajipe) formed a clade (BP = 100 for ML and ≥ 95 for BI) with very low *16S* divergence (0.1%), whereas the specimen from Aralam showed 2.9% *16S* divergence. The *Raorchestes luteolus* from the Madikeri specimen formed a clade (BP = 100 for ML, ≥ 95 for BI, and *16S* divergence = 1.0%) with the *Ra. luteolus* from the Western Ghats specimen, while *Raorchestes* sp. from Talapu (India) formed a clade (BP = 97 for ML and ≥ 95 for BI) with the *Ra. anili* from the Western Ghats (India) specimen with negligible *16S* divergence (1.4%). *Raorchestes* cf. *glandulosus* (Talapu, India) formed a clade (BP = 91 for ML and ≥ 95 for BI) with *Ra. glandulosus* (Western Ghats, India) with only 0.7% *16S* divergence. Of the examined *P. leucomystax* specimens, the Okinawa (introduced) and Univ. Malaya Campus populations showed low *16S* divergence (1.6%) with respect to the topotypic specimen (Java, Indonesia). In contrast, the *P. leucomystax* specimen from Chantaburi (Thailand) diverged *16S* (3.3%) from that of the former topotypic specimen. The *P. pseudocruciger* specimen from Bajipe (India) formed a clade with the Sri Lankan specimen of *P. maculatus* and they have relatively

low (2.2%) *16S* divergence, but this value is high (11.7%) when compared to another Western Ghats specimen of *P. pseudocruciger*. *Rhacophorus malabaricus* of the Madikeri specimen formed a clade (BP = 100 for ML and ≥ 95 for BI) with the Western Ghats specimen with 3.6% *16S* divergence.

3.4. Family Ranidae (Figure 4)

Among the examined ranid specimens, the *16S* divergences are generally low (<0.1%) within each species in a clade. *Odorrana hosii* of the Gombak population formed a clade (BP = 100 for ML and ≥ 95 for BI) and their *16S* divergence varied from 0.1% to 0.2%. Yet, the *16S* of *H. chalconota* specimens from Maelippet Siberut (Indonesia) and *Hylarana* sp. from Langkawi Island (Malaysia) were highly diverged (8.0% and 10.9%, respectively) from that of the topotypic specimen (Java, Indonesia). In *H. erythraea*, *16S* sequences from the Thailand, Vietnam, and Cambodia specimens were highly diverged (5.8%) from those of the Malaysia specimens. We also found that our *H. cf. nicobariensis* (Muara Siberut, Indonesia) specimen was greatly diverged (9.1%) from the Java specimen (Indonesia). *Amolops larutensis* from Malaysia showed 3.6% and 3.8% *16S* divergence with respect to the Narathiwat and Perak populations, respectively.

3.5. Family Microhylidae (Figure 5)

Microhyla okinavensis of the Okinawa population formed a clade (BP = 100 for ML, ≥ 95 for BI, and *16S* divergence = 4.5%) with *M. okinavensis* of the Amami population. The *M. okinavensis* specimen from Ishigaki (Japan) was found to be diverged (7.1%) from the topotypic specimen (Okinawa, Japan). The type locality of *M. heymonsi* is Taiwan (Vogt, 1911). Our examined *M. heymonsi* (Univ. Malaya Campus, Malaysia) shows great *16S* divergence with respect to the Thailand and China specimens (4.8% and 7.3%, respectively). Similarly, it was found that our Indian *M. ornata* specimens showed great divergence in *16S* sequence. The *16S* divergence between the Karnoor/Bajipe/Talagini populations and Dharwad/Mudigere populations is 7.7%. The *M. mymensinghensis* specimen from Bangladesh formed a clade (BP = 95 for ML and ≥ 95 for BI) with the *M. ornata* specimen from the Karnoor/Bajipe/Talagini populations, and their *16S* divergence is 3.2%. However, our Indian *M. ornata* (Talapu) formed a clade with *M. superciliaris* (Pahang, Malaysia) with a high BP value (BP = 98 for ML and ≥ 95 for BI) and a significant *16S* divergence (11.3%). *Microhyla berdmorei* of the Gombak population formed a clade (BP = 82 for ML, ≥ 95 for BI, and *16S* divergence = 2.9%) with *M. berdmorei* of the Kalimantan population. It is interesting that *Kaloula pulchra*, found across a wide range of Asian countries (Thailand, China, Bangladesh, and Indonesia), did not show high genetic divergence (average *16S* divergence = 0.74%).

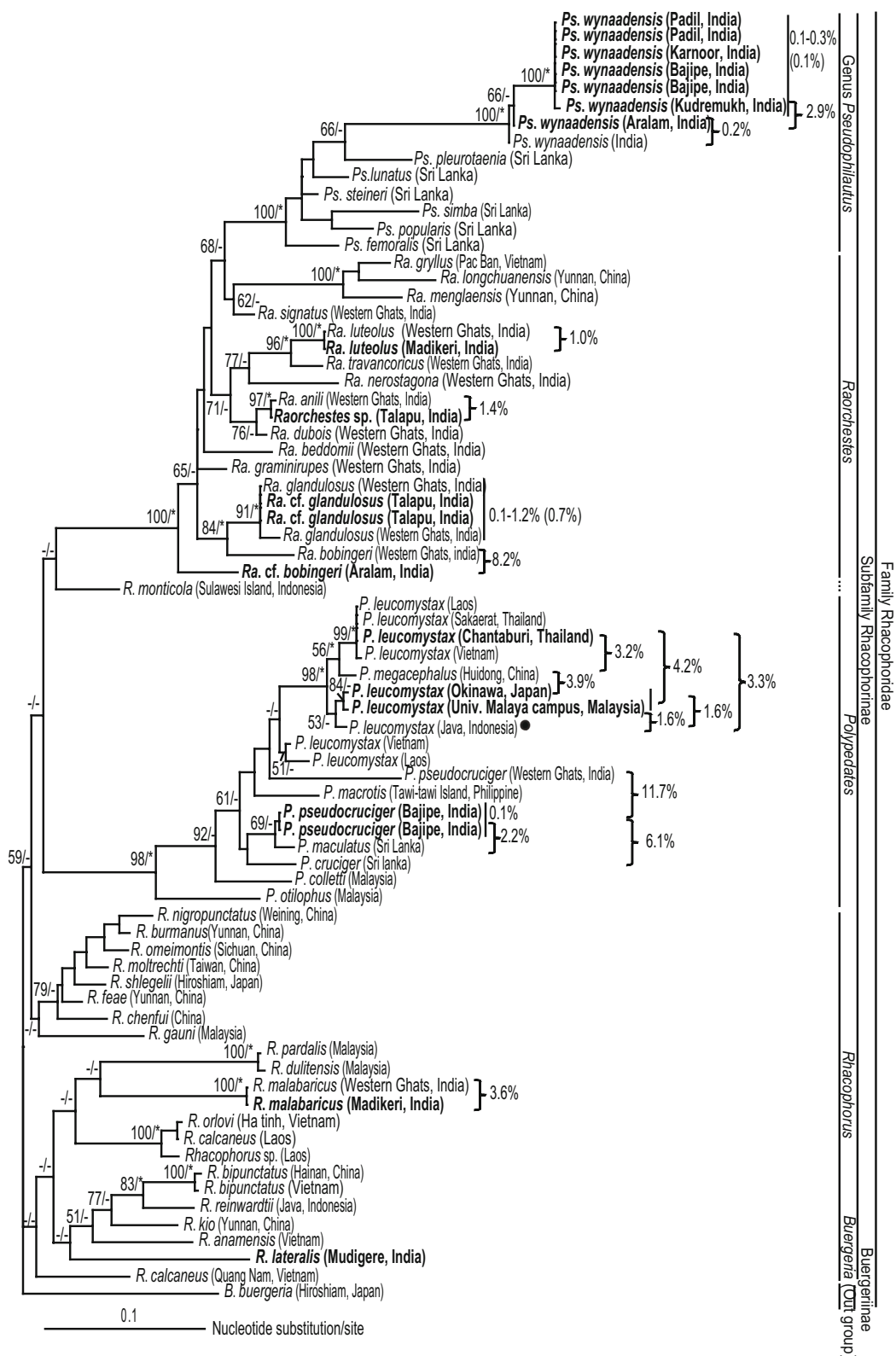


Figure 3. Maximum likelihood (ML) tree of rhacophorid frogs based on nucleotide sequences of the mitochondrial 16S rRNA gene with *Buergeria buergeria* as an outgroup. Bootstrap support (>50%) is indicated at nodes in the order of ML (500) replicates. Asterisks represent Bayesian posterior probability (BPP; * $\geq 95\%$). Specimens examined in this study are indicated by boldface type and closed circle (●) means samples taken from type locality area.

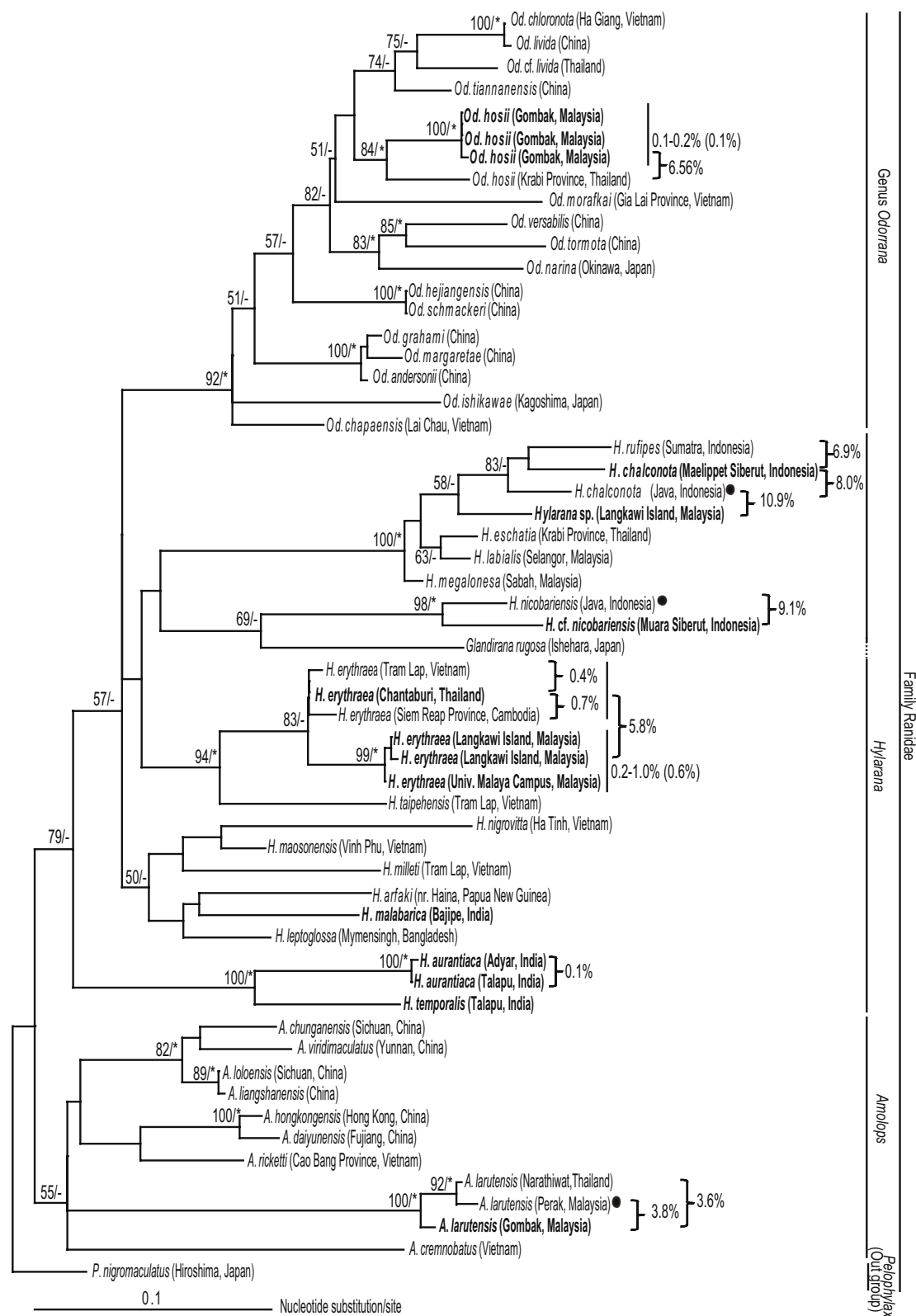


Figure 4. Maximum likelihood (ML) tree of ranid frogs based on nucleotide sequences of the mitochondrial 16S rRNA gene with *Pelophylax nigromaculatus* as an outgroup. The bootstrap support (>50%) is indicated at nodes in the order of ML (500) replicates. Asterisks represent Bayesian posterior probability (BPP; * ≥95%). Specimens examined in this study are indicated by boldface and closed circle (●) means samples taken from type locality area.

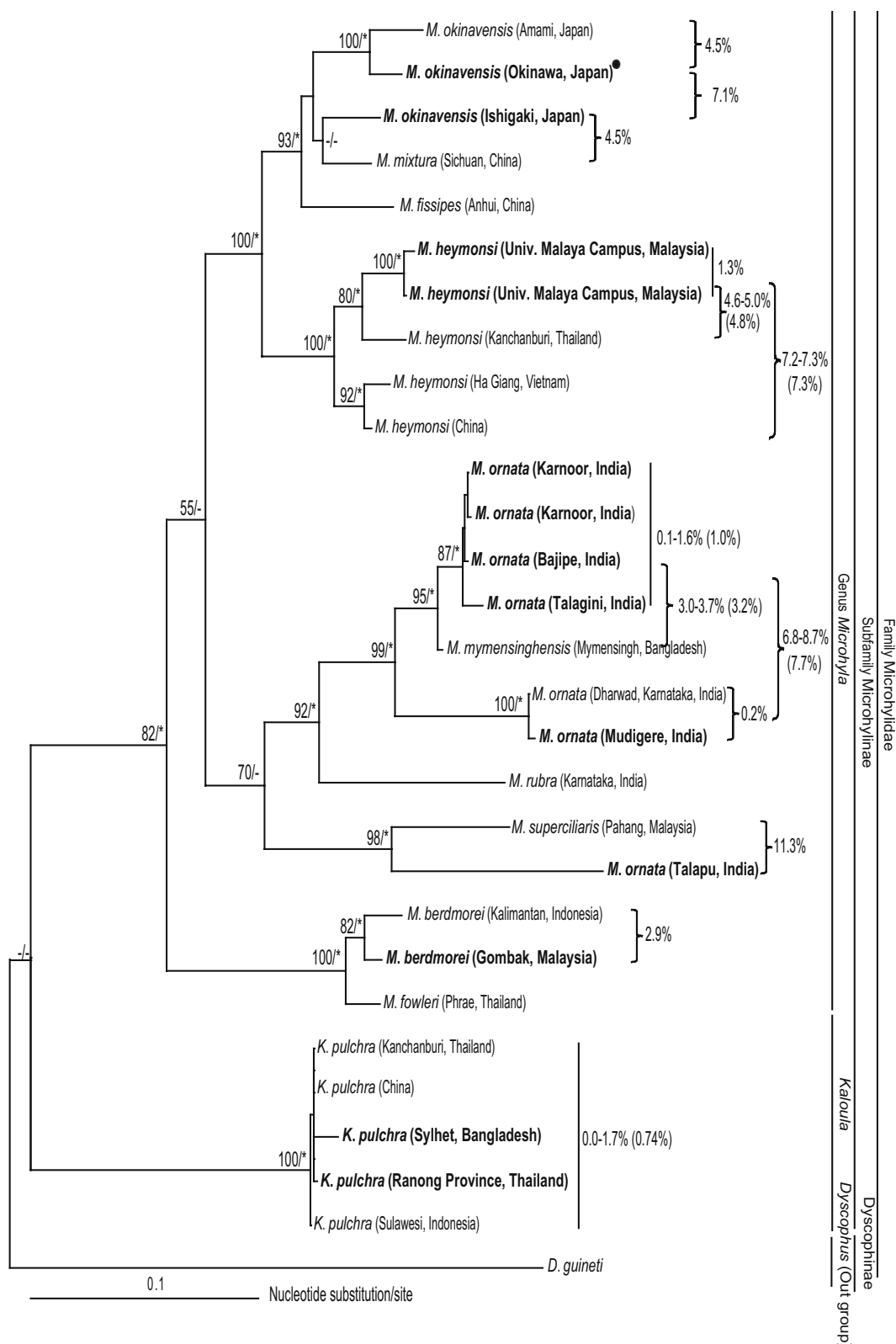


Figure 5. Maximum likelihood (ML) tree of microhylid frogs based on nucleotide sequences of the mitochondrial 16S rRNA gene with *Dyscophus guineti* as an outgroup. Bootstrap support (>50%) is indicated at nodes in the order of ML (500) replicates. Asterisks represent Bayesian posterior probability (BPP; * ≥95%). Specimens examined in this study are indicated by boldface type and closed circle (●) means samples taken from type locality area.

3.6. Family Bufonidae (Figure 6)

In our ML tree, we found that *D. melanostictus* from the Univ. Malaya Campus (Malaysia), Hà Tĩnh (Vietnam), China, Gia Lai (Vietnam), Ranong Province (Thailand), and Penang Island (Malaysia) formed a clade (BP = 62 for ML) with low sequence divergence (0.7%). The other specimens from the Univ. Malaya Campus (Malaysia) and the Gangetic Plain (India) formed a clade (BP = 97 for ML and ≥ 95 for BI) with a 2.8% 16S divergence. Between these 2 clades, the 16S divergence was 4.3%. The *D. melanostictus* specimen from Indonesia formed a clade with *D. melanostictus* specimens from India except that from Bajipe/Shirva (India), which has close affinity with the species *D. brevirostris* (Western Ghats, India) with high statistic support (BP = 100 for ML and ≥ 95 for BI) and low 16S divergence (0.1%). *Duttaphrynus scaber* from Mudigere and the Western Ghats formed a clade (BP = 95 for ML and ≥ 95 for BI) with low 16S divergence (0.4%). Our specimens of *Phrynoidis aspera* from Langkawi Island and Gombak formed a clade (BP = 92 for ML, ≥ 95 for BI, and average 16S divergence = 0.1%) with *Ph. aspera* from Java. *Ingerophrynus parvus* from the Gomabak population formed a single clade (BP = 100 for ML and ≥ 95 for BI) with low 16S divergence (0.4%).

4. Discussion

The members of the genus *Fejervarya* are diverse and widely distributed in Asia. Several researchers (Kosuch et al., 2001; Kurabayashi et al., 2005; Frost et al., 2006; Sumida et al., 2007; Kotaki et al., 2010; Hasan et al., 2012a) have argued that members of the genus *Fejervarya* could be separated into 2 distinct groups—Southeast and South Asian—based on molecular data. This view was further supported by researchers who clearly showed complete hybrid inviability between these 2 groups by crossing experiments (Sumida et al., 2007; Djong et al., 2007; Islam et al., 2008b). In this study, we found that the divergence value of these 2 groups is considerably high (16S diversity = 11.8%), which is in keeping with the previous studies mentioned above. Howlader (2011) recently proposed that South Asian members of *Fejervarya* can be treated as a new genus, *Zakerana*, based on a review of some morphological comparisons that we consider to be peripheral, insufficient, and incorrect. For example, the range of snout vent length (SVL) and the description on tibia length relative to SVL given by Howlader (2011) as evidence of a clear distinction between the 2 genera are erroneous. Moreover, our findings, along with those of other researchers (Kuramoto et al., 2007; Hasan et al., 2012a), found *Mi. sahyadris* nested in a clade composed of South Asian members of *Fejervarya*.

Ohler et al. (2009) claimed that the species of *Fejervarya* studied have tended to evolve small body size (SVL in mm),

based on the results of Kurabayashi et al. (2005). They also mentioned that Southeast Asian *Fejervarya* contains mid-size frog species, while South Asian *Fejervarya* contains both mid- and small-size frog species. The rectal gland is a character common in *Mi. sahyadris*, but absent in the South and South Asian members of *Fejervarya* (Dubois et al., 2001). The common origin of *Fejervarya* and *Minervarya* lineages of small size found in South Asia needs to be explored with a large number of data sets. In this study, we present novel molecular data on *Mi. sahyadris* and show the phylogenetic relationships with its congeners. Considering these facts, the *Fejervarya*–*Minevarya*–*Zakerana* complex needs to be examined further. If South Asian members of *Fejervarya* are treated as belonging to a different genus, the generic name *Minervarya* (Dubois et al., 2001), which has precedence over *Zakerana* (Howlader, 2011), should be used until a number of data sets, such as nuclear gene data, ecological characteristics, and postmating isolation mechanisms, have been collected and analyzed for all small size frogs from the Indian subcontinent.

In the present study, the member of the genus *Fejervarya* and *Microhyla* (sensu lato) clearly separated between 2 distinct clades corresponds to South Asia (Indian subcontinent) and Southeast Asia (Figure 2). Such a pattern of divergence in South Asia also apparently can be found in other vertebrates, i.e. mammals (Kurup, 1974), birds (Gaston and Zacharias, 1996), freshwater fish (Jayaram, 1974), and *Hemidactylus* geckos (Bansal and Karanth, 2010). Moyle et al. (2005) advocated that even comparatively well studied groups such as birds occasionally yield multiple well-differentiated lineages within a widespread species in Borneo. It is generally proposed that India separated from Gondwanaland ~ 195 Mya, and lastly collided with Asia in the Late Eocene. India could have acted as a dinghy, carrying taxa from Africa to Asia, which could spread over Southeast Asia and West Malesia after collision. During its rift, it came in connection with northward moving Sumatra, which means that previous exchange of components of fauna could have taken place. Probably, during the close contact between Sumatra and India, India became colonized by Southeast Asian elements of fauna (Turner et al., 2001). However, aridification of central India, i.e. formation of dry and wet zones, occurred between 10 and 1.6 Mya (Karanth 2003), and blocked the gene flow of West India and Southeast Asian areas (Alam et al., 2008).

Fouquet et al. (2007) reported that a 3% divergence of 16S matches the species threshold in a neotropic anuran taxon, and similar cases are found in other anurans (e.g., Vieites et al., 2009; Kotaki et al., 2010; Hasan et al., 2012a). Thus, we use this value to assign candidate species (16S data of targeted taxa were compared with those of the topotypic specimen) and possible candidate species (16S

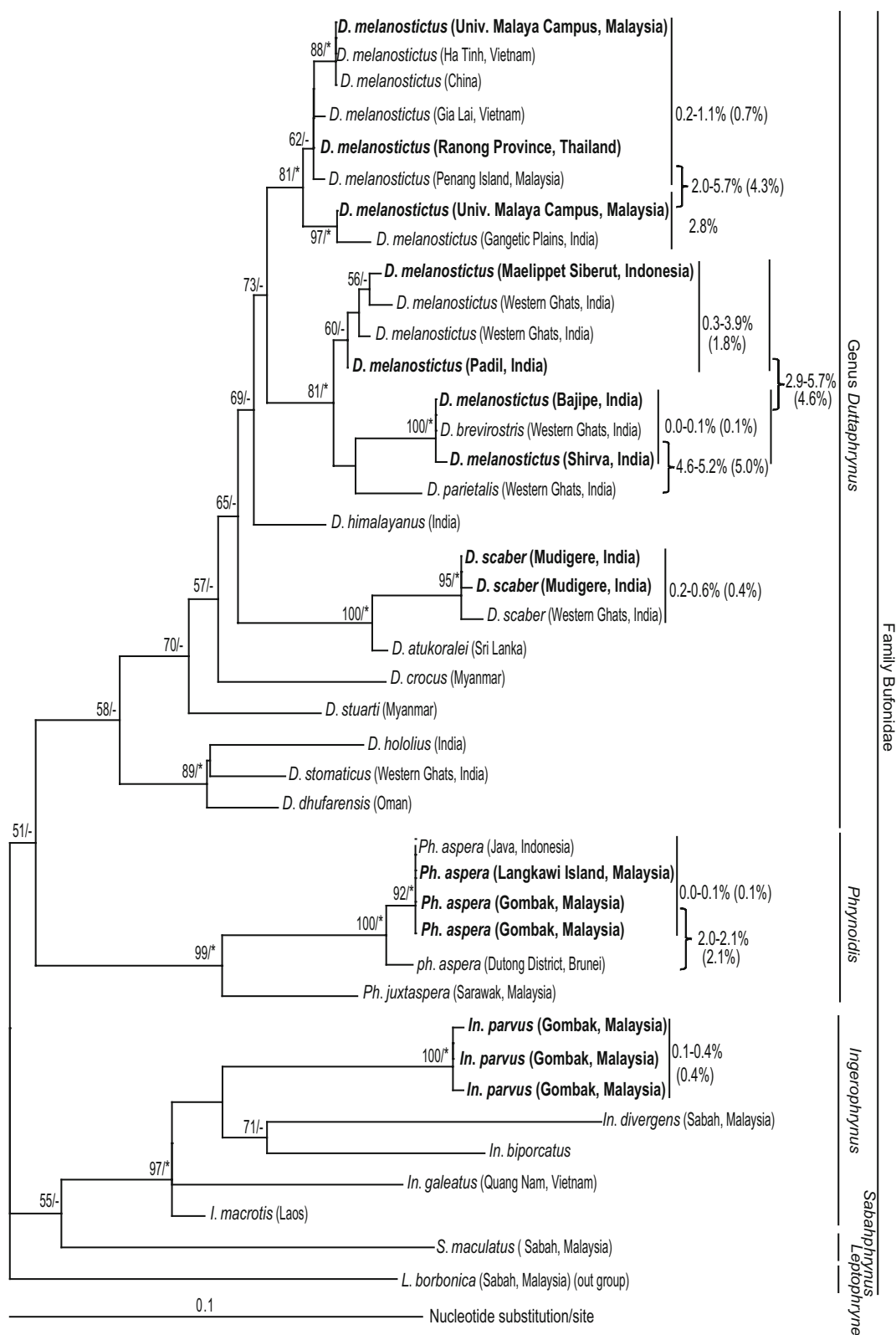


Figure 6. Maximum likelihood (ML) tree of bufonid frogs based on nucleotide sequences of the mitochondrial 16S rRNA gene with *Leptophryne borbonica* as an outgroup. The bootstrap support (>50%) is indicated at nodes in the order of ML (500) replicates. Asterisks represent Bayesian posterior probability (BPP; * $\geq 95\%$). Specimens examined in this study are indicated by boldface type.

data of targeted taxa were not compared with those of the topotypic specimen) in the following sections.

4.1. Taxonomic status of rhacophorid frogs

Our analyses revealed that the 16S sequence of *P. leucomystax* specimens from Japan and Malaysia are greatly different (4.2%) from that of the Thailand specimen. The type locality of *P. leucomystax* is Java, Indonesia. This species is considered to be distributed in eastern India and Nepal, Myanmar, southern China to the Philippines, Sumatra, and Borneo, and has been introduced to the southern Ryukyus, Japan (Frost, 2013). Our results suggest that the Okinawa population of *P. leucomystax* likely originated from southern Malaysia. This finding is congruent with Kuraishi et al. (2009), who noted that the Ryukyu population seems to have originated from somewhere around the Philippines, and Brown et al. (2010) mentioned that the southern Philippines, northern Borneo, and Peninsular Malaysia specimens fall into a single major haplotype clade, *P. leucomystax*. Although the specimens from Japan and Malaysia are very close to the topotypic specimen with respect of the 16S sequence (1.6%), substantial 16S divergence between the specimens from Thailand and elsewhere, including topotypic samples, indicates that the Thailand population represents a candidate species. Our *P. pseudocruciger* specimen from Bajipe diverged from another Western Ghats specimen (Meenakshi et al., 2009, Accession no. GU136109). The type locality of *P. pseudocruciger* is Kanyakumari District, Tamil Nadu State, southern India (Das and Ravichandran, 1998 [1997]), and so at present it is difficult to say which specimen is the definitive *P. pseudocruciger* (sensu stricto) or whether neither of them corresponds to a nominal species. Similarly, *R. malabaricus* showed high 16S divergence between the Madikeri specimen and one Western Ghats specimen, and so we could not assume which specimen corresponded to the definitive *R. malabaricus* (sensu stricto) without obtaining 16S data for a topotype sample from Malabar, India.

4.2. Taxonomic status of ranid frogs

The genus *Hylarana* currently comprises 84 nominal species, and 75 are distributed across Asia and northern Australia (Frost, 2013). Seven *Hylarana* taxa were examined in this study. The 16S sequence of *H. chalconota* from the Maelippet Siberut (Indonesia) population highly diverged from the type locality specimen (Java, Indonesia), indicating the involvement of one candidate species in our "*H. chalconota*" specimens. In contrast, *Hylarana* sp. from Langkawi Island was very close to the specimen from Peninsular Malaysia, which was treated as an "unnamed lineage" by Inger et al. (2009). The 16S divergence between our *Hylarana* sp. and this latter specimen was 0.8%, indicating a candidate species. In addition, the *H. cf. nicobariensis* specimen from Muara Siberut (Indonesia)

has a fair degree of divergence in 16S sequence from the Java specimen (topotype sample) of Indonesia, which suggests the presence of another candidate species in the examined *Hylarana* taxa. Similarly, the 16S divergence (5.8%) of *H. erythraea* specimens between the Langkawi Island/Univ. Malaya Campus (Malaysia) and Chantaburi (Thailand) populations is notably higher than the species threshold value (3%). The type locality of *H. erythraea* is both Java and Sumatra, Indonesia, and we could not obtain any 16S data of the topotypic specimen. Thus, we cannot presently determine which population is the definitive *H. erythraea* (sensu stricto) or whether any of these populations correspond to the nominal species. Our results may therefore indicate an occurrence of possible candidate species in the currently recognized *H. erythraea*. The exact type locality of *H. temporalis* is not clear but was probably described from specimens found in the mountains in south India, although the original type specimen was lost by Jerdon (Frost, 2013). We did not find any 16S data of the type specimen but compared our specimen with one Indian sample (unknown locality; Bossuyt et al., 2006, Accession no. DQ346963) and found that our *H. temporalis* specimen diverged (16S divergence = 11.6%) from it. Therefore, it is difficult at present to determine which specimens correspond to the definitive *H. temporalis* (sensu stricto) without obtaining the 16S data of the topotype specimen. In contrast, the type locality of *A. larutensis* is Perak, Malaysia, and we found that the Gombak specimen largely diverged (16S divergence = 3.8%) from the topotype specimen, which convinced us of the involvement of another candidate species in our examined *Hylarana* taxa.

4.3. Taxonomic status of nyctibatrachid frogs

We found that our "*Nyctibatrachus major*" specimens greatly diverged (0.1%–7.4%) in 16S sequence between populations, although their SVL values are very similar (46.9–48.6 mm). We did not find any corresponding 16S sequence with respect to our *Nyctibatrachus* specimens in the DDBJ, EMBL, and GenBank databases. However, very recently Biju et al. (2011) exclusively revised the taxonomy of the genus *Nyctibatrachus* and described 12 new species from the same sampling area used in our study (Western Ghats, India). Thus, our "*Nyctibatrachus major*" specimens may correspond to some of these new species.

4.4. Taxonomic status of microhylid frogs

The genus *Microhyla* consists of 31 nominal species that are widely distributed throughout South and Southeast Asia (Frost, 2013). Among these nominal microhylid species, 3 species (*M. heymonsi*, *M. fissipes*, and *M. okinavensis*) have been described from Taiwan and the Ryukyu Archipelago. Inger (1947) treated microhylid frogs from the entire Taiwan-Ryukyu region as a single species, *Microhyla ornata*, and later Dubois (1987) proposed *M. okinavensis* as

a valid name for the Ryukyu specimens. Matsui et al. (2005) mentioned the population from the Indian subcontinent as the definitive *M. ornata* (sensu stricto), with the Thailand, Laos, and China populations, previously recognized as “*M. ornata*”, corresponding to *M. fissipes*, and the populations from the Ryukyu Archipelago corresponding to *M. okinavensis*. In this study, we found that the *M. okinavensis* specimens from 2 Ryukyu Islands, Okinawa and Ishigaki, have highly diverged 16S sequences (7.1%). Furthermore, in our ML tree, *M. okinavensis* from Okinawa Island became a clade with *M. okinavensis* from Amami Island, Japan, as reported by Matsui et al. (2005), while the “*M. okinavensis*” specimen from Ishigaki Island became a sister taxon related to *M. mixtura* from Sichuan (16S diversity = 4.5%). Because the type locality of this species is Okinawa Island, the Ishigaki population is a candidate species. Matsui et al. (2011) mentioned that *M. okinavensis* from Amami Island is a sister species of *M. mixtura* with a fair 16S divergence (6.4%), which is congruent with the results of the present study. In addition, our *M. heymonsi* of the Univ. Malaya campus population diverged greatly in 16S sequence from the Chinese specimen, which is not far from the type locality. Therefore, it is our assumption that our *M. heymonsi* specimen from the Univ. Malaya Campus (Malaysia) population is a possible candidate species.

The Karnoor, Bajipe, and Talagini (India) populations of *M. ornata* were found to be monophyletic with a low 16S divergence (0.1%–1.6%). The *M. mymensinghensis* specimen from Bangladesh is closely related to the former Indian samples, but the 16S divergence is considerable (3.2%). The 16S sequence of “*M. ornata*” from Dharwad and Mudigere highly diverged from other Indian *M. ornata* and Bangladesh *M. mymensinghensis* specimens. The *M. ornata* specimens from Talapu (India) significantly diverged from the other Indian populations. Apparently, “*M. ornata*” from Talapu (India) is a different species with a distinct phylogenetic position from the other “*M. ornata*”. The 16S divergence between “*M. ornata*” from Talapu and *M. superciliaris* is notably high (11.3%). Consequently, in the examined Indian “*M. ornata*” taxa, there are 3 distinct species—one corresponding to the Karnoor/Bajipe/Talagini (India) populations, another to the Mudigere (India) population, and the last to the Talapu (India) population. The type locality of *M. ornata* is Côte Malabar, India (Duméril and Bibron, 1841), and our Indian specimens were taken from localities close to the type locality. Because Mudigere and Talapu are situated in the Western Ghats, and because Bajipe and Karnoor are situated in coastal lowlands, we suppose that the latter corresponds to the nominal species. All the same, the present results demonstrate the occurrence of at least 2 possible candidate species from our “*M. ornata*” specimens (Figure 5).

4.5. Taxonomic status of bufonid frogs

The genus *Duttaphrynus* consists of 30 nominal species distributed in temperate Eurasia and Africa. *Duttaphrynus melanostictus* is distributed widely in South and East Asia. The type locality of this species has not been perfectly traced but was described as ‘India Orientali’ (Dutta, 1997; Frost, 2013), and it is likely that more than one species is currently being referred to by this name (Dubois and Ohler, 1999). “*Duttaphrynus melanostictus*” in the present study was largely divided into 2 distinct groups: one consisting of specimens from the Univ. Malaya Campus (Malaysia) and Ranong Province (Thailand), and the other comprising the Maelippet Siberut (Indonesia), Padil, Bajipe, and Shirva (India) specimens (Figure 6). The first group is further split into 2 clades. One clade consists of the Univ. Malaya Campus (Malaysia), Hà Tĩnh, Gia Lai (Vietnam), China, Ranong Province (Thailand), and Penang Island (Malaysia) populations, while the other clade comprises the Univ. Malaya Campus (Malaysia) and Gangetic Plains (India) populations. The 16S divergence between these 2 clades is higher (4.3%) than the species threshold. Furthermore, specimens of both clades were collected from the same Malaysian locality (Univ. Malaya Campus). The sympatric distribution of these genetically diverged specimens clearly indicates the distinct species status of “*D. melanostictus*” from the Univ. Malaya Campus (Malaysia). The second “*D. melanostictus*” group, which consists of the Indonesian and Indian (Padil, Bajipe, and Shirva) specimens, is further divided into 2 clades. One clade comprises the Maelippet Siberut (Indonesia) and 3 distinct Indian populations (Western Ghats and Padil), while the other clade consists of samples from Bajipe and Shirva (India). The 16S divergence between these 2 clades is 4.6%, and the latter clade has a closer affinity with *D. brevirostris* (16S divergence = 0.1%) than with the other “*D. melanostictus*”. Usually, *D. brevirostris* specimens are smaller (SVL = 27 mm) than *D. melanostictus* specimens (SVL = 165 mm) (Chanda, 2002). Our specimens from Bajipe and Shirva were the same: SVL = 58 mm. The type locality of *D. brevirostris* is Hassan District of Karnataka State, India, and this species is distributed only in this particular area (Dutta, 1997; Frost, 2013). Due to the confusing taxonomic status of “*D. melanostictus*”, it is difficult to say whether our specimens from Bajipe and Shirva correspond to the definitive *D. brevirostris* (sensu stricto) or not. Further taxonomic study is necessary to resolve this problem. However, excluding the Bajipe and Shirva (India) populations, the present results indicate the presence of 3 possible distinct species in the “*D. melanostictus*” taxa recognized here. Of these 3 species, fairly diverged specimens from the Univ. Malaya Campus (Malaysia) correspond to 2 possible candidate species, whereas the Maelippet Siberut (Indonesia) and

Padil (India) populations need further taxonomic study to confirm whether this specimen corresponds to the definitive *D. melanostictus* (sensu stricto) or not.

In the present study, we showed at least 6 candidate and 6 possible candidate species from our specimens and that hidden anuran diversity remains in South and Southeast Asia. Although an integrative taxonomic approach (adding morphology and ecology data) will be necessary to determine the range of the “true” species and their taxonomic status, our information will contribute to future studies.

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- ### Appendix 1
- List of the 16S sequence data obtained from a public DNA database (GenBank).
- ### Appendix 2
- Haplotypes distribution maps of South and Southeast Asian frogs (candidate and/or possible candidate species): A) *P. leucomystax*, B) *H. erythraea*, *H. nicobarriensis*, *H. chalconota*, *H. sp.* and *A. larutensis*, C) *M. heymonsi*, *M. ornata* and *M. okinavensis*, and D) *D. melanostictus*.
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Appendix 1. List of the 16S sequence data obtained from public DNA database (GenBank).

Species	Voucher/Tissue Ref.	Locality	Accession no.	Source
<i>Pseudophilautus wynaadensis</i>	—	India	AF249059	Bossuyt and Milinkovitch, 2000
<i>Ps. pleurotaenia</i>	WHT 3176	Sri Lanka	FJ788165	Meegaskumbura et al., 2009
<i>Ps. lunatus</i>	WHT 3283	Sri Lanka	FJ788169	Meegaskumbura et al., 2009
<i>Ps. steineri</i>	WHT 3210	Sri Lanka	FJ788157	Meegaskumbura et al., 2009
<i>Ps. simba</i>	WHT 3221	Sri Lanka	FJ788167	Meegaskumbura et al., 2009
<i>Ps. popularis</i>	WHT 3191	Sri Lanka	FJ788168	Meegaskumbura et al., 2009
<i>Ps. femoralis</i>	WHT 2779	Sri Lanka	AY141833	Meegaskumbura et al., 2002
<i>Raorchestes gryllus</i>	ROM 30288	Vietnam: Pac Ban	GQ285674	Lie et al., 2009
<i>Ra. longchuanensis</i>	5Rao	China: Yunnan	GQ285675	Lie et al., 2009
<i>Ra. menglaensis</i>	060821286Rao	China: Yunnan	GQ285676	Lie et al., 2009
<i>Ra. signatus</i>	BNHS 4489	India: Western Ghats	EU450000	Biju and Bossuyt, 2009
<i>Ra. luteolus</i>	Paratype A9866	India: Western Ghats	AY753560	Gururaja et al., 2007

Appendix 1. (Continued).

Species	Voucher/Tissue Ref.	Locality	Accession no.	Source
<i>Ra. travancoricus</i>	BNHS 4557	India: Western Ghats	EU450029	Biju and Bossuyt, 2009
<i>Ra. nerostagone</i>	BNHS 4244	India: Western Ghats	EU450012	Biju and Bossuyt, 2009
<i>Ra. anili</i>	—	India: Western Ghats	EU450024	Biju and Bossuyt, 2009
<i>Ra. dubois</i>	BNHS5285	India: Western Ghats	EU449996	Biju and Bossuyt, 2009
<i>Ra. beddomii</i>	—	India: Western Ghats	EU449998	Biju and Bossuyt, 2009
<i>Ra. graminirupes</i>	BNHS 4266	India: Western Ghats	EU450015	Biju and Bossuyt, 2009
<i>Ra. glandulosus</i>	—	India: Western Ghats	EU450006	Biju and Bossuyt, 2009
<i>Ra. glandulosus</i>	—	India: Western Ghats	EU450020	Biju and Bossuyt, 2009
<i>Ra. bobingeri</i>	BNHS 4273	India: Western Ghats	EU450014	Biju and Bossuyt, 2009
<i>Polypedates leucomystax</i>	FMNH 255296	Laos	GQ204700	Meegaskumbura et al., 2010
<i>P. leucomystax</i>	POLE 13	Thailand: Sakaerat	HM359101	Sheridan et al., 2010
<i>P. leucomystax</i>	FMNH 253086	Vietnam	GQ204699	Meegaskumbura et al., 2010
<i>P. megacephalus</i>	SCUM 0607116L	China: Huidong	EU215550	Lie et al., 2008
<i>P. leucomystax</i>	ZRC1.1.5269	Indonesia: Java (Topotype)	GQ204693	Meegaskumbura et al., 2010
<i>P. leucomystax</i>	FMNH 253029	Vietnam	GQ204698	Meegaskumbura et al., 2010
<i>P. leucomystax</i>	FMNH 256451	Laos	GQ204701	Meegaskumbura et al., 2010
<i>P. pseudocruciger</i>	PM 3001	India: Western Ghats	GU136109	Meenakshi et al., 2009
<i>P. macrotis</i>	ELR 0180	Philippine: Tawi-tawi Island	HM770138	Brown et al., 2010
<i>P. maculatus</i>	WHTKANT	Sri Lanka	GQ204694	Meegaskumbura et al., 2010
<i>P. cruciger</i>	WHT 2640	Sri Lanka	GQ204687	Meegaskumbura et al., 2010
<i>P. colletti</i>	FMNH 242765	Malaysia	GQ204697	Meegaskumbura et al., 2010
<i>P. otitophus</i>	FMNH 239147	Malaysia	GQ204696	Meegaskumbura et al., 2010
<i>Rhacophorus monticola</i>	RMB 1236	Indonesia: Sulawesi Island	AY326060	Darst and Cannatella, 2004
<i>R. nigropunctatus</i>	SCUM 070657L	China: Weining, Guizhou	EU215533	Lie et al., 2008
<i>R. taronensis</i>	SCUM 060614L	China: Mt. Gaoligong, Yunnan	EU215537	Lie et al., 2008
<i>R. omeimontis</i>	SCUM 0606137L	China: Pengxian, Sichuan	EU215535	Lie et al., 2008
<i>R. moltrechti</i>	SCUM 061106L	China: Lianhuachi, Taiwan	EU215543	Lie et al., 2008
<i>R. schlegelii</i>	—	Japan: Hiroshima	AB202078	Sano et al., 2005
<i>R. feae</i>	SCUM 050642W	China: Hekou, Yunnan	EU215544	Lie et al., 2008
<i>R. chenfui</i>	FMNH 232964	China	GQ204712	Meegaskumbura et al., 2010
<i>R. gauni</i>	FMNH 235047	Malaysia	GQ204714	Meegaskumbura et al., 2010
<i>R. pardalis</i>	FMNH 231366	Malaysia	GQ204711	Meegaskumbura et al., 2010
<i>R. dulitensis</i>	FMNH 235741	Malaysia	GQ204715	Meegaskumbura et al., 2010
<i>R. malabaricus</i>	M 3003	India: Western Ghats	GU136112	Meenakshi et al., 2009
<i>R. orlovi</i>	AMNHA 161405	Vietnam, Ha Tinh	DQ283049	Frost et al., 2006
<i>R. calcaneus</i>	FMNH 256465	Laos	GQ204719	Meegaskumbura et al., 2010
<i>Rhacophorus</i> sp.	FMNH 255280	Laos	GQ204718	Meegaskumbura et al., 2010
<i>R. bipunctatus</i>	SN 030035	China: Hainan	EU215529	Lie et al., 2008
<i>R. bipunctatus</i>	FMNH 253114	Vietnam	GQ204716	Meegaskumbura et al., 2010
<i>R. reinwardtii</i>	ZRC 1.1.5273	Java	GQ204720	Meegaskumbura et al., 2010
<i>R. kio</i>	SCUM 37941C	China: Yunnan	EU215532	Lie et al., 2008
<i>R. anamensis</i>	FMNH 253934	Vietnam	GQ204717	Meegaskumbura et al., 2010

Appendix 1. (Continued).

Species	Voucher/Tissue Ref.	Locality	Accession no.	Source
<i>R. calcaeus</i>	AMNHA 163749	Vietnam: Quang Nam	DQ283380	Frost et al., 2006
<i>Buergeria buergeria</i>	—	Japan: Hiroshima	AB127977	Sano et al., 2004
<i>Odorrana chloronota</i>	AMNH A163935	Vietnam: Ha Giang	DQ283394	Frost et al., 2006
<i>Od. livida</i>	SCUM 050518CHX	China: Yunnan	EF453748	Cai et al., 2007
<i>Od. cf. livida</i>	FMNH 265922	Thailand: Loei Province	DQ650566	Stuart et al., 2006
<i>Od. tiannanensis</i>	SCUM 050510CHX	China: Yunnan	EF453751	Cai et al., 2007
<i>Od. hosii</i>	FMNH 268778	Thailand: Krabi Province	DQ650596	Stuart et al., 2004
<i>Od. morafkai</i>	FMNH 253837	Vietnam: Gia Lai Province	DQ650616	Stuart et al., 2006
<i>Od. versabilis</i>	HNNU-A0019L	China: Hainan	EF453752	Cai et al., 2007
<i>Od. tormota</i>	SCUM 052069	China: Anhui	EF453754	Cai et al., 2007
<i>Od. narina</i>	alive	Japan: Okinawa	AB511287	Kurabayashi et al., 2010
<i>Od. hejiangensis</i>	SCUM 0405180CJ	China: Sichuan	EF453747	Cai et al., 2007
<i>Od. schmackeri</i>	CIB-WU37990	China: Sichuan	EF453750	Cai et al., 2007
<i>Od. grahami</i>	SCUM 0405186CJ	China: Sichuan	EF453746	Cai et al., 2007
<i>Od. margaretae</i>	SCUM 045830HX	China: Sichuan	EF453749	Cai et al., 2007
<i>Od. andersonii</i>	KIZ-RD 02YNJD01	China: Yunnan	EF453745	Cai et al., 2007
<i>Od. ishikawae</i>	IABHU-5275	Japan: Kagoshima	AB511282	Kurabayashi et al., 2010
<i>Od. chapaensis</i>	AMNH A161439	Vietnam: Lai Chau	DQ283372	Frost et al., 2006
<i>Hylarana rufipes</i>	FMNH 268588	Indonesia: Sumatra	EF487449	Inger et al., 2009
<i>H. chalconota</i>	UTA 53665	Indonesia: Java	DQ650428	Stuart et al., 2006
<i>H. eschatia</i>	FMNH 268869	Thailand: Krabi Province	EF487483	Inger et al., 2009
<i>H. labialis</i>	FRIM 1123	Malaysia: Selangor	EF487520	Inger et al., 2009
<i>H. megalonesa</i>	FMNH 230956	Malaysia: Sabah	EF487456	Inger et al., 2009
<i>H. arfaki</i>	AMSR 114913	Papua New Guinea: nr. Haia	DQ283203	Frost et al., 2006
<i>H. leptoglossa</i>	IABHU 3897	Bangladesh: Mymensingh	AB530526	Hasan et al., 2012
<i>H. erythraea</i>	ROM 7296	Vietnam: Tram Lap	AF206475	Chen et al., 2005
<i>H. erythraea</i>	FMNH 257285	Cambodia: Siem Reap Province	DQ283138	Frost et al., 2006
<i>H. taipehensis</i>	ROM 7193	Vietnam: Tram Lap	AF206495	Chen et al., 2005
<i>H. nicobariensis</i>	TNHC 59856	Indonesia: Java	AY326062	Darst and Cannatella, 2004
<i>H. nigrovittata</i>	AMNHA 161280	Vietnam: Ha Tinh	DQ283371	Frost et al., 2006
<i>H. maosonensis</i>	AMNHA 161487	Vietnam: Vinh Phu	DQ283373	Frost et al., 2006
<i>H. milleti</i>	ROM 7240	Vietnam: Tram Lap	AF206490	Chen et al., 2005
<i>Glandirana rugosa</i>	—	Japan: Isehara	AB430340	Sekiya et al., 2010
<i>Amolops hongkongensis</i>	KUZ 30210	China: Hong Kong	AB211473	Matsui et al., 2006
<i>A. daiyunensis</i>	C93075	China: Fujian	AB211474	Matsui et al., 2006
<i>A. ricketti</i>	ROM 26365	Vietnam: Cao Bang Province	DQ204486	Ngo et al., 2006
<i>A. larutensis</i>	KUHE 23166	Thailand: Narathiwat	AB211485	Matsui et al., 2006
<i>A. larutensis</i>	KUHE15488	Malaysia: Perak	AB211484	Matsui et al., 2006
<i>A. chunganensis</i>	KUHE 27699	China: Sichuann province	AB211477	Matsui et al., 2006
<i>A. viridimaculatus</i>	C-green 05	China: Yunnan	AB211480	Matsui et al., 2006
<i>A. loloensis</i>	C18	China: Sichuan	AB211478	Matsui et al., 2006
<i>A. liangshanensis</i>	SCUM045807HX	China: Sichuan	EF453743	Cai et al., 2007

Appendix 1. (Continued).

Species	Voucher/Tissue Ref.	Locality	Accession no.	Source
<i>A. cremnobatus</i>	ROM 14528	Vietnam: Nghe An	DQ204477	Ngo et al., 2006
<i>Pelophylax nigromaculatus</i>	—	Japan: Hiroshima	AB043889	Sumida et al., 2001
<i>Microhyla heymonsi</i>	KUHEK1845	Thailand: Kanchanburi	AB201190	Matsui et al., 2005
<i>M. heymonsi</i>	AMNHA 163850	Vietnam: Ha Giang	DQ283382	Frost et al., 2006
<i>M. heymonsi</i>	Living animals	China	AY458596	Zhang et al., 2005
<i>M. okinavensis</i>	KUHE 12840	Japan: Amami Island	AB201184	Matsui et al., 2005
<i>M. fissipes</i>	KUHE 32943	China: Anhui	AB201185	Matsui et al., 2005
<i>M. mixtura</i>	CIB: 20070248	China: Sichuan	AB634669	Matsui et al., 2011b
<i>M. berdmorei</i>	MZB Amp 15270	Indonesia: Kalimantan	AB634661	Matsui et al., 2011b
<i>M. fowleri</i>	KUHE 21992	Thailand: Phrae	AB634667	Matsui et al., 2011b
<i>M. mymensinghensis</i>	IABHU F5012	Bangladesh: Mymensingh	AB530529	Hasan et al., 2012
<i>M. ornata</i>	ZSIK-A9119	India: Dharwad, Karnataka	AB201188	Matsui et al., 2005
<i>M. rubra</i>	released	India: Karnataka	AB201192	Matsui et al., 2005
<i>M. superciliaris</i>	KUHE 52558	Malaysia: Pahang	AB634682	Matsui et al., 2011b
<i>Kaloula pulchra</i>	KUHE 35171	Thailand: Kanchanaburi	AB201194	Matsui et al., 2005
<i>K. pulchra</i>	Living animals	China	AY458595	Zhang et al., 2005
<i>K. pulchra</i>	IABHU 3781	Bangladesh: Sylhet	AB530543	Hasan et al., 2012
<i>Dyscophus guineti</i>	RdS	—	DQ283434	Frost et al., 2006
<i>Duttaphrynus melanostictus</i>	AMNH A161135	Vietnam: Ha Tinh	DQ283333	Frost et al., 2006
<i>D. melanostictus</i>	Living animals	China	AY458592	Zhang et al., 2005
<i>D. melanostictus</i>	ROM 33163	Vietnam: Gia Lia Province	AF160793	Liu et al., 2000
<i>D. melanostictus</i>	KUHE 39029	Malaysia: Penang Island	AB435318	Matsui et al., 2010
<i>D. melanostictus</i>	—	India: Gangetic Plains	EU367009	Singh et al., 2006
<i>D. melanostictus</i>	BM 1011	India: Western Ghats	GU136100	Meenakshi et al., 2009
<i>D. melanostictus</i>	VUB 0052	India: Western Ghats	FJ882791	Vocxler et al., 2009
<i>D. brevivirostris</i>	SDB 4714	India: Western Ghats	FJ882786	Vocxler et al., 2009
<i>D. parietalis</i>	SDB 10100	India: Western Ghats	FJ882784	Vocxler et al., 2009
<i>D. himalayanus</i>	SDB 4566	India	FJ882790	Vocxler et al., 2009
<i>D. scaber</i>	SDB 532	India: Western Ghats	FJ882785	Vocxler et al., 2009
<i>D. atukoralei</i>	VUB 0101	Sri Lanka	FJ882835	Vocxler et al., 2009
<i>D. crocus</i>	CAS 220193	Myanmar	FJ882789	Vocxler et al., 2009
<i>D. stuarti</i>	CAS 221485	Myanmar	FJ882788	Vocxler et al., 2009
<i>D. hololius</i>	SDB 4240	India	FJ882781	Vocxler et al., 2009
<i>D. stomaticus</i>	SDB 4020	India: Western Ghats	FJ882787	Vocxler et al., 2009
<i>D. dhufarensis</i>	CAS 227584	Oman	FJ882837	Vocxler et al., 2009
<i>Phrynowidius aspera</i>	TNHC 53891	Indonesia: Java	AY680266	Pauly et al., 2004
<i>Ph. aspera</i>	FMNH 248147	Brunei: Dutong District	DQ283148	Frost et al., 2006
<i>Ph. juxtaspera</i>	KUHE 12363	Malaysia: Borneo, Sarawak	AB331713	Matsui et al., 2007
<i>Ingerophrynus divergens</i>	BORNEENSIS09169	Malaysia: Sabah	AB331715	Matsui et al., 2007
<i>In. biporcatus</i>	TNHC 61079	—	AY325987	Darst and Cannatela, 2004
<i>In. galeatus</i>	AMNH A163648	Viet Nam: Quang Nam	DQ283376	Frost et al., 2006
<i>In. macrotis</i>	FMNH 255318	Laos	DQ158468	Pramuk, 2006
<i>Sabahphrynus maculatus</i>	BORNEENSIS 08425	Malaysia: Sabah	AB331718	Matsui et al., 2010

Appendix 2.

