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Genetic Divergence and Evolutionary Relationship in *Fejervarya cancrivora* from Indonesia and Other Asian Countries Inferred from Allozyme and MtDNA Sequence Analyses

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To elucidate genetic divergence and evolutionary relationship in *Fejervarya cancrivora* from Indonesia and other Asian countries, allozyme and molecular analyses were carried out using 131 frogs collected from 24 populations in Indonesia, Thailand, Bangladesh, Malaysia, and the Philippines. In the allozymic survey, seventeen enzymatic loci were examined for 92 frogs from eight representative localities. The results showed that *F. cancrivora* is subdivided into two main groups, the mangrove type and the large- plus Pelabuhan ratu types. The average Nei's genetic distance between the two groups was 0.535. Molecular phylogenetic trees based on nucleotide sequences of the 16S rRNA and Cyt *b* genes and constructed with the ML, MP, NJ, and BI methods also showed that the individuals of *F. cancrivora* analyzed comprised two clades, the mangrove type and the large plus Pelabuhan ratu / Sulawesi types, the latter further split into two subclades, the large type and the Pelabuhan ratu / Sulawesi type. The geographical distribution of individuals of the three *F. cancrivora* types was examined. Ten individuals from Bangladesh, Thailand, and the Philippines represented the mangrove type; 34 individuals from Malaysia and Indonesia represented the large type; and 11 individuals from Indonesia represented the Pelabuhan ratu / Sulawesi type. Average sequence divergences among the three types were 5.78–10.22% for the 16S and 12.88–16.38% for Cyt *b*. Our results suggest that each of the three types can be regarded as a distinct species.

Key words: genetic divergence, evolutionary relationship, *Fejervarya cancrivora*, allozyme, mtDNA

INTRODUCTION

Since Gravenhorst (1829) first described *Rana cancrivora*, the name *R. cancrivora* has been applied to distinguish *R. cancrivora* from *R. limnocharis*. According to Gravenhorst's analysis, *R. cancrivora* is "larger" than *R. limnocharis*, and the name *R. cancrivora* has been consistently applied to large individuals in the *R. limnocharis* complex occurring in Java and neighboring regions. The type specimen of *Rana cancrivora* stored in the Breslau Museum is considered to be lost, and another specimen collected at Cianjur's Rice Field (06°49'S, 107°08'E, West Java, Indonesia), traditionally recognized under this name, was designated as the neotype by Dubois and Ohler (2000). Several genera, including *Limnonectes* and *Fejervarya*, were recently resurrected from their former status as synonyms of the very large, widespread genus *Rana* (Frost, 2007). Thus, *Rana cancrivora* became *Fejervarya cancrivora* (Iskandar, 1998; Dubois and Ohler, 2000).

The crab-eating frog, *F. cancrivora* is one of the most widely distributed frog species in the Asian region, extending

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from Guangxi and the northeastern coast of Hainan Island, China, through to Vietnam, the Andaman and Nicobar Islands (India), Peninsular Thailand, Malaya, Singapore, the Greater Sundas, the Philippines, and the Lesser Sundas as far as Flores (Frost, 2007). It has also been introduced to Sorong and Jayapura, Papua, Indonesia. Nutphund (2001) and Taylor (1962) reported that *Fejervarya raja* (previously *Rana raja*) occurs in southern Thailand, while Iskandar (1998) asserted that *F. raja* from Thailand could be described as an extra-large specimen of *F. cancrivora*. Some of the records of *F. cancrivora* from Peninsular Malaya may refer to this species.

Inger (2005) proposed that *F. cancrivora* on Sulawesi Island may have been introduced from Kalimantan Island across the Wallace line, although available data are not sufficient to support this. *Fejervarya cancrivora* from Thailand and the Philippines has been studied using allozyme and mitochondrial gene markers (Nishioka and Sumida, 1990; Sumida et al., 2002). A preliminary study of *F. cancrivora* showed a significant divergence between populations in Thailand or the Philippines and that in the type locality in Indonesia (Kurniawan, 2008). This suggests that *F. cancrivora* might include several cryptic species of difficult to identify morphologically. Until now, no study has been conducted to elucidate genetic divergence in the wide-ranging *F. cancrivora*.

Allozyme analyses continue to provide valuable information on the relatedness of populations and the amount of genetic variability within and between populations (Bader, 1998; Islam et al., 2008a). Sequences of the 16S rRNA gene are now widely used for barcoding vertebrates (Vences et al., 2005); sequences of the Cyt *b* gene have proven useful in resolving relationships among closely related taxa, and are widely used in phylogenetic (Sumida et al., 1998; Parson et al., 2000; Igawa et al., 2006; Djong et al., 2007b) or phylogeographic studies (Fouquet et al., 2007; Gamble et al., 2008).

The aim of this study was to elucidate genetic divergence and evolutionary relationships in *F. cancrivora* from Indonesia and other Asian countries by using allozyme and mitochondrial gene markers. Our results indicate that there may be several cryptic species in this group.

MATERIAL AND METHODS

Allozyme analysis

Fejervarya cancrivora specimens were collected from eight localities of Indonesia, the Philippines, Malaysia, Thailand, and Bangladesh (Fig. 1). Ninety-two frogs, comprising 40 males, 38 females, and 14 immature individuals, were included in the allozyme analysis (Table 1). Three distinct types were found among *F. cancrivora* specimens based on SVL (snout vent length) and ecological characteristics. The first type has an average SVL of 54.1 mm in males and 68.0 mm in females, and inhabits mainly brackish water areas such as shrimp ponds or mangroves. The second type has an average SVL of 68.1 mm in males and 86.7 mm in females, and inhabits mainly rice fields. The third type has an average SVL of 43.5 mm in male and 50.7 mm in females, and inhabits rice fields. Here we refer to these types as the large, mangrove, and Pelabuhan ratu types, respectively. *Fejervarya iskandari* was used as the outgroup.

Seventeen enzymes extracted from skeletal muscle tissue were analyzed by means of starch-gel electrophoresis (Table 2). Horizontal starch-gel electrophoresis was carried out by using Sigma starch at a concentration of 12.5%, as described by Nishioka et al. (1980, 1992). Each locus was detected by using the agar-overlay method outlined by Harris and Hopkinson (1976), with slight modification (Nishioka et al., 1992). Genetic distance (D) and genetic identity (I) values were calculated following Nei (1972) with the software POPGENE (Yeh et al., 1997). A neighbor-joining (NJ) tree was constructed based on Nei's genetic distances to infer phylogenetic relationships among populations (Saitou and Nei, 1987). Bootstrap values were calculated from 1000 pseudoreplicates by using the software PHYLIP 3.65 (Felsenstein, 2005).

Molecular analysis

In the molecular analysis, we included 55 individuals from 24 populations at 22 localities in five countries, including 16 localities in Indonesia, three in Thailand, one at Selangor, Malaysia, one at Manila, the Philippines, and one at Khulna, Bangladesh (Table 1). Specimens from Cianjur, Indonesia (06°49'S, 107°08'E), the type locality of the neotype, were also included. One individual *F. iskandari* from Cianjur, Indonesia, was used as the outgroup. Five additional 16S sequences retrieved from the GenBank were included in the 16S analyses (Table 3).

DNA extraction, PCR, and sequencing

Total genomic DNA was extracted from clipped toes using a DNeasy Tissue Kit (QIAGEN) according to the manufacturer's instructions. Two pairs of primers, F51 (5'-CCC GCC TGT TTA CCA AAA ACA T-3') and R51 (5'-GGT CTG AAC TCA GAT CAC GTA -3') (Sumida et al., 2002), and Fow 1-1 (5'-ACM GGH YTM TTY YTR GC ATR CAY TA -3') and Rev-1 (5'-TAD GCR AAW AGR

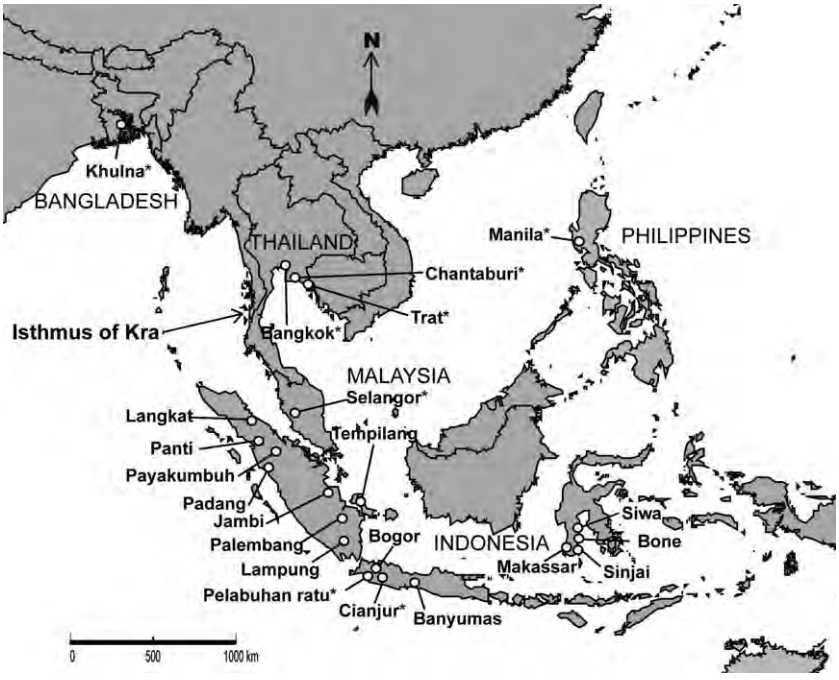

Fig. 1. Map showing the collection localities for *F. cancrivora* samples included in this study. Samples for allozyme analysis were collected from the localities indicated by asterisks.

Table 1. Samples included in this study.

Type	Collecting Station			Number of frogs					
	Country	Locality	(Population)	Allozyme					Molecular Work
				Male	(SVL; mean) (mm)	Female	(SVL; mean) (mm)	Immature	
Mangrove	Philippines	Manila	(Mani)	3	(45.0–55.0; 50.3)	5	(49.0–68.0; 55.4)	0	3
	Thailand	Bangkok	(Bang)	6	(48.0–60.0; 53.3)	4	(59.0–71.0; 65.0)	0	2
		Trat	(Trat)	10	(47.3–62.4; 55.5)	7	(51.3–78.3; 66.0)	14	1
		Chantaburi	(Chan)	0		1	(74.2)	0	1
	Bangladesh	Khulna	(Khul)	7	(46.6–60.6; 54.5)	6	(46.6–83.6; 70.5)	0	3
Large	Malaysia	Selangor	(Sela)	3	(59.3–71.2; 64.9)	6	(58.0–87.5; 73.4)	0	4
	Indonesia	Cianjur (A)[1], Java	(Cian-A)	10	(62.7–76.2; 69.7)	7	(87.6–112.0; 98.6)	0	2
		Cianjur (B)[2], Java	(Cian-B)	0		0		0	1
		Pelabuhan ratu (B)[3], Java	(Pela-B)	0		0		0	3
		Bogor, Java	(Bogo)	0		0		0	2
		Banyumas, Java	(Bany)	0		0		0	2
		Langkat, Sumatra	(Lang)	0		0		0	3
		Padang, Sumatra	(Pada)	0		0		0	3
		Payakumbuh, Sumatra	(Paya)	0		0		0	2
		Panti, Sumatra	(Pant)	0		0		0	3
		Jambi, Sumatra	(Jamb)	0		0		0	4
		Palembang, Sumatra	(Pale)	0		0		0	2
		Lampung, Sumatra	(Lamp)	0		0		0	2
		Tempilang, Bangka	(Temp)	0		0		0	1
Pelabuhan ratu/ Sulawesi	Indonesia	Pelabuhan ratu (A)[4], Java	(Pela-A)	1	(43.5)	2	(48.7–52.7; 50.7)	0	3
		Makassar, Sulawesi	(Maka)	0		0		0	3
		Sinjai, Sulawesi	(Sinj)	0		0		0	2
		Siwa, Sulawesi	(Siwa)	0		0		0	2
		Bone, Sulawesi	(Bone)	0		0		0	1
	Total			40		38		14	55

[1] Collected by Nia Kurniawan in 2007.
[2] Collected from type locality by Nia Kurniawan in 2008.
[3] Collected by Nia Kurniawan in 2008.
[4] Collected and provided by Professor H. Ota in 1996.

Table 2. Enzymes analyzed and number of phenotypes and alleles for each locus detected in this study. T-C, Tris citrate buffer; TEB, Tris-EDTA-borate buffer; E.C., Enzyme Commission; *, considering both ingroup and outgroup taxa.

Enzyme	Abbreviation	E.C. No.	No. of loci	Locus	Sample	Buffer system	Number of phenotypes*	Number of alleles*
Aspartate aminotransferase	AAT	2.6.1.1	2	AAT-1	Muscle	T-C pH 7.0	2	2
				AAT-2	Muscle	T-C pH 7.0	1	1
Adenosine deaminase	ADA	3.5.4.4	1	ADA	Muscle	T-C pH 7.0	3	3
Adenylate kinase	AK	2.7.4.3	1	AK	Muscle	T-C pH 7.0	3	3
Aldolase	ALD	4.1.2.13	1	ALD	Muscle	T-C pH 7.0	3	2
Creatine kinase	CK	2.7.3.2	1	CK	Muscle	TEB pH 8.0	1	1
Fumarase	FUM	4.2.1.2	2	FUM-1	Muscle	TEB pH 8.0	3	3
				FUM-2	Muscle	TEB pH 8.0	1	1
α-Glycerophosphate dehydrogenase	α-GDH	1.1.1.8	1	α-GDH	Muscle	T-C pH 6.0	2	2
Glucose-6-phosphate isomerase	GPI	5.3.1.9	1	GPI	Muscle	TEB pH 8.0	4	3
Isocitrate dehydrogenase	IDH	1.1.1.42	2	IDH-1	Muscle	T-C pH 7.0	3	3
				IDH-2	Muscle	T-C pH 7.0	1	1
Lactate dehydrogenase	LDH	1.1.1.27	2	LDH-1	Muscle	T-C pH 6.0	5	4
				LDH-2	Muscle	T-C pH 6.0	2	2
Malate dehydrogenase	MDH	1.1.1.37	2	MDH-1	Muscle	T-C pH 6.0	4	4
				MDH-2	Muscle	T-C pH 6.0	2	2
Malic enzyme	ME	1.1.1.40	2	ME-1	Muscle	T-C pH 7.0	5	4
				ME-2	Muscle	T-C pH 7.0	4	3
Mannose-6-phosphate isomerase	MPI	5.3.1.8	1	MPI	Muscle	T-C pH 7.0	8	5
Peptidase	PEP	3.4.3.1	4	PEP-A	Muscle	TEB pH 8.0	1	1
				PEP-B	Muscle	TEB pH 8.0	5	4
				PEP-C	Muscle	TEB pH 8.0	2	2
				PEP-D	Muscle	TEB pH 8.0	2	2
6-Phosphogluconate dehydrogenase	6-PGD	1.1.1.44	1	6-PGD	Muscle	T-C pH 7.0	5	4
Phosphoglucomutase	PGM	5.4.2.2	1	PGM	Muscle	TEB pH 8.0	3	3
Superoxide dismutase	SOD	1.15.1.1	1	SOD	Muscle	TEB pH 8.0	4	4

Table 3. List of the genes, types, haplotype names, populations, and accession numbers used in this study. –, no data.

Type	Haplotype name		Population	Accession number[2]		Source	
	16S	Cyt *b*		16S	Cyt *b*	16S	Cyt *b*
Mangrove	M-I	M-1a	Mani	(AB070738)	AB444706	(Sumida et al., 2002)	This study
		M-1b	Bang	AB444691	AB444707	This study	This study
		–	Negros Island (Philippines)	(AF206473)	–	(Chen et al., 2005)	–
		–	Hainan Island (China)	(DQ458252)	–	(Che et al., 2007)	–
	M-II	M-2	Trat, Chan	AB444692	AB444708	This study	This study
	M-III	M-3	Khul	(AB372018)	(AB372070)	(Islam et al., 2008b)	(Islam et al., 2008b)
	M-IV	–	Orissa (India)	(AY841754)	–	(Guha et al., unpublished)	–
Large	L-I	L-1a	Bogo	AB444689	AB444702	This study	This study
		L-1b	Cian-A	AB444684	AB444695	This study	This study
		L-1c	Cian-B, Pela-B-1, Sela-1	AB444684	AB444696	This study	This study
		L-1d	Lang	AB444684	AB444694	This study	This study
		L-1e	Temp	AB444684	AB444698	This study	This study
	L-II	L-1e	Pale	AB444686	AB444698	This study	This study
		L-2a	Lamp	AB444686	AB444699	This study	This study
		L-2b	Jamb	AB444687	AB444700	This study	This study
		L-2c	Bany	AB444690	AB444703	This study	This study
	L-III	L-3	Pada, Paya, Pant	AB444685	AB444697	This study	This study
	L-IV	L-4	Sela-2	AB444688	AB444701	This study	This study
	L-V	–	Kalimantan Island (Indonesia)	(AF346810)	–	(Veith et al., 2001)	–
	L-VI	–	Jiadong (Taiwan)	(EU365387)	–	(Hsu et al., unpublished)	–
	–	L-5	Paya-2	–	AB444704	–	This study
	–	L-6	Pela-B-2	–	AB444705	–	This study
Pelabuhan ratu/Sulawesi	PS-I	PS-1a	Pela-A, Maka	AB444693	AB444709	This study	This study
		PS-1b	Bone, Sinj, Siwa	AB444693	AB444710	This study	This study

[1]We used *F. iskandari* as the outgroup: accession number AB277303 (Kotaki et al., 2008) for 16S; accession number AB296085 (Djong et al., 2007) for Cyt *b*.
[2]Accession numbers in parentheses were retrieved from Genbank.

AAR TAY CAY TCN GG-3'), were used for amplifying and sequencing the 5' portions of the 16S rRNA and Cyt *b* genes, corresponding to positions 6189–6761 and 16662–17491, respectively, in *Fejervarya limnocharis* (Liu et al., 2005). A PCR mixture in final volumes of 50 μl was prepared by using the TaKaRa Ex Taq Kit. Cycling conditions for both genes were 35 cycles of 10 s at 98°C, 30 s at 47.5°C, and 80 s at 72°C. Purified amplicons were directly sequenced by using the BigDye Terminator Cycle Sequencing Kit (ABI) and a 3100-Avant automated DNA Sequencer (ABI). Sequences obtained have been deposited in the DDBJ database under accession nos. AB444684–AB 444710).

Sequence data analysis

DNA sequences were aligned by using CLUSTAL W (Thompson et al., 1994). Gaps and ambiguous sites were excluded by using GBlocks 0.91b (Castresana, 2000) at the default settings. Gaps were found only in the 16S sequences. Two sequence alignments (16S and Cyt *b*) were used for phylogenetic analyses. Sequence divergence was calculated as uncorrected "p" distances, while phylogenetic relationships were estimated by the maximum-likelihood (ML), Bayesian inference (BI), maximum-parsimony (MP), and neighbor-joining (NJ) methods. Nucleotide substitution models for ML, BI, and NJ analyses were selected for both 16S and Cyt *b* based on the Akaike information criterion implemented in the program Kakusan 3.0 (Tanabe, 2003).

ML analyses were performed with 1000 bootstrap replicates by using TREEFINDER (Jobb et al., 2004). MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) was used for BI analyses. For BI analyses, Markov chain Monte Carlo (MCMC) was run for = one million generations with a sampling frequency of 100. The number of MCMC generations and the burn-in size for both 16S and Cyt *b* were determined by checking the convergence of -log likelihood (-ln*L*) and tree length against generation number by using Tracer ver. 1.4 (Rambaut and Drummond, 2007); the first 100,000 generations were discarded as burn-in. All MCMC runs were repeated twice to confirm consistent approximation of the posterior probability distribution. The MP and NJ analyses were performed with 1000 bootstrap replicates by using PAUP* 4.0b10 (Swofford, 2003). A median-joining haplotype network tree was constructed for Cyt *b* by using Network 4.502 (Bandelt et al., 1999; available at http://www.fluxus-engineering. com).

RESULTS

Allozyme data

Based on the electrophoretic patterns, 17 enzymes were presumed to be encoded by genes at 26 loci. One to eight phenotypes (an average of 3.0 phenotypes) were produced by one to five alleles (an average of 2.7 alleles). The loci AAT-2, CK, FUM-2, IDH-2 and PEP-A were monomorphic, and *F. cancrivora* and *F. iskandari* shared the same phenotype. The MPI locus was found to be most polymorphic, and five alleles gave rise to eight phenotypes.

Table 4 give the allele frequencies for all 26 loci for each population of *F. cancrivora* and the outgroup, *F. iskandari.* Alleles distinguishing *F. cancrivora* populations from *F. iskandari* were found at 14 loci: ADA, AK, FUM-1, α-GDH, IDH-1, LDH-A, LDH-B, MDH-1, MDH-2, ME-2, PEP-B, PEP-C, PEP-D, and PGM (Table 4).

At the ADA locus, allele *a* was observed in the large type and the Pelabuhan ratu type; allele *c* was observed in the mangrove type; and allele *b* was exclusive to the outgroup (Fig. 2). The same pattern was found at another two

Table 4. Allele frequencies at 26 loci for eight populations of *F. cancrivora* and one population of the outgroup *F. iskandari*.

Locus	Mangrove type					Large type		Pelabuhan ratu type	Outgroup
	Mani	Bang	Trat	Chan	Khul	Sela	Cian-A	Pela-A	Fisk
AAT-1	b	b	b	b	b	b	b	a	a
AAT-2	a	a	a	a	a	a	a	a	a
ADA	c	c	c	c	c	a	a	a	b
AK	a	a	a	a	a	b	b	b	c
ALD	b	b	a(0.02) b(0.98)	b	b	a(0.12) b(0.88)	b	b	a
CK	a	a	a	a	a	a	a	a	a
FUM-1	a(0.19) c(0.81)	c	a(0.02) c(0.98)	a(0.50) c(0.50)	c	c	c	c	b
FUM-2	a	a	a	a	a	a	a	a	a
α-GDH	b	b	b	b	b	b	b	b	a
GPI	a	a	a(0.95) b(0.05)	a	a	b	b(0.88) c(0.12)	b	a
IDH-1	b	b	a(0.02) b(0.98)	b	b	b	b	b	c
IDH-2	a	a	a	a	a	a	a	a	a
LDH-A	a	a	a	a	a	a	a	a	b
LDH-B	d	d	c(0.02) d(0.98)	d	d	c	c	c	a(0.33) b(0.67)
MDH-1	d	d	d	d	d	d	d	c	a(0.67) b(0.33)
MDH-2	b	b	b	b	b	b	b	b	a
ME-1	a(0.13) b(0.87)	b	a(0.02) b(0.98)	b	b	c(0.78) d(0.22)	c	c(0.84) d(0.16)	b
ME-2	a(0.75) b(0.25)	a	a(0.98) b(0.02)	a	a(0.96) b(0.04)	b	b	a(0.17) b(0.83)	c
MPI	c(0.38) d(0.62)	d(0.95) e(0.05)	d(0.92) e(0.08)	d	d	b(0.61) c(0.27) d(0.12)	b(0.35) c(0.23) d(0.42)	d	a(0.17) b(0.50) c(0.33)
PEP-A	a	a	a	a	a	a	a	a	a
PEP-B	c(0.13) d(0.87)	d	d	d	d	c	c	b	a
PEP-C	b	b	b	b	b	b	b	b	a
PEP-D	b	b	b	b	b	b	b	b	a
6-PGD	b	b	a(0.08) e(0.92)	b	c	d	d	c	a
PGM	c	c	c	c	c	b	b	b	a
SOD	d	d	d	d	d	b	a(0.03) b(0.97)	b	c(0.50) d(0.50)

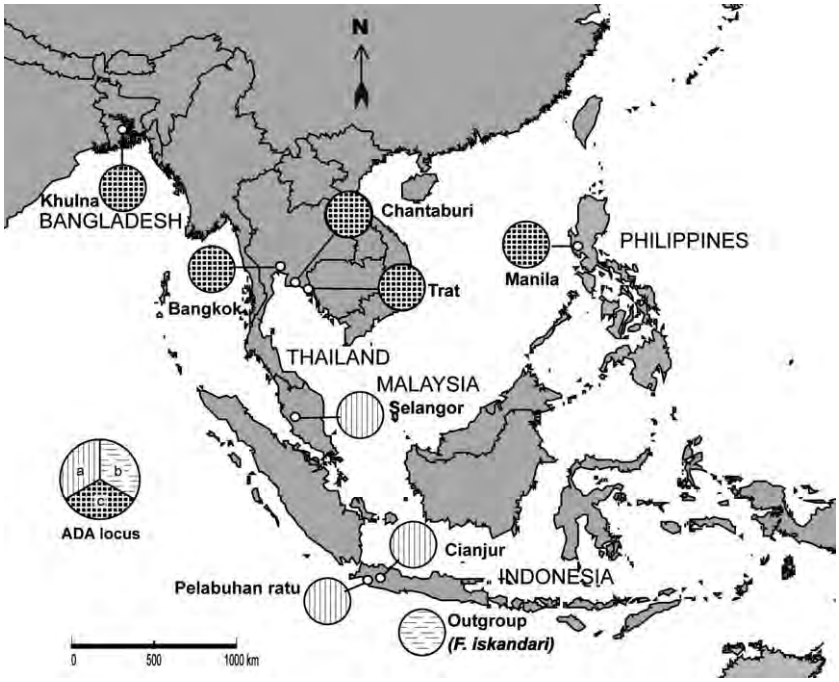

Fig. 2. Geographical distribution of ADA alleles in *F. cancrivora* and *F. iskandari* from Indonesia and other Asian countries. The large type (Selangor and Cianjur) shared the same alleles with the Pelabuhan ratu type.

loci, AK and PGM (Table 4). An almost complete separation of *F. cancrivora* into three groups was observed at the PEP-B locus, with allele *d* dominant in the mangrove type, allele *c* observed in the large type, allele *b* observed in the Pelabuhan ratu type, and allele *a* belonging to the outgroup. The Manila population was partly differentiated from the other populations of the mangrove type by allele *c* at the PEP-B locus (Fig. 3). At the MDH-1 locus, the Pelabuhan ratu type had allele *c*, while the mangrove type and the large type had allele *d*, and the outgroup had alleles *a* and *b*. At the MPI locus, the Cianjur and Selangor populations (corresponding to the large type) shared the same alleles, *b*, *c*, and *d*. The other populations were dominated by allele *d*. Allele *d* of the MPI locus was dominant in the mangrove type and the Pelabuhan ratu type, while allele *b* in addition to alleles *a* and *c* was dominant in the outgroup (Fig. 4). The ALD locus almost completely distinguished *F. cancrivora* populations from the outgroup *F. iskandari*: allele *b* dominated in the former, while allele *a* dominated in the latter (Table 4). At the AAT-1 locus, allele *a* was observed in the Pelabuhan ratu type and the outgroup, whereas allele *b* was observed in the mangrove type and the large type. At the GPI locus, the outgroup and mangrove type had allele *a*, while the Trat population corresponding to the mangrove type was dominated by allele *a* in addition to allele *b*. The large type and the Pelabuhan ratu type had allele *b*, while the Cianjur population corresponding to the large type was dominated by allele *b* in addition to allele *c* at the GPI locus (Table 4). At the ME-1 locus, the outgroup and mangrove type had allele *b*, while the Manila and Trat populations corresponding to the mangrove type were dominated by allele *b* in addition to allele *a*. The large type had allele *c*, while the Selangor population corresponding to the large type was dominated by allele *c* in addition to allele *d*. The Pelabuhan ratu type was also dominated by allele *c* in addition to allele *d* at the ME-1 locus (Table 4).

In summary, within *F. cancrivora* types, clear differences in allele frequencies distinguishing the mangrove type from the large and Pelabuhan ratu / Sulawesi types were found at the ADA, AK, GPI, LDH-B, ME-1, PGM, and SOD loci; clear differences in allele frequencies distinguishing the Pelabuhan ratu / Sulawesi type from the mangrove and large types were found at the AAT-1 and MDH-1 loci; and a clear difference distinguishing each type from the

outgroup was found at the PEP-B locus (Table 4).

Nei's genetic distance (D) and genetic identity (I) values among populations of *F. cancrivora* and the outgroup are listed in Table 5. *Fejervarya cancrivora* was divided largely into two groups, the mangrove type, and the large plus Pelabuhan ratu types. The average genetic distance (D) between the two groups was 0.535. Genetic distances (D) within the mangrove-type populations ranged from 0 to 0.050 (mean, 0.023 ± 0.019), and that within the large-type populations was only 0.006 (Table 6). Genetic distances (D) between the mangrove type and large type ranged from 0.468 to 0.553 (mean, 0.510 ± 0.025); between the mangrove type and Pelabuhan ratu type, from 0.525 to 0.623 (mean, 0.586 ± 0.036); and between the large type and Pelabuhan ratu type, from 0.188 to 0.205 (mean, 0.197 ± 0.012) (Tables 5, 6). Genetic distances (D) between *F. cancrivora* and the outgroup ranged from 1.153 to 1.545 (mean, 1.300 ± 0.16) (Tables 5). Genetic distances (D) were smaller within the ingroup (*F. cancrivora*) than between the ingroup and the outgroup (Tables 5, 6).

The dendrogram based on Nei's genetic distances shows *F. cancrivora* divided largely into two groups, the mangrove type (BP = 86.5%) and the large plus Pelabuhan ratu types (BP = 88.5%), the latter was further subdivided into two subclades, the large type (BP = 97.8%) and the Pelabuhan ratu type (Fig. 5).

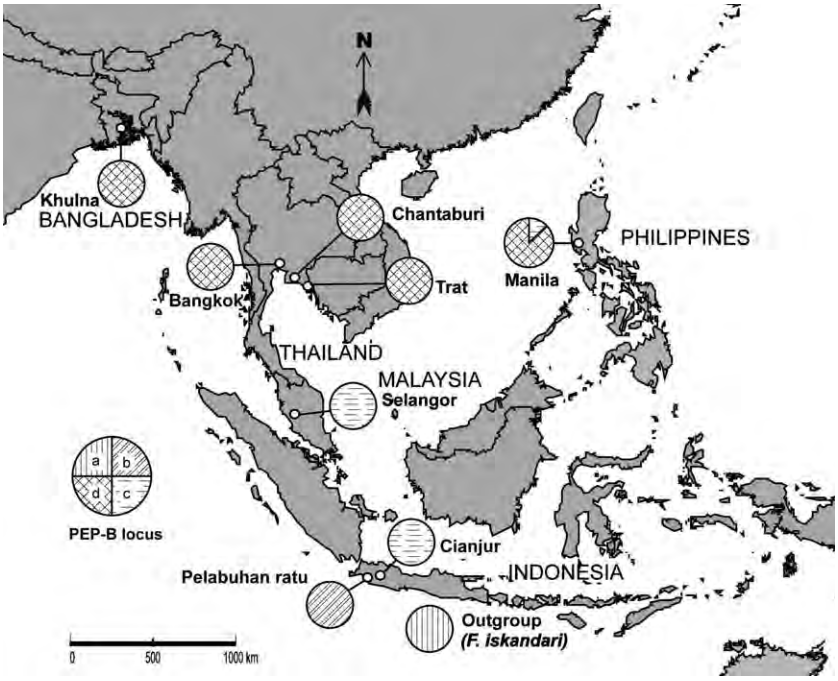

Fig. 3. Geographical distribution of PEP-B alleles in *F. cancrivora* and *F. iskandari* from Indonesia and other Asian countries. With the exception of Manila, where allele *c* was present at low frequency, we found exclusive alleles (i.e., allele *a* for *F. iskandari*; allele *b* for the Pelabuhan ratu type; allele *c* for the large type; allele *d* for the mangrove type).

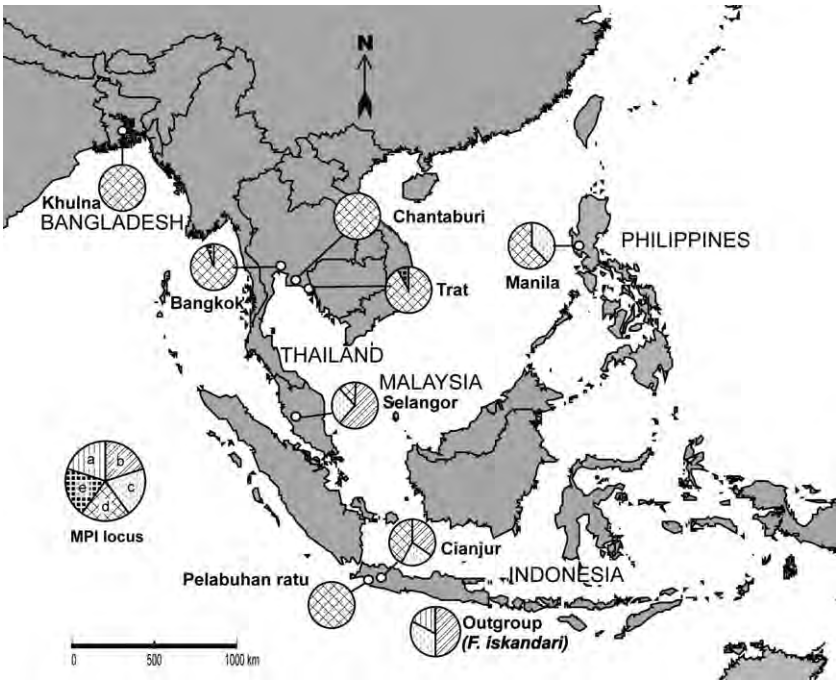

Fig. 4. Geographical distribution of MPI alleles in *F. cancrivora* and *F. iskandari* from Indonesia and other Asian countries.

Nucleotide sequence data

The 16S and Cyt *b* genes were sequenced for 55 specimens. Alignments of these sequences revealed 10 haplotypes for a 497-bp segment of 16S and 17 haplotypes for a 557-bp segment of Cyt *b* (Table 3). In the 16S analysis, we included the 10 haplotypes we obtained and seven additional haplotypes retrieved from GenBank. A new 16S alignment 389 bp long revealed 12 haplotypes, including the outgroup (Table 7). The 389-bp 16S segment contained 83 variable sites, of which 42 were parsimony-informative. The 557-bp Cyt *b* segment contained 180 variable sites, of which 125 were parsimony-informative. The best-fit substitution model for 16S was J2 + G, with a Gamma distribution shape parameter (G) of 0.2393. J2 + G was used as the substitution model for the ML, BI, and NJ analyses. For the ML analyses, the empirical base frequencies were T = 0.2340, C = 0.2705, A = 0.2929, and G = 0.2026. For the Cyt *b* data set, the best-ft model was HKY + G, with a Gamma distribution shape parameter (G) of 0.5244. HKY + G was used for the ML and NJ analyses. For the ML analyses, the empirical base frequencies were T = 0.3094, C = 0.2778, A = 0.2460, and G = 0.1668.

For 16S, sequence divergences between *F. iskandari* and 11 haplotypes of *F. cancrivora* ranged from 14.14% to 16.20% (mean, 14.96 ± 0.76%) (Tables 7,

Table 5. Nei's genetic distances (D) (below diagonal) and genetic identity values (I) (above diagonal) among eight populations of *F. cancrivora* and one outgroup population of *F. iskandari*, based on allozyme data. See Table 1 for population abbreviations.

Type	No.	Population	1	2	3	4	5	6	7	8	9
Mangrove	1	Mani	–	0.992	0.992	0.986	0.952	0.617	0.626	0.552	0.316
	2	Bang	0.008	–	1.000	0.990	0.961	0.590	0.604	0.550	0.301
	3	Trat	0.008	0.000	–	0.990	0.964	0.598	0.611	0.556	0.305
	4	Chan	0.014	0.010	0.010	–	0.951	0.575	0.590	0.537	0.303
	5	Khul	0.049	0.040	0.037	0.050	–	0.592	0.606	0.592	0.301
Large	6	Sela	0.483	0.527	0.514	0.553	0.525	–	0.994	0.815	0.223
	7	Cian-A	0.468	0.505	0.493	0.528	0.501	0.006	–	0.829	0.213
Pelabuhan ratu	8	Pela-A	0.595	0.598	0.588	0.623	0.525	0.205	0.188	–	0.243
Outgroup	9	Fisk	1.153	1.201	1.187	1.194	1.202	1.501	1.545	1.416	–

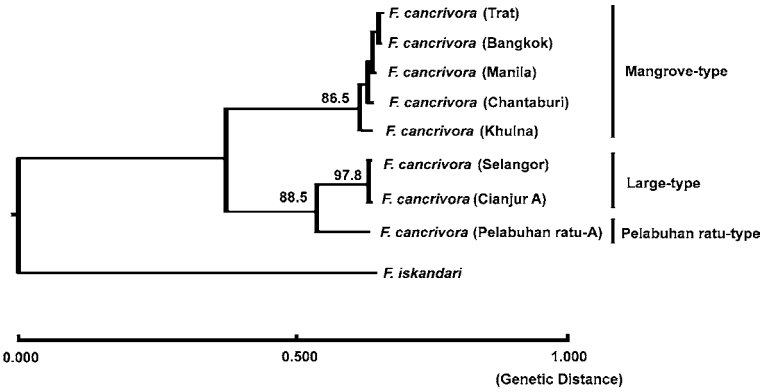

Fig. 5. Neighbor-joining tree based on Nei's genetic distances (Saitou and Nei, 1987) calculated from allelic frequencies at 26 loci from eight populations of *F. cancrivora* and one population of *F. iskandari* (outgroup). Values above branches are bootstrap values > 50% from analysis of 1000 pseudoreplicates. The scale bar indicates the branch length in terms of Nei's genetic distance.

8). Sequence divergences among mangrove-type haplotypes ranged from 0.26% to 1.80% (mean, 1.12 ± 0.68%), while those among large-type haplotypes ranged from 0.26% to 0.51% (mean, 0.43 ± 0.12%). Sequence divergences between mangrove-type and large-type haplotypes ranged from 8.74% to 9.51% (mean, 9.10 ± 0.25%) (Tables 7, 8). Sequence divergences between the mangrove-type and the Pelabuhan ratu / Sulawesi-type haplotypes ranged from 10.03% to 10.54% (mean, 10.22 ± 0.24%) (Tables 7, 8). Sequence divergences between the large-type and Pelabuhan ratu / Sulawesi-type haplotypes ranged from 5.40% to 5.91% (mean, 5.78 ± 0.21%) (Tables 7, 8).

In the 16S gene tree, *F. cancrivora* formed two main clades, a mangrove-type clade and a large and Pelabuhan ratu / Sulawesi-type clade (Fig. 6). The mangrove-type clade consisted of four haplotypes, and the large-type clade consisted of six haplotypes. The Pelabuhan ratu / Sulawesi type had one haplotype (Table 3; Fig. 6).

In the mangrove type, haplotype M-I from Manila, Bangkok, Negros Island, and Hainan Island joined with haplotype M-II from Trat and Chantaburi, while haplotype M-III from Khulna joined with haplotype M-IV from Orissa. Haplotypes M-I and M-II were divergent from haplotypes M-III and M-IV, with strong bootstrap support (98/100/100/100) (Fig. 6). In the large type, haplotype L-I from Cianjur, Langkat, Selangor, Pelabuhan ratu, Tempilang, and Bogor was separated from other haplotypes with moderate bootstrap support (81/99/100/96). The other five haplotypes in the large type were haplotype L-II from Palembang, Lampung, Jambi, and Banyumas; haplotype L-III

Table 6. Average Nei's genetic distances (D) within and among three *F. cancrivora* types and the outgroup, *F. iskandari*, based on allozyme data.

Type	No.	1	2	3
Mangrove	1	0.023 ± 0.019		
Large	2	0.510 ± 0.025	0.006	
Pelabuhan ratu	3	0.586 ± 0.036	0.197 ± 0.012	–
F. iskandari	4	1.187 ± 0.02	1.153 ± 0.031	1.416

Table 8. Uncorrected "p" distances (mean values ± SD, in percent) for 16S haplotypes within and among three *F. cancrivora* types and the outgroup *F. iskandari*.

Type	No.	1	2	3
Mangrove	1	1.12 ± 0.68		
Large	2	9.10 ± 0.25	0.43 ± 0.12	
Pelabuhan ratu/Sulawesi	3	10.22 ± 0.24	5.78 ± 0.21	–
F. iskandari	4	15.88 ± 0.25	14.48 ± 0.21	14.14

Table 7. Uncorrected "p" distances among 16S haplotypes from *F. cancrivora* populations and the outgroup *F. iskandari*.

Type	No.	Haplotype	Population	1	2	3	4	5	6	7	8	9	10	11	12
Mangrove	1	M-I	Mani, Bang, Negros Isl.(Philippines), Hainan Isl.(China)	–											
	2	M-II	Trat, Chan	0.26	–										
	3	M-III	Khul	1.54	1.29	–									
	4	M-IV	Orissa (India)	1.80	1.54	0.26	–								
Large	5	L-I	Cian-A, Cian-B, Lang, Sela-1, Pela-B, Temp, Bogo	9.00	9.00	9.25	9.51	–							
	6	L-II	Pale, Lamp, Jamb, Bany	8.74	8.74	9.00	9.25	0.26	–						
	7	L-III	Pada, Paya, Pant	8.74	8.74	9.00	9.25	0.51	0.26	–					
	8	L-IV	Sela-2	9.00	9.00	9.25	9.51	0.51	0.26	0.51	–				
	9	L-V	Kalimantan Isl. (Indonesia)	9.00	9.00	9.25	9.51	0.51	0.26	0.51	0.51	–			
	10	L-VI	Jiadong (Taiwan)	9.00	9.00	9.25	9.51	0.51	0.26	0.51	0.51	0.51	–		
Pelabuhan ratu/Sulawesi	11	PS-I	Pela-A, Maka, Bone, Sinj, Siwa	10.03	10.03	10.28	10.54	5.40	5.66	5.91	5.91	5.91	5.91	–	
Outgroup	12	*F. iskandari*		15.68	15.68	15.94	16.20	14.65	14.40	14.40	14.65	14.65	14.14	14.14	–

from Padang, Payakumbuh, and Panti; haplotype L-IV from Selangor; haplotype L-V from Kalimantan Island; and haplotype L-VI from Jiadong (Fig. 6). The Pelabuhan ratu / Sulawesi type contained haplotype PS-I from Pelabuhan ratu, Makassar, Bone, Sinjai, and Siwa. Haplotype PS-I joined with the large type with low bootstrap support (65/63/82/68) (Fig. 6).

For the Cyt *b* gene (Tables 9, 10), sequence divergences between the outgroup and 18 haplotypes of *F. cancrivora* ranged from 19.57% to 21.90% (mean, 20.24 ± 0.73%). Sequence divergences among mangrove-type haplotypes ranged from 0.54% to 5.03% (mean, 2.78 ± 1.58%); those among large-type haplotypes ranged from 0.18% to 1.62% (mean, 0.76 ± 0.33%); and that beween the Pelabuhan ratu- / and Sulawesi-type haplotypes was 0.36% (Tables 9, 10). Sequence divergences between large-type and mangrove-type haplotypes ranged from 13.82% to 16.16% (mean, 14.64 ± 0.59%). Sequence divergences between large-type and Pelabuhan ratu / Sulawesi-type haplotypes ranged from 12.39% to 13.47% (mean, 12.88 ± 0.28%). Sequence divergences between mangrove-type and Pelabuhan ratu / Sulawesi-type haplotypes ranged from 15.98% to 17.24% (mean, 16.38 ± 049%) (Tables 9, 10).

In the phylogenetic tree for Cyt *b* (Fig. 7), *F. cancrivora* comprised two main clades, a strongly supported mangrove-type clade (BP = 98/100/100/100) and a poorly supported large plus Pelabuhan ratu / Sulawesi-types clade (BP = 66/67/78/80), comprising a strongly supported large-type subclade (BP = 85/99/100/100) and a strongly supported Pelabuhan ratu / Sulawesi-type subclade (BP = 100/100/100/100). In the mangrove-type clade, haplotypes M-1a, M-1b, and M-2 formed a subclade (BP = 87/95/99/97) (Fig. 7).

A haplotype network including 18 Cyt *b* haplotypes from 55 individuals (Fig. 8A) revealed three haplogroups of *F. cancrivora*, corresponding to three subclades observed in the phylogenetic trees based on allozyme and molecular data (Figs. 5, 6, 7). The mangrove-type haplogroup was separated from the large-type haplogroup and from the Pelabuhan ratu / Sulawesi-type haplogroup by 44 and 69 nucleotide substitutions, respectively. In the mangrove haplogroup, each population had only one haplotype: the Manila, Bangkok, and Khulna populations had M-1a, M-1b, and M-3, respectively. The Trat and Canthaburi populations had M-2 (Figs. 7, 8). In 17 populations of the large and Pelabuhan ratu / Sulawesi haplogroups, 13 populations had only one haplotype each, whereas the other four populations had two or three haplotypes (Fig. 8B). Among these populations, the Pelabuhan ratu population had three haplotypes (PS-1a, L-1c, and L-6) in the Pelabuhan ratu / Sulawesi haplogroup, and the Selangor, Payakumbuh, and

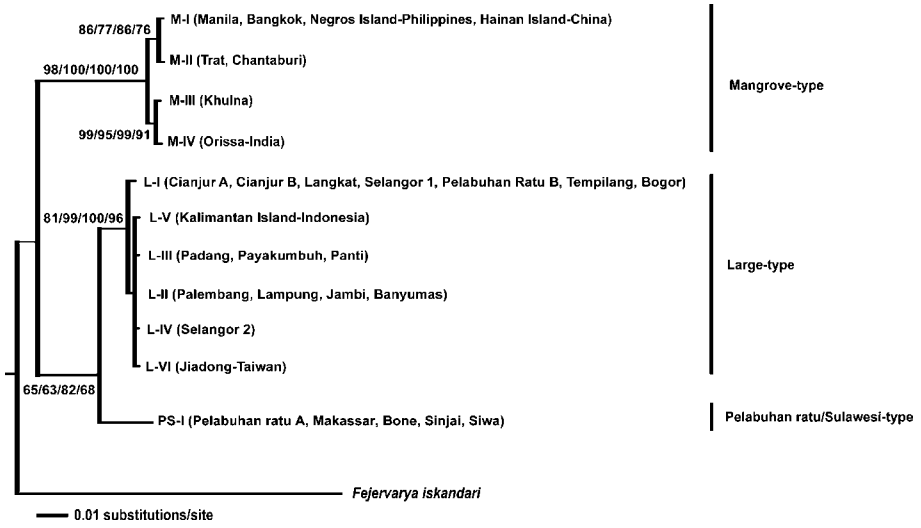

Fig. 6. Maximum-likelihood tree based on a 389-bp segment of the mitochondrial 16S rRNA gene, including 17 haplotypes from *F. cancrivora* and one haplotype from *F. iskandari* (outgroup). Bootstrap support values are listed in order for the ML/MP/NJ/BI analyses.

Table 9. Uncorrected "p" distances among Cyt *b* haplotypes from *F. cancrivora* populations and the outgroup *F. iskandari*.

Type	No.	Haplotype	Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Mangrove	1	M-1a	Mani	–																		
	2	M-1b	Bang	0.54	–																	
	3	M-2	Trat, Chan	2.33	1.80	–																
	4	M-3	Khul	3.77	5.03	3.23	–															
Large	5	L-1a	Bogo	14.36	15.62	14.18	14.54	–														
	6	L-1b	Cian-A	14.54	15.80	14.36	14.72	0.18	–													
	7	L-1c	Cian-B, Pela-B-1, Sela-1	14.54	15.80	14.36	14.72	0.18	0.36	–												
	8	L-1d	Lang	14.90	16.16	14.72	15.08	0.54	0.72	0.72	–											
	9	L-1e	Temp, Pale	14.00	15.08	13.82	14.18	1.08	1.26	1.26	1.62	–										
	10	L-2a	Lamp	14.18	15.44	14.00	14.36	0.36	0.54	0.54	0.90	1.08	–									
	11	L-2b	Jamb	14.00	15.08	13.82	14.18	0.72	0.90	0.90	1.26	0.72	0.72	–								
	12	L-2c	Bany	14.36	15.44	14.18	14.54	0.72	0.90	0.90	1.26	0.72	0.72	0.36	–							
	13	L-3	Pada, Paya-1, Pant	14.18	15.26	14.00	14.36	0.54	0.72	0.72	1.08	0.54	0.54	0.18	0.18	–						
	14	L-4	Sela-2	14.18	15.08	14.00	14.36	0.90	1.08	1.08	1.44	0.90	0.90	0.36	0.54	0.36	–					
	15	L-5	Paya-2	14.36	15.44	14.18	14.54	0.72	0.90	0.90	1.26	0.72	0.72	0.18	0.36	0.18	0.54	–				
	16	L-6	Pela-B-2	14.54	15.80	14.36	14.72	0.54	0.36	0.72	1.08	1.26	0.54	0.72	0.90	0.72	1.08	0.90	–			
Pelabuhan ratu/	17	PS-1a	Pela-A, Maka	16.16	17.06	15.98	15.98	12.93	13.11	13.11	13.47	12.75	12.93	12.57	12.75	12.57	12.39	12.75	13.11	–		
Sulawesi	18	PS-1b	Bone, Sinj, Siwa	16.34	17.24	16.16	16.16	12.93	13.11	13.11	13.47	12.75	12.93	12.57	12.75	12.57	12.39	12.75	13.11	0.36	–	
Outgroup	19	*F. iskandari*		21.19	21.90	21.01	20.47	19.57	19.75	19.75	19.57	19.93	19.75	19.75	19.57	19.75	19.75	19.93	20.11	21.19	21.19	–

Table 10. Uncorrected "p" distances (mean values ± SD, in percent) for Cyt *b* haplotypes within and among three *F. cancrivora* types and the outgroup *F. iskandari*.

Type	No.	1	2	3
Mangrove	1	2.78 ± 1.58		
Large	2	14.64 ± 0.59	0.76 ± 0.33	
Pelabuhan ratu/Sulawesi	3	16.38 ± 0.49	12.88 ± 0.28	0.36
F. iskandari	4	21.14 ± 0.59	19.78 ± 0.17	21.9

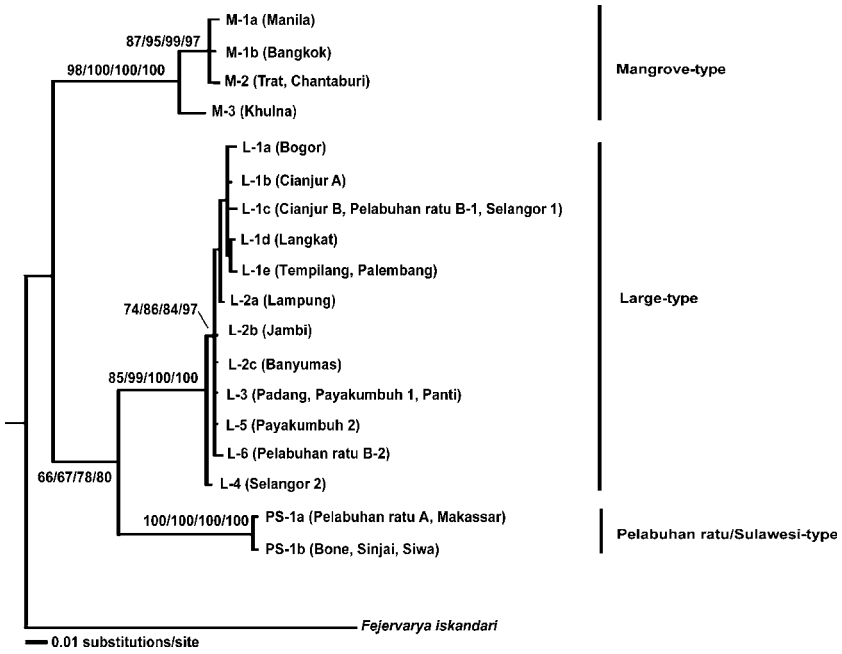

Fig. 7. Maximum-likelihood tree based on a 557-bp segment of the mitochondrial Cyt *b* gene, including 18 haplotypes from *F. cancrivora* and one from *F. iskandari* (outgroup). Bootstrap support values are listed in order for the ML/MP/NJ/BI analyses.

Cianjur populations each had two haplotypes (L-1c and L-4, L-3 and L-5, and L-1b and L-1c, respectively) in the large haplogroup (Fig. 8B).

DISCUSSION

Genetic distances and divergences among the three types of *F. cancrivora*

Genetic distances and percent sequence divergences for 16S and Cyt *b* among the three types of *F. cancrivora* were as follows (See Tables 6, 8, 10). The values between the mangrove type and large type were 0.510, 9.10%, and 14.64% for allozymes, 16S, and Cyt *b*, respectively; those between the mangrove type and Pelabuhan ratu /Sulawesi type were 0.586, 10.22%, and 16.38%; and those between the large type and Pelabuhan ratu / Sulawesi type were 0.197, 5.78%, and 12.88%.

Genetic distance and divergence have been reviewed at the species level for many frog species. Sasa et al. (1998) suggested a lower threshold of Nei's genetic distance (D = 0.30) for the evolution of hybrid inviability, based on data from 116 crosses involving 46 frog species. Vences et al. (2004) reported that differentiation among conspecific populations in African Malagasy frogs does not exceed 2.0% for 16S. Sumida et al. (1998) found that Cyt *b* sequence divergence in Japanese pond frogs ranges from 10.4% to 12.4% at the species level, and from 3.68% to 4.62% at the subspecies level. In Palearctic pond

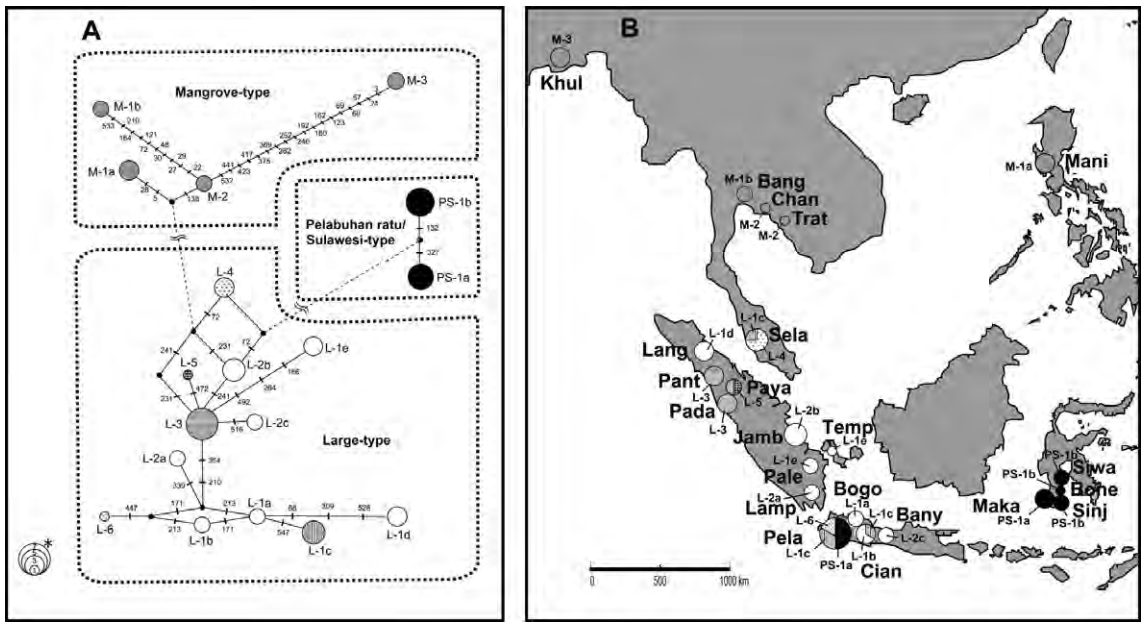

Fig. 8. **(A)** Haplotype network for 18 Cyt *b* haplotypes from 55 individuals. The haplotypes are represented by circles, with the number of individuals indicated by circles of different sizes; in the asterisked key to circle sizes at bottom left, the inner to outer circles represent 1, 3, 5, and 7 individuals, respectively. The transverse dashes on branches indicate substitutions between haplotypes, labeled with the position in the Cyt *b* gene alignment. Haplotype abbreviations correspond those in Table 3: M, mangrove type; L, large type; PS, Pelabuhan ratu / Sulawesi type. **(B)** Geographic distribution and frequency of the Cyt *b* haplotypes. A pie chart indicates the haplotype frequencies for each population.

frogs, Cyt *b* sequence divergences range from 9.50% to 20.45% at the species level (Sumida et al., 2000).

Toda et al. (1998) were the first to recognize two syntopically occurring, genetically divergent species (Nei's genetic distance = 0.458) in the *Fejervarya limnocharis* complex, in Java, Indonesia. Later, upon similarly recognizing these two species in sympatry in Java, Veith et al. (2001) described a new taxon known exclusively from Java, *F. iskandari*. Though hardly distinguishable morphologically, these two species (*F. limnocharis* and *F. iskandari*) showed substantial genetic differentiation, with a Nei's genetic distance of 0.316 and a 16S sequence divergence of 13.5% in studies by Veith's group. Djong et al. (2007a, b) recently found that these two species are isolated by complete hybrid inviability at the tadpole stage, with a genetic distance of 0.628–0.749, and sequence divergences of 10.8–11.0% for 16S and 18.6–18.8% for Cyt *b*. Islam et al. (2008a, b) observed three morphologically divergent species in sympatry in Mymensingh, Bangladesh, with substantial genetic divergence among them: Nei's genetic distance of 0.739–1.628; sequence divergences of 5.5–17.1% for 16S and 18.0–25.0% for Cyt *b*. These species are reproductively isolated by hybrid inviability or hybrid sterility. In a study of *Fejervarya*, Sumida et al. (2007) found members of the *Fejervarya* species complex to be diverged into two groups, the South Asian and East-Southeast Asian groups. These two groups had a Nei's genetic distance of 1.185–1.898, had sequence divergences of 19.3–21.9% for the 16S and 12S rRNA genes, and were reproductively isolated by complete hybrid inviability at the embryonic stage.

Based on these above, we can roughly conclude that a Nei's genetic distance greater than 0.3, and sequence divergences greater than 5.5% for 16S and 9.50% for Cyt *b*, are good indicators of species-level differences.

The genetic distances and percent sequence divergences among the three types of *F. cancrivora* in our study were mostly higher than the values above sufficient for the recognition of species differentiation. Our findings imply that each of the three types can be considered an independent species. This did not apply, however, to the genetic distance between the large type and the Pelabuhan ratu / Sulawesi type.

The large type as nominal *F. cancrivora*

Dubois and Ohler (2000) described a neotype for *F. cancrivora*, based on an examination of an individual male sample (No. FMNH 256688) with a snout vent length (SVL) of 68.2 mm, collected from a rice field in Cianjur, Java, Indonesia. If we compare this neotype with the large type, we find that the average male body size of the former (68.2 mm) is close to that of the latter (69.7 mm for the Cianjur population and 64.9 mm for the Selangor population) (Table 1). In the morphological description, Iskandar (1998) reported that *F. cancrivora* from the Java type locality reaches a body length of up to 120 mm, but is usually only about 100 mm long. In our data, large-type females from the Cianjur population range from 87.6 to 112.0 mm in length (Table 1). The neotype has a glandular fold and glandular warts on the back and upper parts. The skin texture of the large type is similar to that described for the neotype. The neotype was originally taken from a rice field in Cianjur, West Java (Dubois and Ohler, 2000), and one of our large-type populations was also sampled from a rice field in the same region. Based on similarities in morphology, habitat, and locality, we conclude that the *F. cancrivora* neotype is the same as our large-type *F. cancrivora*. This implies that the other two types might be undescribed species.

Mangrove type

Taylor (1962) described *F. cancrivora* based on an individual female sample (No. 34294) with an SVL of 68 mm, collected from the seashore at Anghin, Chon Buri, Thailand. The dorsal surface of skin from this female has numerous glandular warts, some forming elongate ridges and others forming small tubercles. This frog also had a strong tolerance for salt water (Taylor, 1962). In addition, Nutphund (2001) mentioned that *F. cancrivora* from mangrove swamps and brackish areas has smooth skin covering the chin, venter, and underside of the thighs. In view of its smaller body size and confinement to the mangrove habitat, we conclude that Taylor's *F. cancrivora* is not *F. cancrivora* sensu Dubois and Ohler (2000). In light of the above information, we assume that our mangrove type is close to Taylor's *F. cancrivora* (1962) and distinct from the *F. cancrivora* neotype described by Dubois and Ohler (2000). Given the great morphological and ecological differences between the mangrove type and neotype, we speculate that the mangrove type is not *F. cancrivora*, but an undescribed species.

Possible synonymy of *F. raja* with *F. cancrivora*

Iskandar (1998) also provided information on *F. raja* from Thailand, describing it on the basis of an extra-large specimen of *F. cancrivora*. According to Nutphund (2001), the natural habitat of *Fejervarya raja* (previously *Rana raja*) is rice fields, and it is distributed in Patthalung, Satun, and Songkhla Provinces in southern Thailand, and on the Malay Peninsula. Taylor (1962) reported *F. raja* is distributed in Pattani, Songkla, and Phatthalung in Thailand, and from Kuala Lumpur in Malaysia. The natural habitat of large-type *F. cancrivora* of the Cianjur and Selangor populations is rice fields. Selangor is near Kuala Lumpur in the Malay Peninsula, in what is thought to be the distributional region of *F. raja*. Nutphund (2001) reported that *F. raja* has a total body length of 120 mm, and Taylor (1962) described *F. raja* as a large species. The SVLs of the largest *F. raja* paratypes in the British Museum reach 62.0 mm for males and 121.0 mm for females. The SVLs of the other paratypes of *F. raja* are 84.0–88.0 mm for males, and 98.0–119.5 mm for females (Taylor, 1962). Among the large-type frogs included in our study, the largest female (from Cianjur, Indonesia) is 112.0 mm long (Table 1). Our largest females were within the size range of the *F. raja* female paratypes. The SVLs of the large-type frogs from these two localities (Cianjur and Selangor) were 57.0–76.2 (mean, 68.6 ± 5.4) mm for males and 58.0–112.0 (mean, 86.9 ± 16.2) mm for females. These values are larger than for the mangrove type and the Pelabuhan ratu /Sulawesi type. Given that the large-type frog is morphologically identical to the neotype of *F. cancrivora*, inhabits rice fields, and is comparable to *F. raja* in body size, it seems likely that the large-type frog is *F. cancrivora*, and that *F. raja* is a synonym of *F. cancrivora*.

Pelabuhan ratu / Sulawesi type

Based on genetic distances and sequence divergence

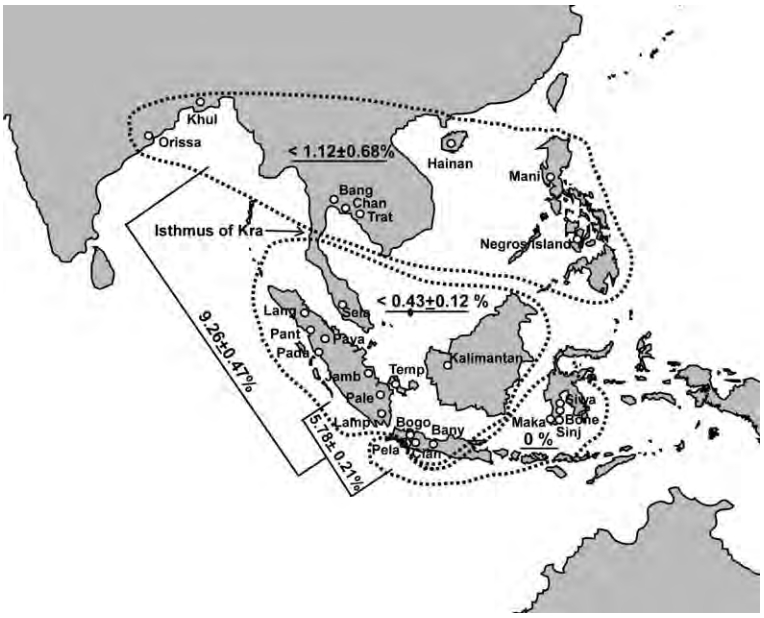

Fig. 9. Geographic distribution of the *F. cancrivora* types in Asia, based on our molecular data.

(Tables 6, 8, 10), the Pelabuhan ratu / Sulawesi type is somewhat closely related to the large type, but is greatly diverged from the mangrove type. The relative closeness of the Pelabuhan ratu / Sulawesi type to the large type is also evidenced by moderate bootstrap values (Figs. 5, 6, 7). The skin texture in the Pelabuhan ratu / Sulawesi type is similar to that in the large type. Additionally, the Pelabuhan ratu /Sulawesi type has mean SVLs of 43.5 mm and 50.7 mm for male and females, respectively, but these values are based on only a very limited sample size. These limited data suggest that this type is smaller than the large type or the mangrove type. According to Ota (personal communication), this type inhabits rice fields near the seashore at Pelabuhan ratu. This suggests that Pelabuhan ratu / Sulawesi type may not be *F. cancrivora*, but may be a distinct species related to the large type.

Haplotype PS-1a of the Pelabuhan ratu / Sulawesi type was found in two localities separated by the sea: at Pelabuhan ratu, Java, and at Makassar, Sulawesi (Figs. 8, 9). Iskandar and Colijn (2000) reported that *F. cancrivora* was introduced into Sulawesi, Ambon, and Papua. Inger (2005) presumed that *F. cancrivora* had been introduced into Sulawesi from Kalimantan Island. In contrast, our study shows that the haplotypes in southern Sulawesi differ genetically from those in Sunda land, including Kalimantan Island (Fig. 9). This suggests that *F. cancrivora* from southern Sulawesi was not introduced from Kalimantan Island. Noting that four localities in southern Sulawesi include the Pelabuhan ratu / Sulawesi type (Figs. 8, 9), we propose that this type originated from Sulawesi. Given that the Pelabuhan ratu and Makassar haplotypes are genetically identical, we believe that frogs from Makassar were also artificially introduced to Pelabuhan ratu. To support this contention, we point out that both Makassar and Pelabuhan ratu have long been port cities for the fisheries trade; even now, fishermen often travel between these cities to trade in fish. These frogs were very likely found accidentally at Pelabuhan ratu in the 1996 sampling, but we did not find them in 2008 at the same locality.

The results of our allozyme and molecular study suggest that it is valid to classify all three types as different species. Further morphological observations, crossing experiments, and the like will be necessary to clearly elucidate the taxonomic status of these three types. Some of these studies are already underway.

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