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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_CANCRI (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 accessions 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)
Euphlyctis cyanophlyctis	India	Madikeri	1	RBRL 030607-02	1(AB530596)
Euphlyctis aloysii		Adyar	1	BNHS 5124	1(AB530594)
Euphlyctis cf. *aloysii*		Mudigere	1	RBRL 050709-39	1(AB530595)
Euphlyctis mudigere		Mudigere	1	BNHS 5128	1(AB530599)
Euphlyctis hexadactylus		Adyar	2	RBRL 050719-01, 02	2(AB53059-98)
Hoplobatrachus tigerinus		Padil	1	Release	1(AB530600)
Hoplobatrachus rugulosus	Thailand	Ranong Province	1	NA	1(AB530609)
Fejervarya limnocharis	Malaysia	University Malaya Campus	1	IABHU 21015	1(AB530625)
Fejervarya rufescens	India	Bajipe	1	RBRL 030526-03	1(AB530601)
		Padil	1	RBRL 040716-1	1(AB530602)
Fejervarya kudremukhensis		Kudremukh	1	BNHS 4654	1(AB530603)
Minervarya sahyadris		Aralam	2	RBRL 050714-01, 02	2(AB530604-05)
Fejervarya caperata		Mudigere	1	BNHS 5060	1(AB530606)
Fejervarya mudduraja		Madikeri	1	BNHS 4646	1(AB530607)
Fejervarya multistriata	China	Sichuan Province	4	Alive, IABHU 3789	2(AB530611-12)
Fejervarya iskandari	Indonesia	Cianjur, Java	2	IABHU 3816, alive	1(AB530613)
Sphaerotheca dobsonii	India	Bajipe	3	RBRL 040706-13, 14, 060720-01	1(AB530608)
Occidozyga cf. *martensii*	Thailand	Ranong Province	1	Alive	1(AB530610)
Occidozyga cf. *lima*	Indonesia	Java	1	Alive	1(AB530619)
Limnonectes shompenorum		Muara Siberut	4	IABHU 20716-20719	1(AB530614)
Limnonectes kuhlii		Desa Munte	7	IABHU 20727- 20732	4(AB530615- 18)
Limnonectes blythii	Malaysia	Langkawi Island	4	IABHU 21101, 21103, 21108, 21118	3(AB530620-22)
Limnonectes laticeps		Gombak	1	IABHU 21020	1(AB530623)
Limnonectes plicatellus		Langkawi Island	1	IABHU 21120	1(1(AB530624))
Polypedates leucomystax	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)
Polypedates pseudocruciger	India	Bajipe	9	RBRL 070721-26, Release	3(AB530550-52)
Rhacophorus lateralis	India	Mudigere	3	RBRL 050709-35, 36, 37	1(AB530548)
Rhacophorus malabaricus		Madikeri	1	Release	1(AB530549)
Raorchestes luteolus		Madikeri	2	RBRL 060709-24, 25	1(AB530553)
		Kudremukh	1	BNHS 4192	1(AB530554)
Raorchestes cf. *glandulosus*		Talapu	2	RBRL 060709-26, 27	2(AB530555-56)
Raorchestes sp.		Talapu	1	RBRL 060709-28	1(AB530557)
Raorchestes cf. *bobingeri*		Aralam	1	RBRL 050714-09	1(AB530558)
Pseudophilautus wynaadensis		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)
Indirana cf. *semipalmata*	India	Shirva	1	RBRL 060715-02	1(AB530593)
Hylarana aurantiaca		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)
Hylarana temporalis		Talapu, Madikeri	4	RBRL 060709-01, 02, 03, Release	1(AB530578)
Hylarana malabarica		Bajipe	2	RBRL 060724-03, Release	1(AB530579)
Hylarana erythraea	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)
Hylarana cf. *nicobariensis*	Indonesia	Muara Siberut	5	IABHU 20707-20711	2(AB530581-82)
Hylarana chalconota		Maelippet Siberut	1	IABHU 20720	1(AB530583)
Hylarana sp.	Malaysia	Langkawi Island	1	IABHU 21104	1(AB530588)
Odorrana hosii		Gombak	3	IABHU 21004-21006	3(AB530589-91)
Amolops larutensis		Gombak	2	IABHU 21007, 21013	1(AB53057892)
Nyctibatrachus major	India	Kudremukh	1	Release	1(AB530571)
		Talapu	2	BNHS 5101, 5103	2(AB530572-73)
Micrixalus saxicola		Madikeri	3	RBRL 040716-37, 38, 39	1(AB530626)
Microhyla heymonsi	Malaysia	University Malaya Campus	2	IABHU 21025-21026	2(AB530636-37)
Microhyla ornata	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)
Microhyla berdmorei	Malaysia	Gombak	1	IABHU 21019	1(AB530638)
Microhyla okinavensis	Japan	Okinawa	1	IABHU 5128	1(AB530634)
		Ishigaki	1	IABHU 5263	1(AB530635)
Kaloula pulchra	Thailand	Ranong Province	1	NA	1(AB530633)
	Indonesia	Makassar, Sulawesi	1	NA	1(AB530639)
Duttaphrynus melanostictus	India	Bajipe	2	BNHS 5021, Release	1(AB530640)
		Padil	1	Release	1(AB530641)
		Shirva	1	Release	1(AB530642)
	Thailand	Ranong Proviance	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)
Duttaphrynus scaber	India	Mudigere	3	BNHS 5023-5025	2(AB530643-44)
Ingerophrynus parvus	Malaysia	Gombak	3	IABHU 21009- 21011	3(AB530649-51)
Phrynoidis aspera		Gombak	2	IABHU 21002-21002	1(AB530652)
		Gombak	1	IABHU 21013	1(AB530654)
		Langkawi Island	5	IABHU 21102, 21114-21117	1(AB530653)
Leptobrachium smithi		Langkawi Island	1	IABHU 21112	1(AB530655)
Xenophrys longipes		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 (–In*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 evoulationary 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

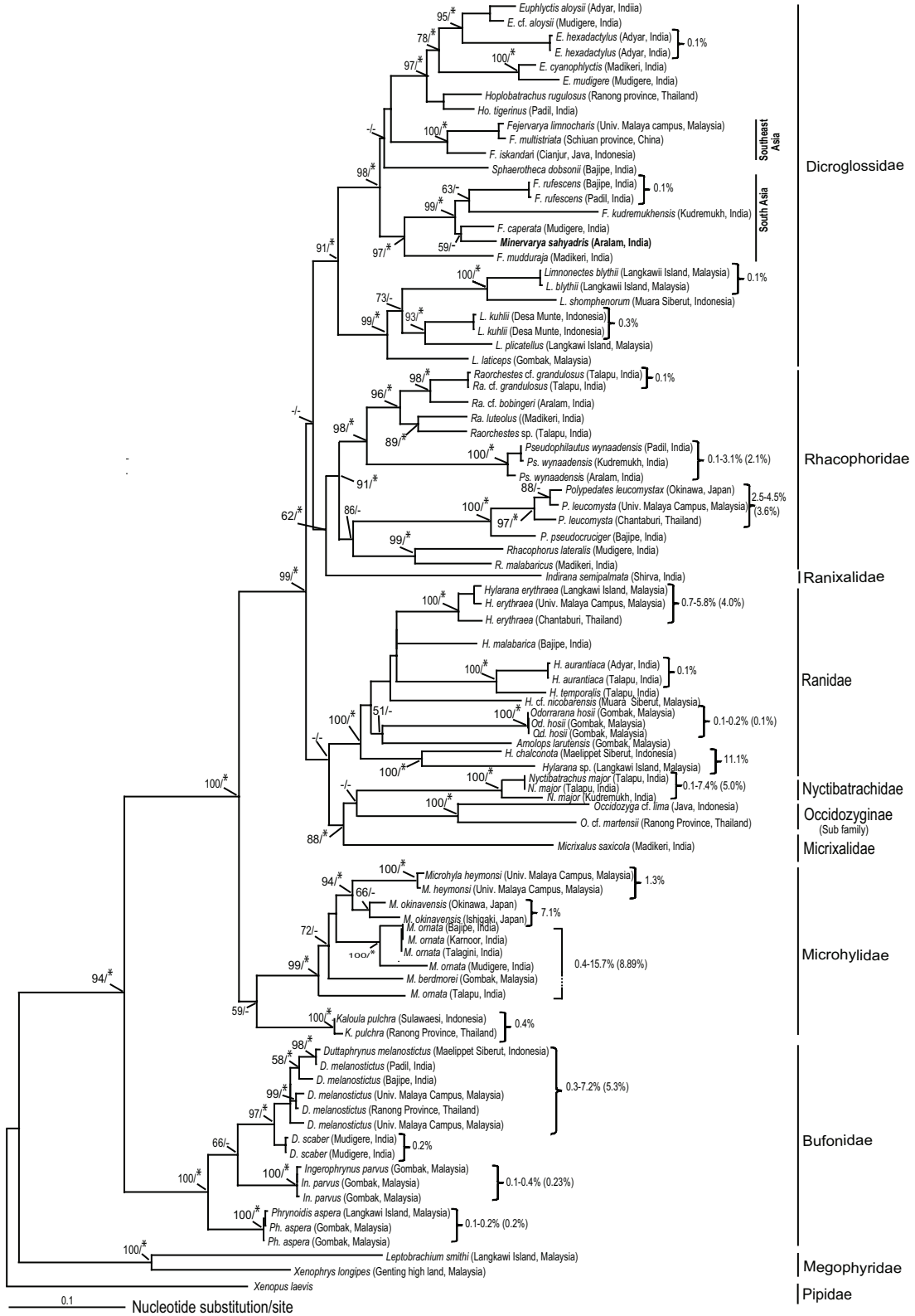

Figure 2. Maximum likelihood (ML) tree based on nucleotide sequences of the mitochondrial 16S rRNA gene from 88 haplotypes of frogs (Table 1), with *Xenopus laevis* as an outgroup. Bootstrap support (>50%) is indicated at nodes in the order of ML (1000) replicates. Asterisks represent Bayesian posterior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

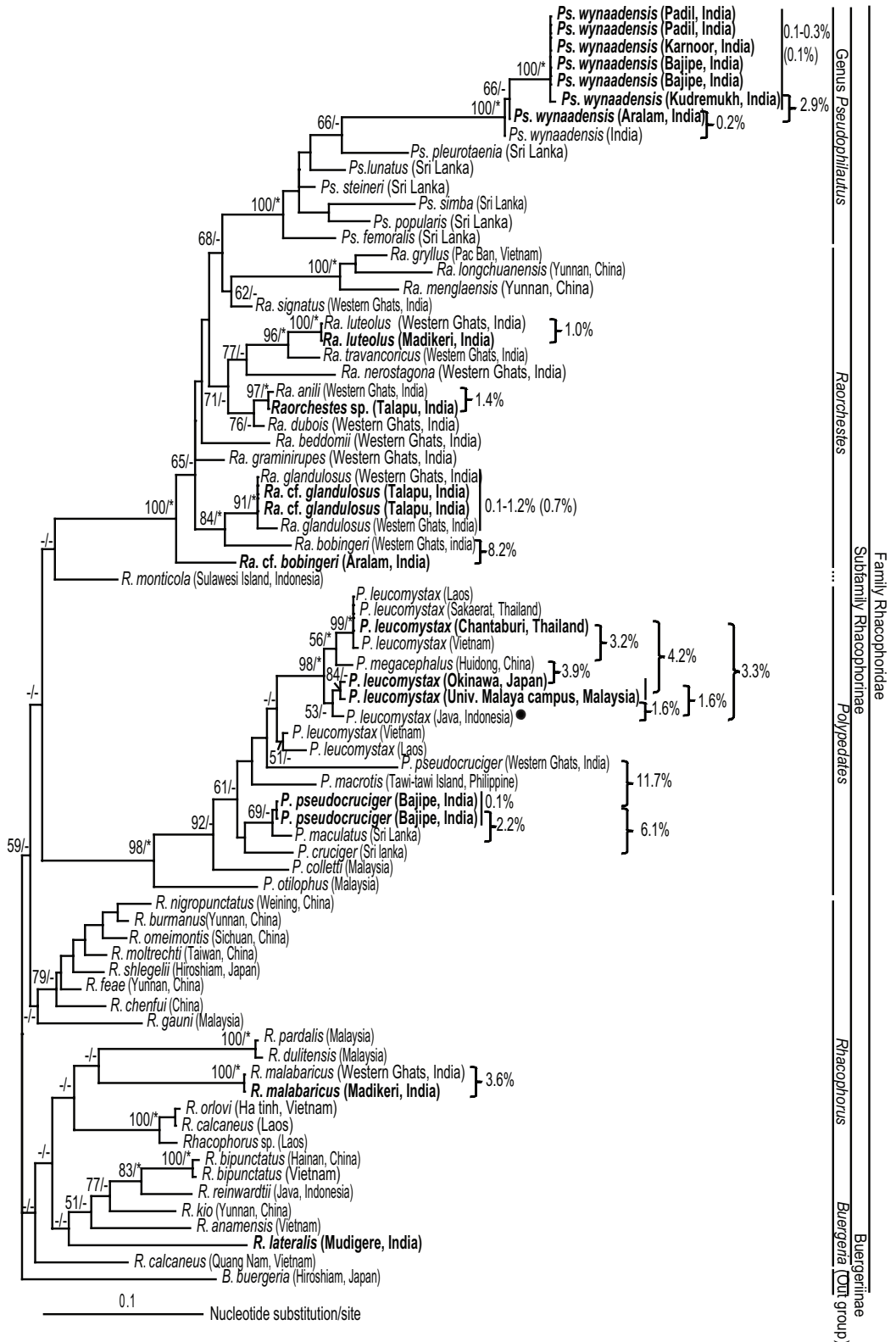

Figure 3. Maximum likelihood (ML) tree of rhacophorid frogs based on nucleotide sequences of the mitochondrial 16S rRNA gene with *Buergeria buergeria* as an outgroup. Bootstrap support (>50%) is indicated at nodes in the order of ML (500) replicates. Asterisks represent Bayesian posterior probability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

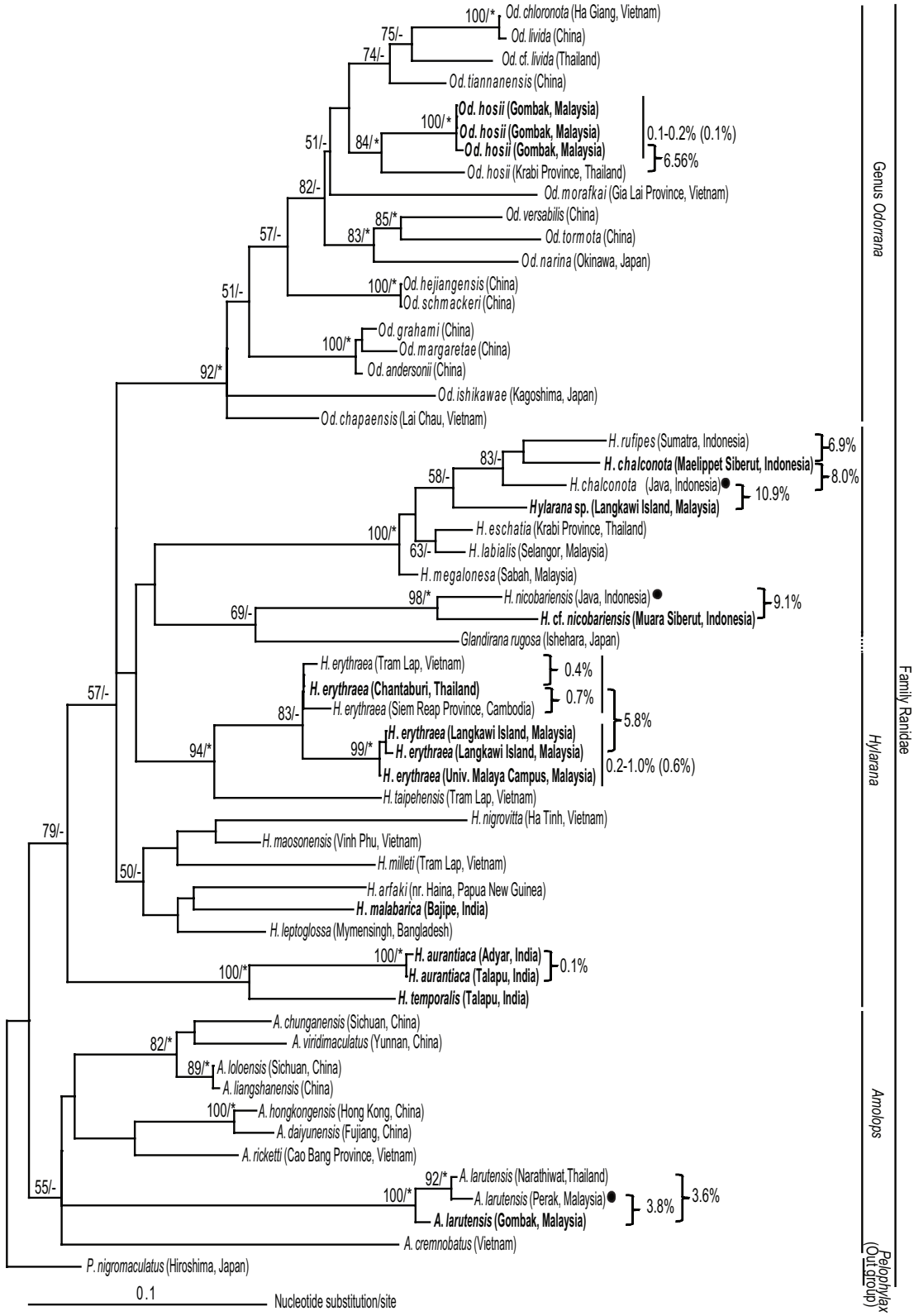

Figure 4. Maximum likelihood (ML) tree of ranid frogs based on nucleotide sequences of the mitochondrial 16S rRNA gene with *Pelophylax nigromaculatus* as an outgroup. The bootstrap support (>50%) is indicated at nodes in the order of ML (500) replicates. Asterisks represent Bayesian posterior probability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

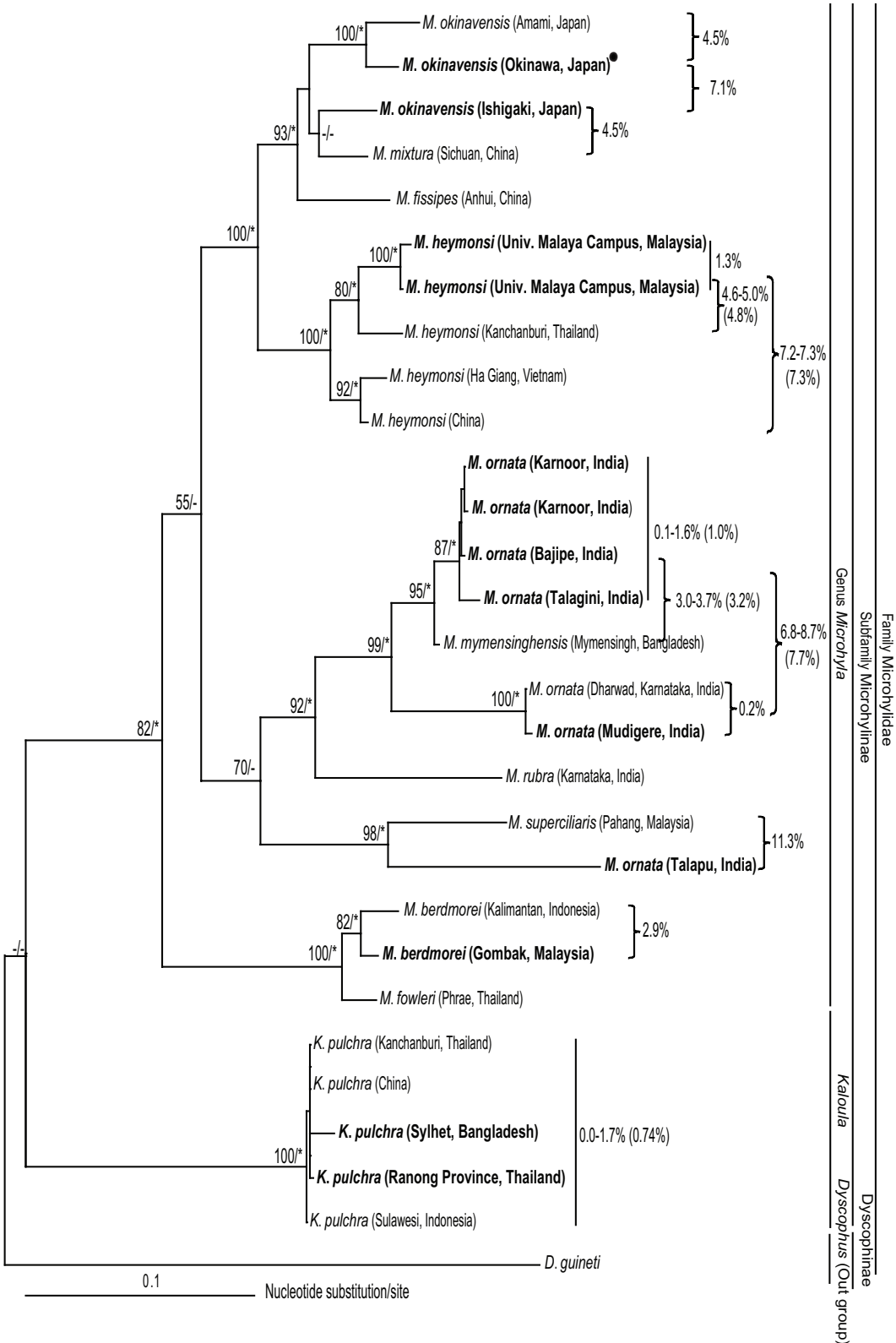

Figure 5. Maximum likelihood (ML) tree of microhylid frogs based on nucleotide sequences of the mitochondrial 16S rRNA gene with *Dyscophus guineti* as an outgroup. Bootstrap support (>50%) is indicated at nodes in the order of ML (500) replicates. Asterisks represent Bayesian posterior probability (BPP; * ≥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 rictal 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 *Fejervaya–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* (senso 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*

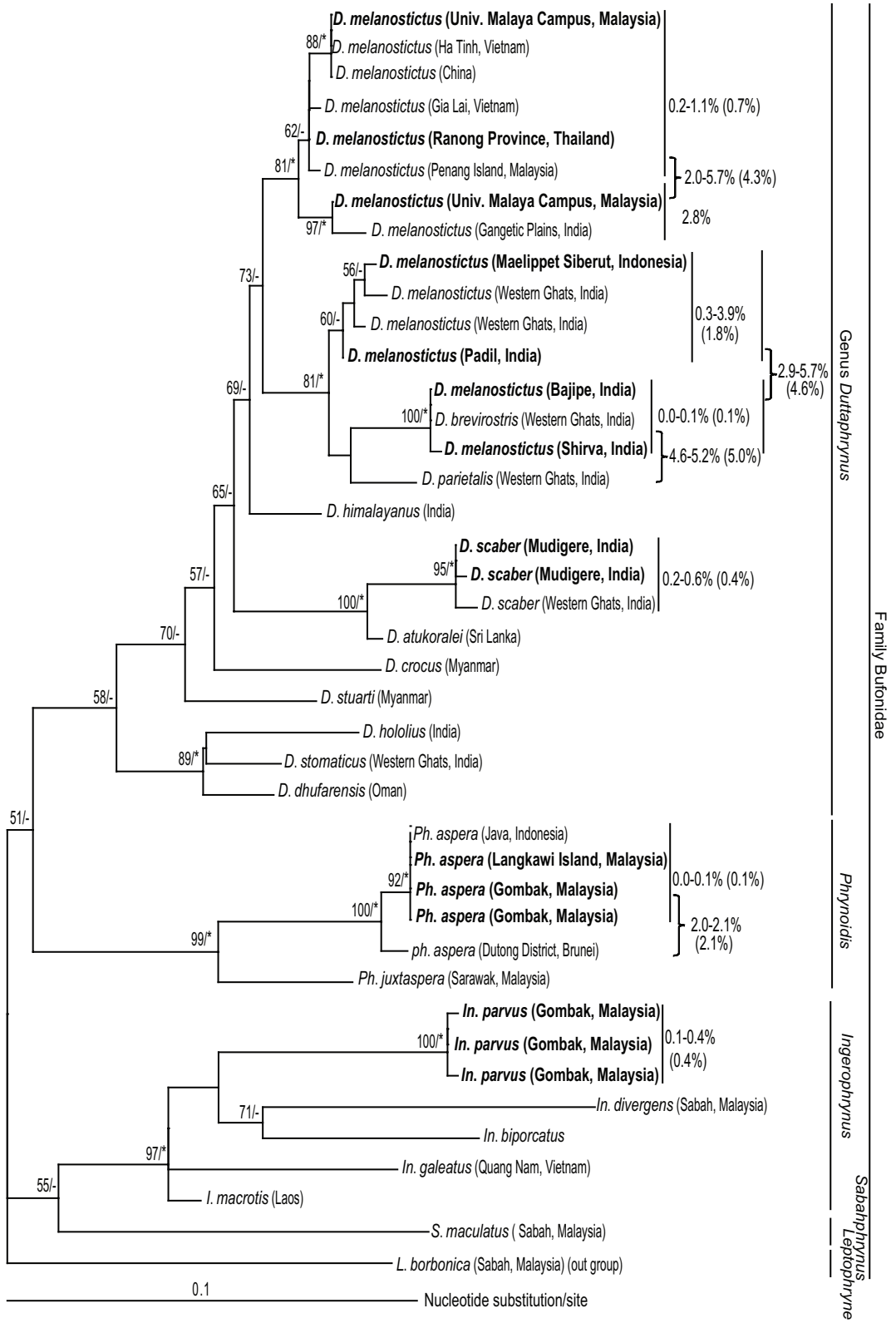

Figure 6. Maximum likelihood (ML) tree of bufonid frogs based on nucleotide sequences of the mitochondrial 16S rRNA gene with *Leptophryne borbonica* as an outgroup. The bootstrap support (>50%) is indicated at nodes in the order of ML (500) replicates. Asterisks represent Bayesian posterior probability (BPP; * ≥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. brevirostis* (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. lecucomystax*, 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
Pseudophilautus wynaadensis	—	India	AF249059	Bossuyt and Milinkovitch, 2000
Ps. pleurotaenia	WHT 3176	Sri Lanka	FJ788165	Meegaskumbura et al., 2009
Ps. lunatus	WHT 3283	Sri Lanka	FJ788169	Meegaskumbura et al., 2009
Ps. steineri	WHT 3210	Sri Lanka	FJ788157	Meegaskumbura et al., 2009
Ps. simba	WHT 3221	Sri Lanka	FJ788167	Meegaskumbura et al., 2009
Ps. popularis	WHT 3191	Sri Lanka	FJ788168	Meegaskumbura et al., 2009
Ps. femoralis	WHT 2779	Sri Lanka	AY141833	Meegaskumbura et al., 2002
Raorchestes gryllus	ROM 30288	Vietnam: Pac Ban	GQ285674	Lie et al., 2009
Ra. longchuanensis	5Rao	China: Yunnan	GQ285675	Lie et al., 2009
Ra. menglaensis	060821286Rao	China: Yunnan	GQ285676	Lie et al., 2009
Ra. signatus	BNHS 4489	India: Western Ghats	EU450000	Biju and Bossuyt, 2009
Ra. luteolus	Paratype A9866	India: Western Ghats	AY753560	Gururaja et al., 2007

Appendix 1. (Continued).

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

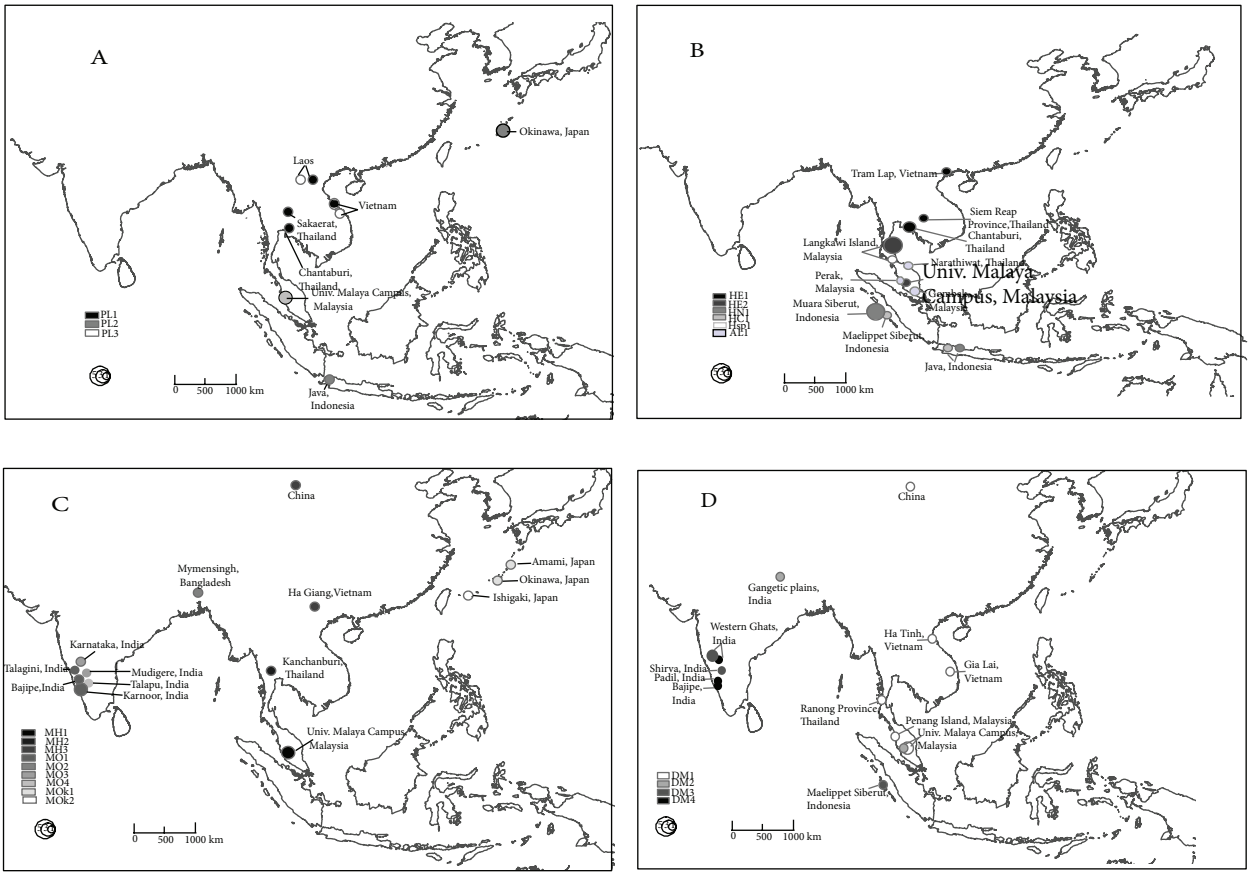
Appendix 1. (Continued).

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

Appendix 1. (Continued).

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

Appendix 2.


A
Okinawa, Japan
Laos
Vietnam
Sakaerat, Thailand
Chantaburi, Thailand
Univ.Malaya Campus, Malaysia
PL1
PL2
PL3
0 500 1000 km
Java, Indonesia
B
Tram Lap, Vietnam
Siem Reap Province,Thailand
Chantaburi, Thailand
Langkawi Island, Malaysia
Narathiwat, Thailand
Perak, Malaysia
Univ. Malaya Campus, Malaysia
Muara Siberut, Indonesia
Maelippet Siberut, Indonesia
Java, Indonesia
HE1
HE2
HN1
HC1
Hap1
AE1
0 500 1000 km
C
China
Amami, Japan
Okinawa, Japan
Ishigaki, Japan
Mymensingh, Bangladesh
Ha Giang,Vietnam
Karnataka, India
Talagini, India
Bajipe,India
Mudigere, India
Talapu, India
Karnoor, India
Kanchanburi, Thailand
Univ. Malaya Campus, Malaysia
MH1
MH2
MH3
MO1
MO2
MO3
MO4
MOk1
MOk2
0 500 1000 km
D
China
Gangetic plains, India
Western Ghats, India
Shirva, India
Padil, India
Bajipe, India
Ha Tinh, Vietnam
Gia Lai, Vietnam
Ranong Province, Thailand
Penang Island, Malaysia
Univ. Malaya Campus, Malaysia
Maelippet Siberut, Indonesia
DM1
DM2
DM3
DM4
0 500 1000 km