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Molecular Phylogeny of the Diversified Frogs of Genus *Fejervarya* (Anura: Dicroglossidae)

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Consensus on the taxonomic system and phylogenetic relationships for the anuran genus *Fejervarya* has yet to be established. Morphological characters in this genus are generally unsuitable for species identification. To carry out molecular species identification and solve phylogenetic problems, we collected 67 *Fejervarya* specimens from 12 Asian countries and sequenced part of the mitochondrial (mt) Cytb gene. We also sequenced the mt 12S and 16S rRNA genes and seven nuclear genes (*BDNF*, *CXCR4*, *NCX1*, *RAG-1*, *RAG-2*, *Rhod*, and *Tyr*) for 25 *Fejervarya* taxa. These molecular markers appear to be adequate for the identification of species. We subjected the molecular data molecular to phylogenetic analyses. In the resulting trees, topotypic *F. limnocharis* and "*F. multistriata*" (from China) formed a clade. On the other hand, neither "*F. limnocharis*" from the Japan mainland nor "*F. limnocharis*" from eastern Taiwan formed a clade with the real *F. limnocharis*, and the genetic divergences were larger than the species threshold for frog taxa proposed in previous studies (> 3% for *16S*). These results may suggest that "*F. multistriata*" is a junior synonym of *F. limnocharis*, or that only some of the populations now recognized as "*F. multistriata*" correspond to *F. limnocharis*. Our results also suggest that several cryptic species may be included among the widely distributed *Fejervarya* species. Finally, our datasets support paraphyly for the genus *Fejervarya*, although alternative phylogenetic topologies, including *Fejervarya* monophyly, were not rejected by KH and SH tests.

Key words: sequence divergence, molecular phylogeny, mitochondrial genes, nuclear genes, *Fejervarya*

INTRODUCTION

The anuran genus *Fejervarya* is widely distributed in Asia (Frost, 1985); 34 nominal *Fejervarya* species are currently known (Frost, 2009). Despite the many morphological and molecular studies conducted (e.g., Stuart et al., 2006; Djong et al., 2007a; Matsui et al., 2007), a consensus on the taxonomic system and phylogenetic relationships related to this genus is far from established. The open controversies have two principal causes. First, the monophyletic nature of this genus is problematic. Frost et al. (2006) showed the nested grouping of *Fejervarya* and several other genera (*Hoplobatrachus*, *Euphlyctis*, *Nannophrys*, and *Sphaerotheca*), and Kotaki et al. (2008) suggested paraphyly for this genus. Second, accurate identification of *Fejervarya* species is difficult for the following reasons. 1) About half of the 34 *Fejervarya* species were described in the 19th century and early 20th century (Frost, 2009), and access to type specimens is difficult. 2) In some cases, only poor morphological diagnoses are available. For some *Fejervarya* groups (e.g., the *Fejervarya limnocharis* complex), there are very few diagnostic morphological features. 3) Several cryptic species have been found from *Fejervarya* populations formerly recognized as single nominal species (Dubois, 1975; Toda et al., 1998; Veith et al., 2001; Sumida et al., 2007; Islam et al., 2008a, 2008b), and there is a chance that many undescribed cryptic species will be found in certain Asian areas, where detailed surveys of the herpetological fauna have yet to be performed (Kuramoto et al., 2007). Several other *Fejervarya* species besides *F. limnocharis* also seem to be confusingly named, and erroneous identifications have been occasionally found (see the Discussion). Some of the errors were discovered from ecological and morphological studies (e.g., Dubois, 1975; Matsui et al., 2007). In the

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majority of cases, however, cryptic species and erroneous identifications were initially recognized through molecular markers (Toda et al., 1998; Veith et al., 2001; Kurabayashi et al., 2005; Djong et al., 2007a; Kuramoto et al., 2007; Sumida et al., 2007; Islam et al., 2008a). Thus, phylogenetic analyses with more abundant molecular data, the accumulation of sequence data from nominal species, and species surveys with specimens from many localities would be good approaches to clear up these problems.

In this study, we collected 67 *Fejervarya* specimens from 12 Asian countries and surveyed genetic divergences among and within populations by determining partial mito-

Table 1. Accession numbers for nucleotide sequences of three mitochondrial and seven nuclear genes. NA, not available.

Species	Collecting station		No. of frogs	Accession Nos.										
	Country	Locality		Cyt b	12S rRNA	16S rRNA	BDNF	CXCR4	NCX1	3' RAG1	5' RAG1	RAG2	Rhodopsin	Tyrosinase
F. cancrivora	Malaysia	Selangor	1	AB488817	AB488859	AB488882	AB500232	AB500240	AB500246	AB500218	AB500226	NA	AB500255	AB500263
F. caperata	India	Mudigere	2	AB488843	AB488871	AB488894	AB489055	AB488912	AB488929	AB488946	AB488970	AB488990	AB489031	AB489010
F. granosa	India	Mudigere	2	AB488844	AB488872	AB488895	AB489056	AB488913	AB488930	AB488947	AB488971	AB488991	AB489032	AB489011
F. greenii	Sri Lanka	Hakgala	1	AB488838	AB488868	AB488891	AB489053	AB488910	AB488927	AB488944	AB488968	AB488988	AB489029	AB489008
F. iskandari	Indonesia	Java	1	AB488813	AB277287[a]	AB277303[a]	AB489045	AB277316[a]	AB277328[a]	AB488954	AB277342[a]	AB488981	AB489021	AB277355[a]
			1	AB488814	AB277287[a]	AB277303[a]	NA	NA	NA	NA	NA	NA	NA	NA
F. kirtisinghei	Sri Lanka	Hakgala	1	AB488836	AB488867	AB488890	AB489052	AB488909	AB488926	AB488943	AB488967	AB488987	AB489028	AB489007
			1	AB488837	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. kudremukhensis	India	Kudremukh	2	AB488849	AB488875	AB488898	AB489059	AB488916	AB488933	AB488950	AB488974	AB488994	AB489035	AB489014
F. limnocharis	Indonesia	Java	1	AB488811	AB277285[a]	AB277292[a]	AB489044	AB277315[a]	AB277327[a]	AB488953	AB277341[a]	AB488980	AB489020	AB277354[a]
			1	AB488812	AB277286[a]	AB277302[a]	NA	NA	NA	NA	NA	NA	NA	NA
F. limnocharis	Japan	Hiroshim	1	AB488832	AB488864	AB488887	AB489050	AB488907	AB488924	AB488941	AB488965	AB488986	AB489026	AB489005
F. limnocharis	Malaysia	Kuala Lumpur	1	AB488815	AB277275[a]	AB277301[a]	NA	NA	NA	NA	NA	NA	NA	NA
			1	AB488828	AB277275[a]	AB277301[a]	NA	NA	NA	NA	NA	NA	NA	NA
		Sabah	1	AB488815	AB277275[a]	AB277292[a]	NA	NA	NA	NA	NA	NA	NA	NA
F. limnocharis	Taiwan	Green Island	2	AB488829	AB488862	AB488885	NA	NA	NA	NA	NA	NA	NA	NA
		Orchard Island	2	AB488829	AB488862	AB488885	AB500233	AB500241	AB500247	AB500219	AB500227	NA	AB500256	AB500264
F. limnocharis	Thailand	Ranong	1	AB488816	AB277278[a]	AB277292[a]	NA	AB277307[a]	AB277321[a]	NA	AB277333[a]	NA	NA	AB277351[a]
		Tha Ton	1	AB488818	AB277275[a]	AB277292[a]	NA	AB277307[a]	AB277322[a]	NA	AB277334[a]	NA	NA	AB277348[a]
		Nakhon Si Thammarat	1	AB488819	AB277275[a]	AB277292[a]	NA	AB277307[a]	AB277321[a]	NA	AB277336[a]	NA	NA	AB277347[a]
F. cf. *limnocharis*	Cambodia		1	AB488818	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			1	AB488833	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. cf. *limnocharis*	Laos	Phongsaly	1	AB488818	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			2	AB488827	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. cf. *limnocharis*	Thailand	Chanta Buri	3	AB488818	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Phang Nga	1	AB488823	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			1	AB488824	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			1	AB488825	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. cf. *limnocharis*	Vietnam	Sapa	3	AB488826	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. mudduraja	India	Madikeri	1	AB488845	AB488873	AB488896	AB489057	AB488914	AB488931	AB488948	AB488972	AB488992	AB489033	AB489012
		Ooty	1	AB488846	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. multistriata	China	Hainan	1	AB488828	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Husa	1	AB488828	AB488861	AB488884	AB500234	AB500242	AB500248	AB500220	AB500228	AB500252	AB500257	AB500265
F. multistriata	Taiwan	Taipei	1	AB488828	AB488861	AB488884	AB500235	AB500243	AB500249	AB500221	AB500229	AB500253	AB500258	AB500266
F. orissaensis	India	Orissa	2	AB488842	AB277288[a]	AB277304[a]	AB500236	AB277317[a]	AB277329[a]	AB500222	AB277343[a]	NA	AB500259	AB277356[a]
F. pierrei	Nepal	Chitwan	2	AB488834	AB488865	AB488888	AB489051	AB488908	AB488925	AB488942	AB488966	AB490160	AB489027	AB489006
F. rufescens	India	Mangalore	1	AB488847	AB488874	AB488887	AB489058	AB488915	AB488932	AB488949	AB488973	AB488993	AB489034	AB489013
			1	AB488848	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. sakishimaensis	Japan	Iriomote Island	1	AB488831	AB488863	AB488886	AB489049	AB488906	AB488923	AB488940	AB488964	AB488985	AB489025	AB489004
		Ishigaki Island	1	AB488830	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. cf. *syhadrensis*	India	Karnool	1	AB488840	AB488870	AB488893	AB489054	AB488911	AB488928	AB488945	AB488969	AB488989	AB489030	AB489009
			1	AB488841	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. cf. *syhadrensis*	Sri Lanka	Bentota	1	AB488839	AB488869	AB488892	AB500237	AB500244	AB500250	AB500223	AB500230	NA	AB500260	AB500267
		Matale	1	AB488839	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. triora	Thailand	Ubon Ratchatani	2	AB488820	AB488860	AB488883	AB489046	AB488905	AB488922	AB488939	AB488963	AB488982	AB489022	AB489003
F. sp. hp2	Thailand	Bangkok	1	AB488821	AB277281[a]	AB277299[a]	NA	AB277307[a]	AB277321[a]	NA	AB277338[a]	NA	NA	AB277350[a]
		Mae Hong Son	1	AB488821	AB277282[a]	AB277299[a]	NA	AB277307[a]	AB277321[a]	NA	AB277335[a]	NA	NA	AB277349[a]
		Three Pagoda Pass	1	AB488821	AB277282[a]	AB277299[a]	AB500238	AB277308[a]	AB277323[a]	AB500224	AB277335[a]	AB500254	AB500261	AB277349[a]
F. sp. hp3	Thailand	Pilok	3	AB488822	AB277284[a]	AB277300[a]	AB489048	AB277312[a]	AB277325[a]	AB488956	AB277340[a]	AB488984	AB489024	AB277352[a]
F. sp. hp4	Nepal	Chitwan	1	AB488835	AB488866	AB488889	AB500239	AB500245	AB500251	AB500225	AB500231	NA	AB500262	AB500268
F. sp. hp5	India	Assam	1	AB488852	AB488877	AB488900	AB489061	AB488918	AB488935	AB488952	AB488976	AB488996	AB489037	AB489016
F. sp. hp6	India	Andaman Island	1	AB488850	AB488876	AB488899	AB489060	AB488917	AB488934	AB488951	AB488975	AB488995	AB489036	AB489015
			1	AB488851	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
E. cyanophlyctis	India	Mangalore	1	AB488853	AB488878	AB488901	AB489062	AB488919	AB488936	AB488957	AB488977	AB488997	AB489038	AB489017
H. tigerinus	India	Mangalore	1	AB488854	AB488879	AB488902	AB489063	AB277319[a]	AB277331[a]	AB488958	AB277345[a]	AB488998	AB489039	AB277358[a]
L. laticeps	Malaysia	Kuala Lumpur	1	AB488856	AB277291[a]	AB277306[a]	AB489065	AB277320[a]	AB277332[a]	AB488960	AB277346[a]	AB489000	AB489041	AB277359[a]
S. dobsoni	India	Bajipe	1	AB488855	AB277290[a]	AB277305[a]	AB489064	AB277318[a]	AB277330[a]	AB488959	AB277344[a]	AB488999	AB489040	AB277357[a]
O. lima	Malaysia	Kuala Lumpur	1	AB488857	AB488880	AB488903	AB489066	AB488920	AB488937	AB488961	AB488978	AB489001	AB489042	AB489018
O. sp.	Malaysia	Kuala Lumpur	1	AB488858	AB488881	AB488904	AB489067	AB488921	AB488938	AB488962	AB488979	AB489002	AB489043	AB489019
Total			73											

[a]Kotaki et al. (2008)

chondrial (mt) cytochrome b gene (*Cytb)* sequences from the samples. *Cytb*, with relatively fast nucleotide substitution rates, has been used as a population- or genus-level molecular marker in dicroglossid frogs, including several *Fejervarya* taxa (e.g., Dojong et al., 2007b; Alam et al., 2008; Islam et al., 2008b). We also determined nucleotide sequences for parts of mt 12S and 16S ribosomal RNA genes (*12S* and *16S*) from 25 *Fejervarya* representatives consisting of 15 nominal and 10 unidentified species, for use in molecular species identification and species-genus level molecular phylogenetic analyses. Although the mt COX1 gene is generally used as a "species tag" in DNA barcoding (Hebert et al., 2003a, b; Hebert et al., 2004a, b), Vences et al. (2005a, b) suggested that this gene has several basic problems as a species tag for amphibians. Alternatively, Vences et al. (2005a, b) considered *16S* to be a suitable molecular tag for amphibian species, and previous research has demonstrated the utility of this gene in phylogenetic analyses and molecular species identification (Bossuyt et al., 2006; Fouquet et al., 2007; Alam et al., 2008; Vieites et al., 2009). Finally, adding to the mt genes, we sequenced from the 25 *Fejervarya* taxa seven additional nuclear genes having relatively slow nucleotide substitution rates (e.g., Hoegg et al., 2004), and tried to elucidate higher level (intra- and intergeneric) relationships for the genus *Fejervarya*.

MATERIAL AND METHODS

Specimens

Sixty-seven *Fejervarya* specimens from 40 localities in 12 countries were used in this study (Fig. 1). Among them, 37 specimens were identified to species (although our results suggested that some specimens may have been cryptic species or incorrectly identified; see the sections below), and the other 30 specimens could not be identified due to either the small size of the tissue samples or morphological characteristics that failed to match other nominal species (Table 1). We also included six dicroglossid species from closely related genera, i.e., *Euphlyctis cyanophlyctis*, *Hoplobatrachus tigerinus*, *Sphaerotheca dobsoni*, *Limnonectes laticeps*, *Occidozyga lima*, and *Occidozyga* sp.

PCR and sequencing

Total genomic DNA for PCR was extracted from muscle tissues by using a DNA extraction kit (DNeasy Tissue Kit, QIAGEN) according to the manufacturer's protocol. The amplification primers we used are listed in Table 2. First, part of the *Cytb* (approximately 650 bp) was amplified and directly sequenced from all 68 *Fejervarya* specimens and the six species in other genera. PCR mixtures were prepared with an Ex-Taq Kit (TaKaRa) according to the manufacturer's protocol. DNA sequencing was performed with an automated sequencer (ABI 3100, ABI). Next, we amplified and sequenced parts of the 12S and 16S rRNA genes (approx. 400 and 600 bp long, respectively) and seven nuclear genes: brain-derived neurotrophic factor (*BDNF*, approx. 700 bp), chemokine receptor 4 (*CXCR4*, approx. 600 bp), Na^+/Ca^{2+} exchanger (*NCX1*, approx. 800 bp), tyrosinase (*Tyr*, approx. 700 bp), rhodopsin (*Rhod*, approx. 400bp), recombination activating gene 2 (*RAG-2*, approx. 1.2 kbp), and 5' and 3' partitions of recombination activating gene 1 (5' and 3' *RAG-1*, approx. 1 kbp and 900 bp) from 25 *Fejervarya* representatives (including 23 specimens with *Cytb* haplotypes and two *F. multistriata* specimens) and six other dicroglossids (Table 1). The procedures for amplifying and sequencing these genes were the same as for *Cytb*. *RAG-2* could not be amplified from *F. cancrivora*, "*F. limnocharis*" from Orchard Island, *F. orissaensis*, *F.* cf. *syhadrensis* from Sri Lanka, and *F.* sp. hp4. The nucleotide sequences determined in this study were deposited in the nucleotide sequence database (accession nos. AB488811–AB489067, AB490160, and AB500218–AB500268) (Table 1). Failing to amplify *RAG-2* from *F. cancrivora*, "*F. limnocharis*" from Orchard Island, *F. orissaensis*, *F.* cf. *syhadrensis* from Sri Lanka, and *F.* sp. hp4, we treated these unamplified regions as missing data in the following phylogenetic analyses.

Phylogenetic analyses

The resultant *Cytb* sequences from the 67 *Fejervarya* and six other discoglossids were aligned by using ClustalW (Thompson et al., 1994). The resultant alignment matrix contained 535 nucleotide sites. Based on the alignment data, a NJ tree was reconstructed with PAUP4.10b (Swofford, 2002), using the GTR + G + I substitution

Table 2. Primers used in this study for PCR amplification.

Gene	Primer name	Sequence (5'-3')	Source
Cyt b	Cyt b_150Fow	ACMGGHYTMTTYYTRGCHATRCAYTA	Kurabayashi and Sumida (2009)
	Cyt b Rev1	CCNGARTGRTAYTTTYCTWTTYGCHTA	This study
	Cyt b Rev2	TTYGCNTAYGCHATYCTNCGMTC	This study
12S rRNA	FS01	AACGCTAAGATGAACCCTAAAAAGTTCT	Sumida et al. (1998)
	R16M1	GGGTATCTAATCCCAGTTTG	Sumida et al. (1998)
16S rRNA	F51	CCCGCCTGTTTACCAAAAACAT	Sumida et al. (2002)
	R51	GGTCTGAACTCAGATCACGTA	Sumida et al. (2002)
CXCR4	CXCR4-Fow1	GTNATGGGCTAYCARAARAA	Kotaki et al. (2008)
	CXCR4-Fow2	ATGACWACAAATACAGRYTGCAYCTNTC	Kotaki et al. (2008)
	CXCR4-Rev1	TTGAAYTTGGCNCCSAGGAARGCRTA	Kotaki et al. (2008)
	CXCR4-Rev2	TAATAAGGMARCCARCAGGYRAARAA	Kotaki et al. (2008)
NCX1	NCX1-Fow1	GARAAGGARATAACNATYAARAARCC	Kotaki et al. (2008)
	NCX1-Fow2	ATTGAAGTKTGTGGCCAYAAYTT	Kotaki et al. (2008)
	NCX1-Rev1	TTTTCATCTTCYTCAAADATRTCRTC	Kotaki et al. (2008)
	NCX1-Rev2	TCCTTCTGKGTCTCACCWGGYTTRAA	Kotaki et al. (2008)
RAG-1	RAG1_Ex1_Fow1	AAATWCTCRGAMTGGAAGTTYAARCT	Kotaki et al. (2008)
	RAG1_Ex1_Rev1	TCACCWYCTTCTTCYTTBTCDGCRAA	Kotaki et al. (2008)
	RAG1_Ex1_Fow2	AACAARGGTGGYMGRCCYCGRCAGCAYCT	This study
	RAG1F	AGCTGCAGYCARTACCAYAARATGTA	This study
	RAG1_R_mod	AARCACCACTGGCTSTAYACATCCAA	This study
RAG-2	RAG2-Fow1	TTWGGNCARAARGGNTGGCC	This study
	RAG2-Rev2	GGNCAYTGGGTNCATKCNCARTGCATGGA	This study
Tyr	Tyr 1A	AGGTCCTCTTRAGCAAGGAATG	Bossuyt and Milinkovitch (2000)
	Tyr 1E	GAGAAGAAAGAWGCTGGGCTGAG	Bossuyt and Milinkovitch (2000)
Rhod	Rhod 1A	ACCATGAACGGAACAGAAGGYCC	Bossuyt and Milinkovitch (2000)
	Rhod 1C	CCAAGGGTAGCGAAGAARCCTTC	Bossuyt and Milinkovitch (2000)
BDNF	BDNF-Fow1	ATGACCATCCTTTTCSTKACNATG	This study
	BDNF-Rev1	ACNATHAARAGGGGMAGATAG	This study

model suggested by the Akaike information criterion (AIC) implemented in MODELTEST ver. 3.06 (Posada and Crandall, 1998). *Hoplobatrachus tigerinus* was used as the outgroup in this analysis.

Alignment data were also prepared for two additional mt genes and eight partitions of seven nuclear genes for the 25 *Fejervarya* representatives with clearly distinct haplotypes and the six other dicroglossids. For these genes, the alignments were revised by using GBlock 0.91b (Castresana, 2000) with the default settings to exclude gaps and ambiguous sites. One concatenated alignment for the three mt and seven nuclear genes (total 6364 bp) was prepared. Based on the concatenated data, phylogenetic analyses were performed by maximum-likelihood (ML) and maximum-parsimony (MP) with PAUP 4.10b (Swofford, 2002). In addition, Bayesian inference (BI) analyses were performed with MrBayes ver. 3.0b4 (Huelsenbeck and Ronquist, 2001). The partition homology test (Farris et al. 1995) rejected the concordance of nucleotide substitution patterns among three mt and seven nuclear genes. Therefore, the data set was treated as different partitions in the BI analyses. The analyses were performed by setting the number of Markov chain Monte Carlo (MCMC) generations at two million, setting the sampling frequency as 10, and discarding the first 200,000 generations. For the ML and BI analyses, best-fit substitution models were chosen by AIC as follows: GTR + I + G for the concatenated nuclear genes data (in ML); HKY for the *Rhod* partition; GTR for the *Cytb*, *16S*, and 3' *RAG-1* partitions; SYM for the *Tyr* partition; TIM for the 5' *RAG-1* partition; and TrN for the *12S rRNA*, *BDNF*, *CXCR4*, *NCX1*, and *RAG-2* partitions (in BI). Two *Occidozyga* species were used as the outgroups in these analyses. The reliabilities of the resultant phylogenetic trees were evaluated with the bootstrap proportion (BP). Bootstrap values were calculated by analysis of 300 and 1000 pseudoreplicates in the ML and MP analyses, respectively. Statistical support for the resultant BI trees was determined with Bayesian posterior probability (BPP). Topologies of resultant trees and several alternative hypotheses were compared by resampling the sitewise log-likelihood (RELL), i.e., the Kishino-Hasegawa (KH; Kishino and Hasegawa, 1989) and Shimodaira-Hasegawa (SH; Shimodaira and Hasegawa, 1999) tests, using PAUP. RELL was conducted with 10,000 resamplings.

RESULTS AND DISCUSSION

Cytb haplotypes and phylogeny of *Fejervarya* species based on mt gene data

The nucleotide sequence of part of the *Cytb* gene (approx. 650 bp) was determined for the 67 *Fejervarya* specimens. Twenty-three major haplotypes were observed among the resultant sequences. The NJ tree based on the *Cytb* data recovered 23 clades of these major haplotypes (Clades 1–23 in Fig. 2). As in previous studies, the clades were divided largely into South- and Southeast-Asian groups (Kurabayashi et al., 2005; Sumida et al., 2007; Kotaki et al., 2008). Among the 23 major haplotype groups, 14 clearly corresponded to nominal *Fejervarya* species (i.e., *cancrivora*, *caperata*, *granosa*, *greenii*, *iskandari*, *kirtisinghei*, *kudremukhensis*, *limnocharis*, *mudduraja*, *orissaensis*, *pierreri*, *rufescence*, *sakishimaensis*, and *triora*). Five haplotypes from unidentified individuals (*F.* sp. hp2 from Thailand, *F.* sp. hp3 from Pilok in Thailand, *F.* sp. hp4 from Nepal, *F.* sp. hp5 from India, and *F.* sp. hp6 from the Andaman Islands) had no affinity to the haplotypes of the nominal species (Fig. 2) and no corresponding sequences in DNA databases (data not shown). The specimens of "*F.* cf. *syhadrensis*" showed two distinct haplotypes (Sri Lanka and Western Ghats, India). While both of the *F.* cf. *syhadrensis* haplotypes belonged to the South-Asian group, the Sri Lankan group had a close affinity to the *F. granosa* clade, and the Indian group was a sister group to the *F. greenii* + *F. kirtisinghei* clade. The *F. multistriata* specimens (from China and Taiwan) were included in the *F. limnocharis* clade (Clade 18 in Fig. 2). In this analysis, we also included 14 other unidentified *Fejervarya* samples (two from Cambodia, three from Laos, six from Thailand, and three from Vietnam). The haplotypes from these samples were very similar to those of *F. limnocharis*, and they were embedded within the *F. limnocharis* clade in the NJ tree (Clade 18). Nucleotide sequence divergences for *Cytb* within the *F. limnocharis* clade (including *F. multistriata* haplo-

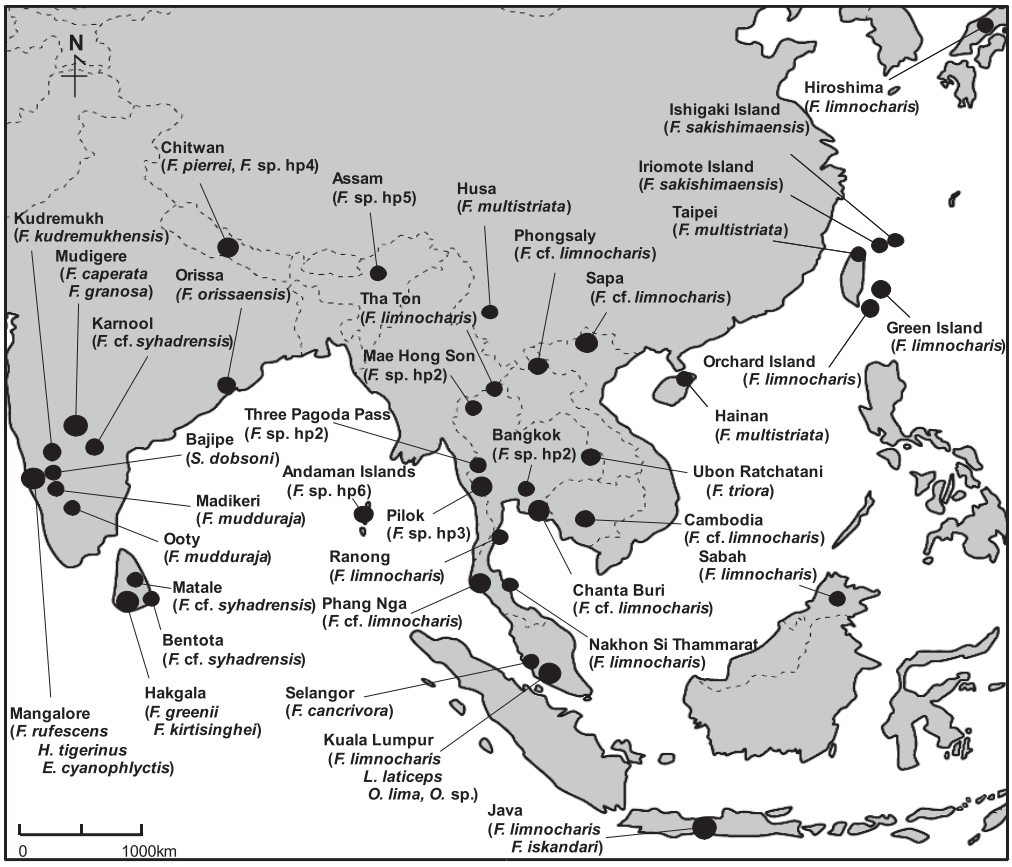

Fig. 1. Map showing collecting localities for frogs included in this study. The size of the circles is proportional to the number of individuals collected at a locality.

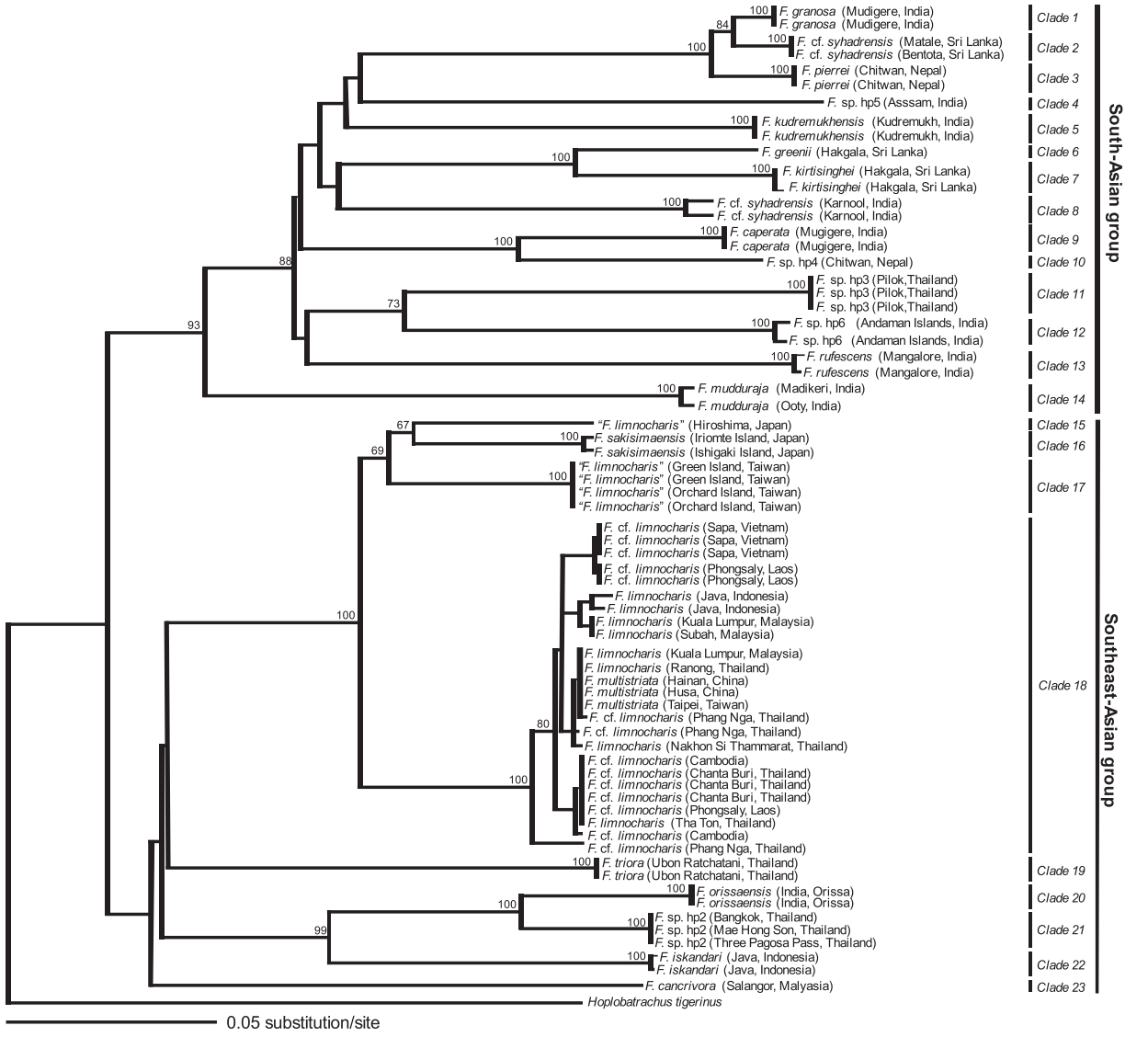

Fig. 2. Neighbor-Joining tree based on 532 bp of the mitochondrial Cytb gene sequenced from 67 frogs. The tree was reconstructed by using PAUP with the heuristic search option and the GTR + I (= 0.50) + G (= 1.41) substitution model, suggested by Modeltest. NJ Bootstrap values are shown near nodes.

types) were very low (1.0%), and this clade included the *F. limnocharis* specimens from the type locality (Java, Indonesia). On the other hand, the specimens from Japan (Hiroshima) and Taiwan (Green and Orchard Islands), traditionally regarded as *F. limnocharis*, comprised clades distinct from the *F. limnocharis* clade (Clades 15 and 17, respectively).

Based on the results of the *Cytb* haplotype analysis, we selected 25 *Fejervarya* individuals (23 major *Cytb* haplotype groups plus two *F. multistriata*) and the six other dicroglossids as the representatives of each haplotype group, and *12S* and *16S* were sequenced from these 31 frogs. Based on the combined mt gene data, we carried out MP, ML, and BI analyses. The resultant ML tree and nodal support values from these analyses (BPs and BPP) are shown in Supplemental Fig. S1. The resultant trees had basically the same topology as the *Cytb* NJ tree, except for the positions of *F.* cf. *syhadrensis* and *Limnonectes* (the former was the sister group to Clade 9 + 10 in all analyses, and the latter grouped with Southeast-Asian *Fejervarya* in the MP and BI trees).

Phylogenetic relationships of *Fejervarya* taxa and closely related genera based on the concatenated data

To elucidate phylogenetic relationships in more detail at both the intra- and inter-generic levels, we additionally determined nucleotide sequences for eight parts of seven nuclear genes (*BDNF*, *CXCR4*, *NCX1*, the 5' and 3' portions of *RAG-1*, *RAG-2*, *Rhod*, and *Tyr*) from the above 25 *Fejervarya* representatives and six species in closely related genera (Table 1). Based on the concatenated alignment (three mt genes and seven nuclear genes; total 6364 bp), we carried out MP, ML and BI analyses. Fig. 3 shows the resultant ML tree (with BP and BPP values for all analyses); the MP and BI analyses recovered the same topology.

In the concatenated tree, the interspecific relationships differed somewhat from those in the *Cytb* NJ tree. The incongru-

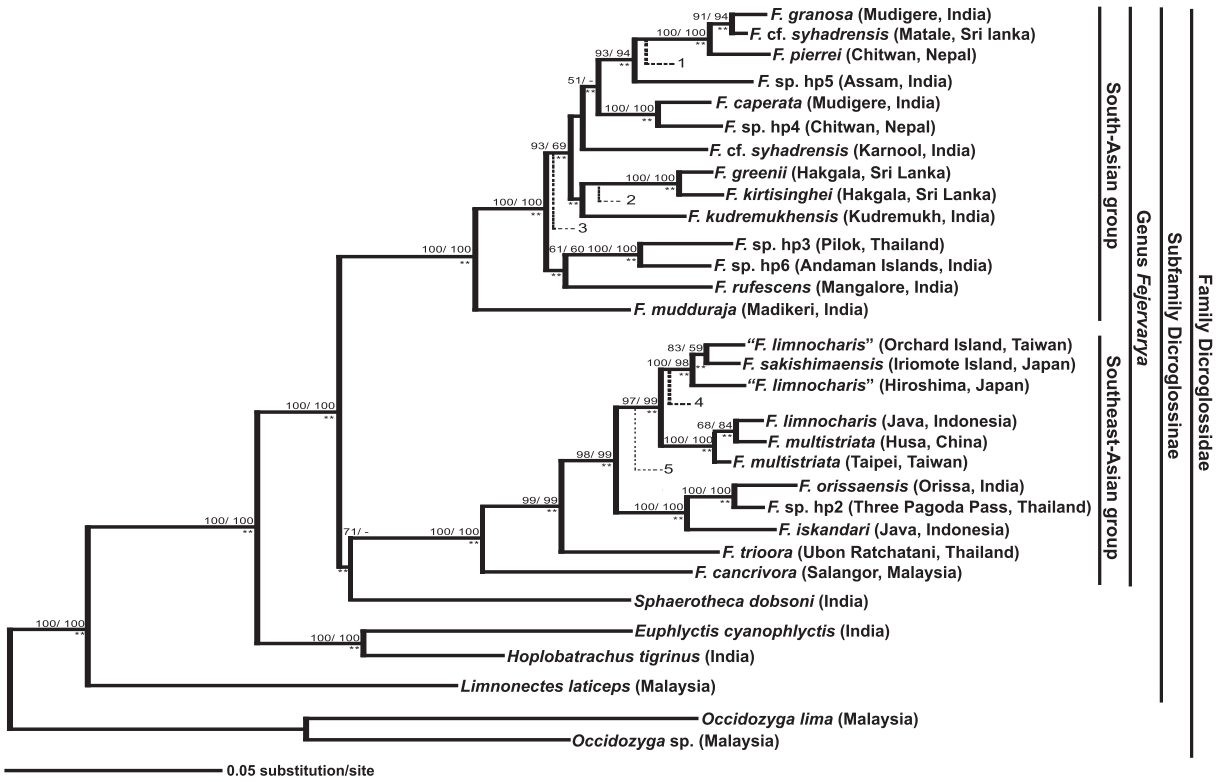

Fig. 3. Maximum-likelihood tree for 31 frogs based on 6364 bp of mitochondrial (*Cytb*, *12S*, and *16S*) and nuclear genes (*BDNF*, *CXCR4*, *NCX1*, *RAG-1*, *RAG-2*, *Rhod*, and *Tyr*). The tree was reconstructed by using PAUP with the heuristic search option and the GTR + I (= 0.48) + G (= 0.54) substitution model, suggested by Modeltest. ML/MP bootstrap values are shown near nodes. Asterisks below branches indicate the Bayesian posterior probability: *, greater than 95%; **, greater than 99%. Locality and country are shown in parenthesis. The dashed lines correspond to branches in the *Cytb* NJ tree: 1, *F. kudremukhensis*; 2, *F.* cf. *syhadrensis* from Karnool, India; 3, the *F. caperata* + *F.* sp. hp4 clade; 4, "*F. limnocharis*" from Orchard Island, Taiwan; 5, *F. triora*).

Table 3. Comparison of log-likelihood scores (KH and SH tests) among alternative tree topologies resulting from analyses of three mitochondrial and seven nuclear genes.

Tree topology	Method	-ln L	-ln L difference	*P*-value	
				KH	SH
(*L. laticeps*,((*H. tigerinus*, *E. cyanophlyctis*),(South Asia,(Southeast Asia, *S. dobsoni*))))	ML, MP, BI based on all combined	35067.68284	best tree	–	–
((*H. tigerinus*, *E. cyanophlyctis*),(*S. dobsoni*,(*L. laticeps*,(Southeast Asia, South Asia))))	ML based on mt combined	35224.82345	157.14061	0.0000*	0.0000*
((*H. tigerinus*, *E. cyanophlyctis*),(*S. dobsoni*,(South Asia,(Southeast Asia, *L. laticeps*))))	MP, BI besed on mt combined	35224.61549	156.93265	0.0000*	0.0000*
(*L. laticeps*,(Southeast Asia,(*S. dobsoni*,(South Asia,(*H. tigerinus*, *E. cyanophlyctis*)))))	mt and nuclear combined (Frost et al., 2006)	35142.31901	74.63617	0.0000*	0.0000*
(*L. laticeps*,(*S. dobsoni*,(South Asia,(Southeast Asia,(*H. tigerinus*, *E. cyanophlyctis*)))))	ML based on mt combined (Kotaki et al., 2008)	35140.50305	72.82022	0.0001*	0.0003*
(*L. laticeps*,((*H. tigerinus*, *E. cyanophlyctis*),(Southeast Asia,(South Asia, *S. dobsoni*))))	MP, BI based on nuclear combined (Kotaki et al., 2008)	35069.25654	1.57371	0.4674	0.7424
(*L. laticeps*,(*S. dobsoni*,((*H. tigerinus*, *E. cyanophlyctis*),(Southeast Asia, South Asia))))	–	35137.63825	69.95541	0.0000*	0.0001*
(*L. laticeps*,((*H. tigerinus*, *E. cyanophlyctis*),(*S. dobsoni*,(Southeast Asia, South Asia))))	–	35068.98452	1.30169	0.5795	0.7688

*Values were not significant (significance level, p < 0.05) among any of the topologies compared.

ent relationships were as follows: the positions of *F. kudremukhensis* (1 in Fig. 3), *F.* cf. *syhadrensis* from Karnool, India (2 in Fig. 3), and the *F. caperata* + *F.* sp. hp4 clade (3 in Fig. 3) in the South-Asian group; and the placements of "*F. limnocharis*" from Orchard Island, Taiwan (4 in Fig. 3) and *F. triora* (5 in Fig. 3) in the South-Asian group. Although the nodal support values in the concatenated tree were far higher than those in the *Cytb* NJ tree, those of the incongruent nodes were not so high, with the exception of the position of *F. triora* (Fig. 3). We tested these incongruent relationships between the *Cytb* and concatenated data using KH and SH tests. These tests rejected the position of *F. triora* suggested by the *Cytb* data (clade 18 + 19 in Fig. 2) (p > 0.05). The other four incongruent relationships, however, were not rejected.

According to Frost et al. (2006), the genus *Fejervarya* may be a paraphyletic rather than monophyletic group with respect to some other dicroglossid genera (e.g., *Euphlyctis*, *Hoplobatrachus*, *Nannophrys*, and *Sphaerotheca*). Similarly, our concatenated analyses recovered a clade containing the Southeast-Asian *Fejervarya* group and another genus, *Sphaerotheca* (ML/MP BPs = 71/–; BPP = 100). Thus, our data suggest paraphyly for *Fejervarya* (Fig. 3). The MP and BI trees from the mt gene data suggested paraphyly for this genus, but with an alternative topology (the

Southeast-Asian group + *Limnonectes*). Our previous analyses also suggested paraphyly for this genus with yet other topologies (South-Asian *Fejervarya* + *Hoplobatrachus* by ML analysis based on the mt gene data, and South-Asian *Fejervarya* + *Sphaerotheca* by MP and BI analyses based on the nuclear gene data). We therefore tested the eight alternative topologies with three monophyletic, four paraphyletic, and one polyphyletic hypotheses for this genus by KH and SH tests based on the concatenated data (Table 3). Five of the eight alternative topologies were rejected ($p > 0.05$), but three hypotheses were not rejected. These non-rejected hypothetical topologies were: 1) Southeast-Asian *Fejervarya* + *Sphaerotheca* (= Fig. 3), 2) South-Asian *Fejervarya* + *Sphaerotheca*, and 3) monophyly of *Fejervarya*.

Given the congruent results from both the mt and concatenated data and the high nodal support in the combined

Table 4. Nucleotide sequence divergences between taxa with different degrees of reproduction isolation and between nominal species, for three mt and seven nuclear genes. Values inside parentheses indicate average sequence divergence.

Taxa compared	Reproductive isolation	Cytb	12S rRNA	16S rRNA	BDNF	CXCR4
Between taxa reproductively isolated South and Southeast-Asian Clades	Complete hybrid inviability (All F1 hybrids died at embryo stage)	16.1–28.3% (23.9%)	15.8–23.1% (19.9%)	12.8–18.3% (15.9%)	1.1–2.4% (1.7%)	6.8–10.4% (8.3%)
F. iskandari vs "*F. limnocharis*" (Hiroshima)	Complete hybrid inviability (All F1 hybrids died at tadpole stage)	17.7–20.6% (19.3%)	11.0–12.6% (11.9%)	11.0–11.7% (11.4%)	0.2–0.4% (0.3%)	1.3–1.6% (1.5%)
F. iskandari vs *F. limnocharis*	Partial hybrid inviability (Most of the F1 hybrids died during tadpole stage)	17.3–20.6% (19.3%)	10.6–12.7% (11.9%)	11.4–12.5% (11.9%)	0.2–0.3% (0.3%)	1.2–1.9% (1.6%)
F. iskandari vs *F.* sp. hp2	Partial hybrid inviability (Small degree abnormal spermatogenesis in F1 hybrids)	12.8–15.6% (13.9%)	6.1–6.3% (6.2%)	5.6–5.9% (5.8%)	0.2–0.3% (0.3%)	1.5–1.8% (1.6%)
Between nominal species		9.5–28.3% (21.7%)	4.0–23.1% (17.1%)	2.7–17.2% (12.9%)	0.3–2.4% (1.3%)	1.3–10.2% (5.3%)
Minimum divergence value and compared taxa		9.5% *F. limnocharis* vs *F. sakishimaensis*	4.0% *F. limnocharis* vs *F. sakishimaensis*	2.7% *F. limnocharis* vs *F. sakishimaensis*	0.2% *F. iskandari* vs *F.* sp. hp2	1.3% *F. limnocharis* vs *F. sakishimaensis*
Between problematic taxa						
"*F. multistriata*" vs *F. limnocharis*		0–1.7% (1.2%)	0.5%	1.1%	0–0.2% (0.1%)	0.3–0.5% (0.4%)
"*F. limnocahris*" (Taiwan) vs *F. limnocharis*		9.0–9.7% (9.4%)	3.8%	3.4%	0.5%	1.1%
"*F. limnocahris*" (Hiroshima) vs *F. limnocharis*		9.7–10.2% (9.9%)	4.8%	3.1%	0.3%	1.4%

Taxa compared	Reproductive isolation	NCX1	5' RAG-1	3' RAG-1	RAG-2	Rhod	Tyr
Between taxa reproductively isolated South and Southeast-Asian Clades	Complete hybrid inviability (All F1 hybrids died at embryo stage)	3.3–5.1% (4.3%)	6.6–9.1% (7.6%)	4.6–7.1% (5.4%)	6.7–8.5% (7.7%)	1.6–4.2% (2.8%)	6.3–10.0% (8.0%)
F. iskandari vs "*F. limnocharis*" (Hiroshima)	Complete hybrid inviability (All F1 hybrids died at tadpole stage)	1.3–2.1% (1.7%)	2.1–4.2% (3.1%)	1.7–2.2% (2.0%)	1.7–2.9% (2.4%)	1.6–1.9% (1.8%)	2.3–3.5% (2.9%)
F. iskandari vs *F. limnocharis*	Partial hybrid inviability (Most of the F1 hybrids died during tadpole stage)	1.1–2.4% (1.8%)	1.9–2.2% (2.1%)	1.1–1.5% (1.3%)	1.3–1.5% (1.4%)	1.6–1.9% (1.7%)	1.8–3.5% (2.7%)
F. iskandari vs *F.* sp. hp2	Partial hybrid inviability (Small degree abnormal spermatogenesis in F1 hybrids)	0.9–1.1% (1.0%)	1.8–2.2% (2.0%)	0.8–0.9% (0.8%)	1.4% (1.4%)	1.0–1.9% (1.5%)	1.6–2.2% (1.8%)
Between nominal species		1.0–4.9% (2.9%)	1.4–9.1% (5.4%)	1.3–7.1% (4.2%)	1.6–9.1% (5.4%)	0.7–4.2% (2.1%)	1.6–9.3% (6.1%)
Minimum divergence value and compared taxa		0.9% *F. iskandari* vs *F.* sp. hp2	1.4% *F. granosa* vs *F. pierrei*	0.8% *F. iskandari* vs *F.* sp. hp2	1.4% *F. iskandari* vs *F.* sp. hp2	0.7% *F. limnocharis* vs *F. sakishimaensis*	1.6% *F. iskandari* vs *F.* sp. hp2
Between problematic taxa							
"*F. multistriata*" vs *F. limnocharis*		1.0%	1.2–1.3% (1.3%)	0.6%	0.8–0.9% 0.8%	0.3–0.6% (0.5%)	0–0.9% (0.5)%
"*F. limnocahris*" (Taiwan) vs *F. limnocharis*		1.0%	4.0%	1.5%	–	0.7%	2.2%
"*F. limnocahris*" (Hiroshima) vs *F. limnocharis*		1.1%	4.0%	1.7%	2.6%	0.7%	1.6%

analyses, our combined analyses well elucidated the intra-generic relationships of the genus *Fejervarya* in most cases. However, there remained four ambiguous intra-generic relationships that were not rejected by statistical tests. We also failed to elucidate the inter-generic relationships, although we condensed this problem to only three alternative hypotheses. In this study, we used relatively long sequence data from multiple loci. Thus, extensive taxon sampling and/or non-sequence-based approaches, i.e., cladistic analyses using retroposon loci (e.g., Okada et al., 2003) or mt gene order information (e.g., Kurabayashi et al., 2008), might be effective in solving the remaining problems.

Taxonomic implications for the unidentified species found in this study

Based on the resultant trees (Figs. 2, 3), some taxa with problematic taxonomic affiliation were brought out (i.e., *F.* "*limnocharis*" from Taiwan and Japan, *F.* "*multistriata*", and *F.* cf. *syhadrensis*; see below). Species identification with nucleotide divergence data has been suggested as an effective procedure for extrapolating the detailed taxonomic status of these problematic taxa (e.g., Sumida et al., 2007; Djong et al., 2007b; Alam et al., 2008). In anurans, *16S* has been considered a usable marker for determining taxonomic affiliations and detecting unconfirmed candidate (i.e., cryptic) species. Data suggest that a 3% divergence in this gene is a species threshold in several different frog taxa (hylids from the Amazonia-Guianas region and all Malagasy frogs, including hyperoliids, microhylids, and mantellids) (Fouquet et al., 2007; Vieites et al., 2009). In our studies, the minimum *16S* divergence among nominal *Fejervarya* species is roughly 3% (= 2.7% between *F. limnocharis* and *F. sakishimaensis)* (Table 4). This confirms the adequacy of this species threshold criterion in *Fejervarya*. We also compared the nucleotide divergences of the other genes among the nominal species and between taxa whose reproductive isolation had been confirmed by artificial crossing experiments (Sumida et al., 2007) (Table 4). Genetic divergences were highly variable among genes, and the genetic divergence of each gene tended to be correlated with both the degree of reproductive isolation and relative phylogenetic positions in the resultant tree. Minimum divergence values occurred among sister nominal species for all genes (Table 4; Figs. 2, 3), and four of these pairs were weakly reproductively isolated (for *BDNF*, *NCX-1*, *RAG-2*, and *Tyr*). These minimum divergence values reflected reproductive isolation levels and/or the resultant phylogeny, and can be regarded as species thresholds for this frog group. The unduly low minimum vales found for the nuclear genes (0.3% for *BDNF* to 1.6% for *Tyr*) are somewhat difficult to use as a basis for species definition. Moreover, few of the genes studied here (excluding *16S*) have been examined to confirm their suitability for use in other frog taxa. Thus, we mainly used the minimum *16S* divergence value (> 3%) to evaluate the taxonomic status of problematic taxa.

Fejervarya multistriata (Hallowell) 1861 is one of the problematic taxa. This species was described from Hong Kong, China. In this study, rather than using topotypic *F. multistriata* specimens, we used individuals from Hainan and Husa, China (approx. 400 and 1500 km from Hong Kong, respectively) and Taipei (mainland), regions from where the species has been reported (Frost, 2009). The *16S* sequence divergence between topotypic *F. limnocharis* and "*F. multistriata*" individuals was only 1.1%, which was much less than the proposed species threshold value (> 3%). Furthermore, in the concatenated tree (Fig. 3), the topotypic *F. limnocharis* was nested in the "*F. multistriata*" clade (i.e., "*F. multistriata*" individuals were paraphyletic with respect to *F. limnocharis*). These results strongly suggest that the *F. limnocharis* and the "*F. multistriata*" specimens are conspecific. Djong et al. (2007b) contended that the name "*F. multistriata*" applies to the populations in China formerly referred to *F. limnocharis*. Our results may support this contention and may suggest that the name "*F. multistriata*" is a junior synonym of *F. limnocharis*.

"*Fejervarya limnocharis*" from the Japan mainland (Hiroshima) and eastern Taiwan (Orchard and Green Islands) populations has been problematic. Sumida et al. (2007) suggested that the Japan mainland populations be regarded as a species distinct from the real *F. limnocharis*. Matsui et al. (2007) pointed out that the eastern Taiwan (+ eastern China) populations are possibly conspecific with *F. sakishimaensis*. In this study, neither "*F. limnocharis*" specimens from the Japan mainland nor "*F. limnocharis*" specimens from eastern Taiwan formed a clade with the topotypic *F. limnocharis*. Furthermore, for many genes, including *16S* (> 3%), the nucleotide divergences between the topotypic *F. limnocharis* and these specimens were equal to or higher than the minimum divergence values among nominal species (Table 4). These results seem to indicate that both the Japan mainland and eastern Taiwan populations are species distinct from *F. limnocharis*, and they seem to support the view of Sumida et al. (2007). The phylogenetic relationships we detected suggest that these populations have a close affinity with *F. sakishimaensis*. However, the *16S* sequence divergences among these taxa were nearly equal to the species threshold values (3.3% between the Japan mainland and eastern Taiwan, 2.8% between the Japan mainland and *F. sakishimaensis*, and 2.7% between eastern Taiwan and *F. sakishimaensis*). Thus, two possibilities can now be considered for these taxa: 1) the Japan mainland and eastern Taiwan populations are conspecific with *F. sakishimaensis* (the assignment of Matsui et al. [2007]), or 2) all three taxa are distinct species.

Fejervarya syhadrensis, a species characterized by small body size, relatively short legs, finger morphology, and the length of hindlimbs (see Kuramoto et al., 2007; Amphibia Web: http://amphibiaweb.org/), has been considered to have a relatively wide distribution range (from Pakistan to Bangladesh). In this study, we used *F. syhadrensis*-like specimens from two different populations (Sri Lanka and the Western Ghats, India). Based on the resultant phylogeny (Fig. 3) and the *16S* sequence divergence between the populations (11.5%), the *F. syhadrensis*-like specimens from these populations are clearly distinct species.

The *16S* sequence from our *F. syhadrensis*-like specimen from the Western Ghats failed to hit any of the other *Fejervarya 16S* data deposited in the DNA databases, while that of our Sri Lanka specimen was almost identical a sequence from "*F. syhadrensis*" from Sri Lanka (Accession No. AY141843). This Sri Lanka *F. syhadrensis* record is doubtful, as the distribution of *F. syhadrensis* in Sri Lanka is

unclear (Frost, 2009). Furthermore, 18 other "*F. syhadrensis*" *16S* sequences have been deposited in the DNA databases. Among them, five sequences were typical for *F. caperata* (AY882951, AY841752, AY882956, AY841755, and AY841753), while the other 13 showed intraspecific levels of nucleotide divergence compared to the sequences of our *F.* cf. *syhadrensis* and the above "*F. syhadrensis*" from Sri Lanka (mainly less than 2.0%, but 2.8% between AY84175 and AY841750, and 3.0% between AY84175 and the Sri Lanka samples). According to Frost (2009), the type locality is the Poona district in India. Before analyzing the type specimen or topotypic specimens of *F. syhadrensis*, it is difficult to specify which data correspond to the real *F. syhadrensis*, or to assert that none of the previous data correspond to this species.

As in the case with *F. syhadrensis*, some *F. cancrivora* sequences in the DNA databases seem to be problematic. According to a recent study by Kurniawan et al. (2009), "*F. cancrivora*" populations are morphologically (and ecologically) divided into three distinct groups (the large, mangrove, and Pelabuhan ratu/Sulawesi types); genetic divergences among these groups clearly correspond to the level of distinct species, and the large type corresponds to topotypic *F. cancrivora.* The *F. cancrivora* specimen we included corresponds to the large type and thus can be regarded as real *F. cancrivora*.

Several unidentified *Fejervarya* samples (*F.* sp. hp2–hp6; only tissue samples were available) were included in this study. None of the nucleotide sequences from these samples hit any sequences from other *Fejervarya* species deposited in the DNA databases. One unidentified sample (*F.* sp. hp4) was from Nepal, a region where four *Fejervarya* species have been reported (Schleich and Kästle, 2002). One of these four species is *F. pierreri* as used in this study, and the other three are *F. nepalensis*, *F. syhadrensis*, and *F. teraiensis*. The *F.* sp. hp4 specimen may correspond to one of the latter three species. Two of the unidentified haplotype groups (*F.* sp. hp2 and hp3) found in this study were from Thailand. According to our previous studies (Sumida et al., 2007; Kotaki et al., 2008), *F.* sp. hp3 from Pilok, Thailand may be an undescribed species, and *F.* sp. hp2 may be the same species as *F. orissaensis* or an undescribed species. On the Andaman Islands, only two *Fejervarya* species (*F. andamanensis* and *F. cancrivora*) have been found (Frost, 2009), and in the current study we used the real *F. cancrivora*. Thus, one unidentified sample, *F.* sp. hp6 from Andaman Island, India, might correspond to *F. andamanensis*.

CONCLUSION

Our phylogenetic trees are the most comprehensive to date for *Fejervarya* species. They provide relatively high-resolution of interspecific relationships and support paraphyly for this genus. Yet despite the relatively abundant molecular data used in this study, several incongruent or ambiguous relationships remain in these trees. To solve these problems, further taxon sampling or a novel approach using different types of molecular makers (e.g., mt gene arrangement or SINEs) will be necessary. We also confirmed the utility of the molecular data, especially *16S* sequences, for species definition. The sequence data provided here are likely to serve as a useful guide for elucidating the taxonomic problems in this frog taxon.

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