ZOOLOGICAL SCIENCE **24**: 360–375 (2007)

Genetic Relationships and Reproductive-isolation Mechanisms among the *Fejervarya limnocharis* Complex from Indonesia (Java) and Other Asian Countries

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In order to elucidate the genetic relationships and reproductive-isolation mechanisms among the *Fejervarya limnocharis* complex from Indonesia and other Asian countries, allozyme analyses and crossing experiments were carried out using 208 individuals from 21 localities in eight Asian countries. The allozyme analyses revealed that 17 enzymes examined were controlled by genes at 27 loci, and that 7.9 phenotypes were produced by 5.2 alleles on average. The two species recognized in *F. limnocharis* sensu lato from Southeast Asia (*i.e.*, *F. limnocharis* sensu stricto and *F. iskandari*) were found to occur sympatrically at three localities (Bogor, Cianjur and Malingping), all on Java, Indonesia. *Fejervaya iskandari* was dominant at each of these localities and showed substantial geographic genetic variation. Laboratory-produced hybrids between *F. limnocharis* and *F. iskandari* from Java became underdeveloped and died at the tadpole stage, suggesting that these species are completely isolated by hybrid inviability. Hybrids between topotypic *F. limnocharis* and the Malaysian and Japanese conspecific populations developed normally to metamorphosis. Likewise, hybrids between topotypic *F. iskandari* and the Thailand and Bangladesh conspecific populations also showed normal viability throughout larval development. The present allozyme analyses and crossing experiments strongly suggested the presence of two distinct forms, the large type and the small type, in the *F. limnocharis* complex from Asia, and further subdivision of the large type into the *F. limnocharis* assemblage and the *F. iskandari* assemblage. The small type was found in samples from India, Thailand, Bangladesh and Sri Lanka, and included at least three different species. The sample from Pilok, Thailand, was considered to represent an undescribed species.

Key words: reproductive isolation, *Fejervarya limnocharis*, Asia, *Fejervarya iskandari*, genetic relationship

INTRODUCTION

Fejervarya limnocharis sensu lato, known as *Rana limnocharis* or *Limnonectes limnocharis* until recently (Dubois and Ohler, 2000), is a common small-sized frog that occurs in and around lowland freshwater habitats including paddy fields. This species is often regarded as one of the most widely distributed frogs in Asia, from Pakistan to Japan through Indonesia and other Southeast Asian countries (Iskandar, 1998; Iskandar and Colijn, 2000; Dubois and Ohler, 2000). A number of different names have been proposed for some populations once assigned to *F.*

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doi:10.2108/zsj.24.360

limnocharis, but most of these names were immediately synonymized to *F. limnocharis* or were used for a long time but only as subspecific names for this species. This situation is partly due to the presence of more-or-less recognizable morphological variations of *F. limnocharis* sensu lato, and partly to the absence of diagnostic features to clearly discriminate certain local populations from other populations (Dubois, 1984, 1987; Inger and Voris, 2001).

Applications of analyses involving non-morphological characters have recently revealed that *F. limnocharis* sensu lato (henceforth referred to as the *F. limnocharis* complex) is a composite of many different species. For example, some populations from Nepal and India, formerly assigned to *F. limnocharis*, were shown to represent several different species chiefly on the basis of differences in their mating call patterns (Dubois, 1975; Dutta, 1997). Moreover, Toda *et al.* (1997, 1998a) provided allozyme data that clearly indicated cryptic taxonomic diversity in the East Asian and Indonesian populations of *F. limnocharis*. Subsequently, Veith *et al.* (2001) described one of the cryptic species detected by Toda *et al.* (1998a) in Java as a new species, *Fejervarya iskandari*, using additional allozyme and mitochondrial DNA sequence data. Although *F. iskandari* is morphologically hardly distinguishable from syntopic populations of *F. limnocharis* sensu stricto, allozyme data reveal a complete absence of gene flow between the two species (Toda *et al*., 1998a; Veith *et al*., 2001). This finding suggests that many more cryptic species may be detected in the *F. limnocharis* complex, if further comprehensive and systematic surveys are carried out for this broadly distributed species complex.

In the present study, we carried out allozyme analyses on samples of the *F. limnocharis* complex from eight Asian countries, including those from the type localities of *F. limnocharis* (Bogor, Java) and *F. iskandari* (Cianjur, Java), to elucidate the genetic relationships among the whole *F. limnocharis* complex from Asia. We also carried out crossing experiments for the available samples from five countries, including those from the type localities of *F. limnocharis* and *F. iskandari*, in order to obtain information regarding the mechanisms responsible for the complete reproductive isolation of the two species (see above) and other populations.

MATERIALS AND METHODS

Allozyme analyses

A total of 208 individuals of the *F. limnocharis* complex (92 male, 70 female and 46 immature frogs) from 21 localities in eight countries were used (Table 1 and Fig. 1). Samples of a congeneric and closely related species, *Fejervarya cancrivora*, were added to the analyses as an outgroup. Based on the snout-vent length (SVL), the specimens of the *F. limnocharis* complex from the eight countries could be classified into two groups, the large type and the small type (in Kruskal Wallis tests of the SVL, the significance level of $P<0.01$ for male $X^2=16.65$ and female $X^2=10.1$). The large type, recognized from 19 localities mainly in Southeast and East Asia, exhibited SVLs ranging from 35.0–55.0 mm (mean, 41.2±4.1 mm) for males and 37.0–56.3 mm (mean, 44.8±5.1 mm) for females. In contrast, the small type, recognized from four localities in South Asia and Pilok of Kanchanaburi, Thailand, had SVLs ranging from 27.0–31.9 mm (mean, 30.2±2.0 mm) for males and 30.8–43.4 mm (mean, 36.4±4.7mm) for females (Table 1).

Seventeen enzymes extracted from skeletal muscles were ana-

Table 1. Sampling localities, numbers of frogs examined, and population abbreviations used

Species	Locality		Number of frogs						Population abbreviation
	Country	Detailed locality	Total	Male	Mean SVL (mm) (range length)	Female	Mean SVL (mm) (range length)	Immature	
F. limnocharis complex	Indonesia	West Java, Bogor*	23	12	42.1 (35.6–47.0)	8	49.0 (46.4–53.8)	3	Bogo(lim), Bogo(isk)
		West Java, Cianjur*	58	30	41.0 (37.1–44.5)	18	45.6 (40.0–55.1)	10	Cian(lim), Cian(isk)
		West Java, Malingping	60	14	37.7 (35.0–40.3)	18	39.3 (37.0–42.7)	28	Mali(lim), Mali(isk)
	Malaysia	Kuala Lumpur, UMC*	3	2	43.4 (41.7–45.0)	1	48.4	0	Kual
		Kota Kinabalu	2	2	41.5 (39.9–41.7)	0	–	0	Kota
	Thailand	Bangkok	4	1	42.0	3	50.3 (48.0–54.1)	0	Bang
		Ranong	2	0	–	2	47.7 (45.2–50.0)	0	Rano
		Pathumthani, Nongsua*	9	2	40.0 (37.0–43.0)	3	43.8 (42.8–45.0)	4	Path
		Saraburi, Muaklek	2	2	38.0 (35.5–40.4)	0	–	0	Sara
		Kanchanaburi, Pilok, large type	2	0	–	2	52.3 (52.0–52.7)	0	Pilo(L)
		Kanchanaburi, Pilok, small type†	2	1	31.9	1	43.4	0	Pilo(S)
		Kanchanaburi, Thongphaphum*	1	1	48.4	0	–	0	Thon
	India	Mangalore, Bajipe†	2	1	28.4	1	31.2	0	Indi
	Sri Lanka	Bentota†	3	2	28.9 (27.0–30.8)	1	30.8	0	SriL
	Bangladesh	Mymensingh, BAU small type†	5	2	31.6 (31.4–31.8)	3	37.7 (37.6–37.8)	0	BAU(S)
		Mymensingh, BAU large type*	5	3	49.7 (48.7–51.0)	2	55.2 (54.1–56.3)	0	BAU(L)
	Taiwan	Chiai	5	5	37.6 (36.1–39.5)	0	–	0	Chia
		Taipei	1	0	–	1	45.6	0	Taip
		Pingdong	1	1	45.0	0	–	0	Ping
	Japan	Hiroshima, Higashihiroshima*	5	1	39.5	4	42.2 (41.6–43.1)	0	Hiro
		Okinawa, Iriomote Island	5	5	50.4 (46.0–55.0)	0	–	0	Irio
		Okinawa, Okinawa Island*	5	4	38.0 (37.0–39.0)	1	38.0	0	Okin
		Okinawa Tsuken Island	3	1	38.0	1	52.3	1	Tsuk
F. cancrivora	Indonesia	West Java, Bogor	3	0	–	0	–	3	Bogo(can)
		Total	211	92		70		49	

UMC, University of Malaya Campus; BAU, Bangladesh Agricultural University Campus.
* Used for crossing experiments.
† Small type

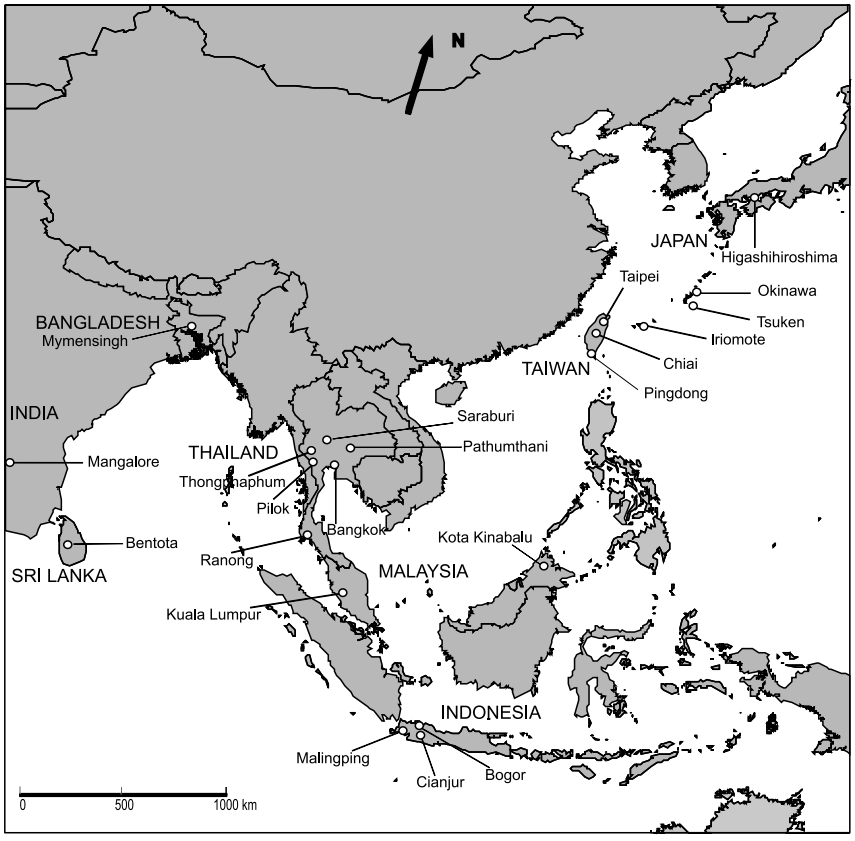

Fig. 1. Map showing sampling localities for 26 populations of the *Fejervarya limnocharis* complex from Asian countries.

Table 2. Enzyme, presumptive locus, enzyme commission number (E.C. No.), tissue and buffer system used for allozyme analysis in the present study

Enzyme	Locus	E.C. No.	Tissue	Buffer system
Aspartate amintrasferase	AAT-1	2.6.1.1	Muscle	T-C pH 7.0
Aspartate amintrasferase	AAT-2	2.6.1.1	Muscle	T-C pH 7.0
Adenosine deaminase	ADA	3.5.4.4	Muscle	T-C pH 7.0
Adenylate kinase	AK	2.7.4.3	Muscle	T-C pH 7.0
Aldolase	ALD	4.1.2.13	Muscle	T-C pH 7.0
Creatine kinase	CK	2.7.3.2	Muscle	TEB pH 8.0
Fumarase	FUM-1	4.2.1.2	Muscle	TEB pH 8.0
Fumarase	FUM-2	4.2.1.2	Muscle	TEB pH 8.0
α-Glycerophospate dehydrogenase	α-GDH	1.1.1.8	Muscle	T-C pH 6.0
Glucose-6-phosphate isomerase	GPI	5.3.1.9	Muscle	TEB pH 8.0
Isocitrate dehydrogenase	IDH-1	1.1.1.42	Muscle	T-C pH 7.0
Isocitrate dehydrogenase	IDH-2	1.1.1.42	Muscle	T-C pH 6.0
Lactate dehydrogenase	LDH-A	1.1.1.27	Muscle	T-C pH 6.0
Lactate dehydrogenase	LDH-B	1.1.1.27	Muscle	T-C pH 6.0
Malate dehydrogenase	MDH-1	1.1.1.37	Muscle	T-C pH 6.0
Malate dehydrogenase	MDH-2	1.1.1.37	Muscle	T-C pH 6.0
Malic enzyme	ME-1	1.1.1.40	Muscle	T-C pH 7.0
Malic enzyme	ME-2	1.1.1.40	Muscle	T-C pH 7.0
Mannose-6-phospate isomerase	MPI	5.3.1.8	Muscle	T-C pH 7.0
Peptidase (valyl-leucine)	PEP-A	3.4.11/13	Muscle	TEB pH 8.0
Peptidase (leucyl-glycyl-glysine)	PEP-B	3.4.11/13	Muscle	TEB pH 8.0
Peptidase (leucyl-alanine)	PEP-C	3.4.11/13	Muscle	TEB pH 8.0
Peptidase (leucyl-proline)	PEP-D	3.4.11/13	Muscle	TEB pH 8.0
6-Phosphogluconate dehydrogenase	6-PGD	1.1.1.44	Muscle	T-C pH 7.0
Phosphoglucomutase	PGM	5.4.2.2	Muscle	TEB pH 8.0
Superoxide dismutase	SOD-1	1.15.1.1	Muscle	TEB pH 8.0
Superoxide dismutase	SOD-2	1.15.1.1	Muscle	TEB pH 8.0

lyzed by horizontal starch gel electrophoresis following a procedure that was previously described in detail (Nishioka *et al*., 1980, 1992). The enzymes analyzed are listed in Table 2, together with the corresponding presumptive loci, enzyme commission numbers (E.C. Nos.), tissues and buffer system names. Each enzyme was detected using the agar–overlay method outlined by Harris and Hopkinson (1976). Multiple alleles at each locus were designated alphabetically in the order of fast to slow anodal mobility.

A locus was considered to be polymorphic when each of multiple alleles existed at a frequency of more than 1% at the locus. The genetic variability in each population was quantified by calculating the mean proportion of heterozygous loci per individual specimen (*H*), proportion of polymorphic loci per population (*P*) and mean number of alleles per locus (*A*) (Lewontin, 1974; Berg and Hamrich, 1997). The syntopic species in one locality was detected based on a diagnostic allele according to Toda *et al*. (1998a) and Veith *et al*. (2001). The genetic distance (D) and genetic identity (I), defined by Nei (1972), were also calculated to evaluate the genetic relationships among populations. Estimates of F-statistics (F_{is}, F_{st} and F_{it}) for groups or multiple populations (Hartl and Clark, 1989) were carried out for each group. All parameters were analyzed using Popgene 1.31 software. The D-value matrices from pairwise comparisons of samples were transferred to PHYLIP 3.65 software in order to build a neighbor-joining (NJ) tree (Saitou and Nei, 1987). Bootstrap (BT) values (Felsenstein, 1985) were also calculated with PHYLIP 3.65, based on the Nei's (1972) genetic distances with 1,000 replicates.

Crossing experiments

Crossing experiments were carried out by artificial insemination among 10 large-type populations from 5 countries (Kawamura *et al.*, 1981) (Table 1). A total of 36 and 19 combinations were carried out in the first and second experiments, respectively (Tables 7 and 8). Ovulation was induced by injecting bullfrog pituitaries into the body cavity of each female. The tadpoles were fed on boiled spinach.

RESULTS

Allozyme analyses

The electrophoretic patterns revealed that the enzymes examined were controlled by genes at 27 presumptive loci (Table 3). Four of these loci (MPI, LDH-B, MDH-1 and ADA) had high numbers of phenotypes and alleles, while five loci (AAT-2, AAT-1, AK, FUM-2 and SOD-1) had low numbers. Among the 27 loci, there were 7.9 phenotypes and 5.2 alleles on average (Table 3).

Analysis of the resultant allelic data for the 27 loci revealed that *F. iskandari* and *F. limnocharis* had diagnostic alleles at 11 loci (AAT-2, FUM-2, LDH-B, MDH-2, ME-2, MPI, PEP-A, PEP-C, PEP-D, 6PGD and PGM), and were present in each of three localities, namely the Bogor, Cianjur and Malingping populations. The allele frequency at each locus is shown in Table 4. The geographic distributions of the PGM and LDH-B alleles among 26 populations of the *F. limnocharis* complex are shown in Fig. 2. Among the 26 populations, the mean proportion of heterozygous loci per individual (*H*) was 7.7% (range, 0–16.7%), the mean proportion of polymorphic loci per population (*P*) was 21.5% (range, 0–63.0%) and the mean number of alleles per locus (*A*) was 1.23 (range, 1.00–2.00) (Table 5).

The F_{st} values calculated among the large-type populations of the *F. limnocharis* complex were 0.766 on average, ranging from 0.010 for the AK locus to 0.938 for the MDH-2 locus. The F_{is} values were low, being –0.090 on average, with a negative value for 18 polymorphic loci and a positive value for 6. The mean F_{it} value was 0.745. The F_{st} values among the small-type populations were 0.843 on average, ranging from 0.130 for the AAT-1 locus to 0.941 for the ME-1 locus. The F_{is} values were low, –0.085 on average, with a negative value for 10 polymorphic loci and a positive value for 3. The mean F_{it} value was 0.830.

Table 3. Numbers of alleles and phenotypes at 27 loci. See Table 2 for abbreviations of loci

Locus	No. of phenotypes	No. of alleles
AAT-1	3	3
AAT-2	2	2
ADA	17	10
AK	3	3
ALD	5	3
CK	4	3
FUM-1	5	4
FUM-2	3	3
α-GDH	6	4
GPI	6	4
IDH-1	4	3
IDH-2	8	7
LDH-A	5	3
LDH-B	20	10
MDH-1	18	12
MDH-2	5	4
ME-1	10	7
ME-2	7	4
MPI	23	14
PEP-A	7	4
PEP-B	6	4
PEP-C	7	6
PEP-D	9	5
6-PGD	11	7
PGM	8	5
SOD-1	3	3
SOD-2	7	4
Average	7.9	5.2

The genetic distances (D) and genetic identities (I) among 26 populations of the *F. limnocharis* complex are shown in Table 6. The D values were 0.846–1.694 between the *F. limnocharis* complex and the outgroup *F. cancrivora*. Furthermore, the D values were 0.628–0.749 between two sympatric species, *F. limnocharis* and *F. iskandari*, from Java; 0.012–0.032 among three populations of *F. iskandari* from Java; 0.470–0.614 between *F. iskandari* from Java and the Malaysia populations; 0.285–0.431 between *F. iskandari* from Java and the Thailand populations; 0.249–0.291 between *F. iskandari* from Java and the Bangladesh populations; and 0.300–0.455 between *F. iskandari* from Java and the Taiwanese-Japanese populations. The distances were 0.046–0.076 among three populations of *F. limnocharis* from Java; 0.410–0.526 between *F. limnocharis* from Java and the Malaysia populations; 0.250–0.572 between *F. limnocharis* from Java and the Thailand populations; 0.509–0.579 between *F. limnocharis* from Java and the Bangladesh populations; and 0.365–0.638 between *F. limnocharis* from Java and the Taiwanese-Japanese populations. The D values among six populations from Thailand were 0.056–0.197, while those among seven populations from Taiwan and Japan were 0.027–0.290. The genetic distances between the small-type populations, including those from

Table 4. Allele frequencies at 27 loci in 26 Asian populations of the *F. limnocahris* complex. See Table 1 and Table 2 for abbreviations for populations and loci, respectively

	Population												
Locus	Bogo (isk)	Cian (isk)	Mali (isk)	Bogo (lim)	Cian (lim)	Mali (lim)	Kota	Kual	Bang	Rano	Path	Sara	Pilo (L)
AAT-1	b (1.000)	a (0.947) b (0.053)	a (0.990) b (0.010)	b (1.000)	b (1.000)	b (1.000)	a (0.250) b (0.750)	a (0.167) b (0.833)	b (1.000)	a (0.250) b (0.750)	a (0.056) b (0.944)	b (1.000)	b (1.000)
AAT-2	b (1.000)	b (1.000)	b (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)
ADA	c (0.088) d (0.088) f (0.794) h (0.029)	d (0.061) f (0.938)	d (0.073) f (0.667) g (0.260)	h (0.917) i (0.083)	h (1.000)	g (0.125) h (0.708) i (0.167)	i (1.000)	h (1.000)	f (0.125) h (0.625 l (0.125) j (0.125)	d (0.250) f (0.250) h (0.500)	f (0.333) h (0.667)	f (0.250) h (0.750)	f (1.000)
AK	c (1.000)	c (1.000)	a (0.010) b (0.990)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	C (1.000)	c (1.000)	c (1.000)
ALD	b (0.970) c (0.030)	a (0.018) b (0.614) c (0.368)	b (0.854) c (0.146)	b (0.093) c (0.917)	c (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	a (0.750) b (0.250)
CK	a (0.030) b (0.970)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)
FUM-1	c (1.000)	a (0.035) b (0.965)	a (0.042) b (0.958)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	b (0.167) c (0.833)	c (1.000)	c (1.000)
FUM-2	c (1.000)	c (1.000)	c (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)
α-GDH	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	b (0.333) c (0.667)	c (0.875)) d (0.125)	c (0.750) d (0.250)	b (0.056) c (0.944)	c (0.750) d (0.250)	b (0.250) c (0.750)
GPI	b (1.000)	b (1.000)	b (0.990) c (0.010)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	c (1.000)	c (1.000)	b (1.000)	b (0.889) c (0.111)	c (1.000)	c (1.000)
IDH-1	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	a (0.056) b (0.833) c (0.111)	b (1.000)	b (1.000)
IDH-2	f (1.000)	f (1.000)	e (0.010) f (0.906) g (0.083)	f (1.000)	f (1.000)	f (1.000)	f (1.000)	f (1.000)	f (1.000)	f (1.000)	a (0.056) f (0.888) g (0.056)	f (1.000)	f (1.000)
LDH-A	b (0.088) c (0.912)	c (1.000)	c (1.000)	a (0.167) b (0.833)	b (1.000)	b (1.000)	a (1.000)	a (1.000)	b (1.000)	b (1.000)	b (1.000)	b (0.750) c (0.250)	b (1.000)
LDH-B	a (0.029) b (0.765) c (0.206)	a (0.105) b (0.456) c (0.439)	b (0.490) c (0.510)	d (0.083) f (0.917)	f (1.000)	e (0.458) f (0.500) g (0.042)	g (1.000)	g (1.000)	c (0.125) e (0.500) f (0.125) j (0.250)	e (1.000)	e (0.722) g (0.111) j (0.167)	e (1.000)	e (0.250) j (0.750)
MDH-1	d (0.412) f (0.176) h (0.323) i (0.083)	d (0.675) f (0.018) h (0.289) l (0.018)	b (0.125) d (0.677) h (0.198)	d (0.833) f (0.167)	d (1.000)	d (1.000)	d (1.000)	d (1.000)	d (0.250) f (0.125) h (0.625)	d (0.500) h (0.500)	b (0.056) d (0.056) e (0.278) f (0.167) h (0.444)	f (0.750) g (0.250)	d (1.000)
MDH-2	c (1.000)	c (1.000)	c (1.000)	a (1.000)	a (1.000)	a (1.000)	a (0.750) b (0.250)	a (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)
ME-1	e (1.000)	e (0.947) f (0.053)	c (0.010) e (0.990)	e (1.000)	e (1.000)	e (1.000)	e (1.000)	e (0.333) g (0.667)	e (0.750) g (0.250)	e (1.000)	e(0.944) g (0.056)	c (0.250) e (0.750)	e (1.000)
ME-2	b (0.942) c (0.029) d (0.029)	b (0.991) d (0.009)	b (0.958) d (0.042)	b (0.083) d (0.917)	d (1.000)	d (1.000)	b (1.000)	b (1.000)	b (1.000)	b (0.750) d (0.250)	a (0.167) b (0.833)	b (1.000)	b (1.000)
MPI	h (0.676) j (0.323)	g (0.017) h (0.597) j (0.386)	h (0.302) j (0.698)	i (0.083) k (0.834) l (0.083)	i (1.000)	i (0.250) k (0.750)	j (1.000)	j (1.000)	h (0.125) l (0.125) k (0.625) l (0.125)	i (0.500) k (0.250) l (0.250)	g (0.111) h (0.333) i (0.222) k (0.278) l (0.056)	l (0.250) j (0.750)	g (0.750) k (0.250)
PEP-A	b (1.000)	b (1.000)	b (1.000)	c (0.750) d (0.250)	c (1.000)	c (0.875) d (0.125)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	a (0.167) b (0.778) c (0.056)	b (0.750) c (0.250)	b (1.000)
PEP-B	c (1.000)	c (0.991) d (0.009)	c (0.375) d (0.625)	c (1.000)	c (1.000)	b (0.333) c (0.667)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)
PEP-C	c (1.000)	c (1.000)	c (1.000)	a (0.083) b (0.917)	b (1.000)	b (1.000)	b (0.250) c (1.000)	b (0.333) c (0.667)	c (1.000)	c (1.000)	b (0.056) c (0.944)	c (1.000)	c (1.000)
PEP-D	b (1.000)	b (0.974) c (0.026)	b (1.000)	c (0.750) d (0.250)	c (1.000)	c (1.000)	d (1.000)	d (1.000)	c (1.000)	c (1.000)	c (0.833) d (0.167)	c (1.000)	c (1.000)
6PGD	d (1.000)	d (0.895) e (0.105)	d (0.990) e (0.010)	c (1.000)	c (1.000)	c (1.000)	b (0.250) c (0.750)	c (1.000)	d (1.000)	c (1.000)	c (0.056) d (0.944)	d (1.000)	d (1.000)
PGM	a (1.000)	a (1.000)	a (0.958) b (0.042)	b (1.000)	b (1.000)	b (1.000)	c (1.000)	b (0.833) c (0.167)	c (0.875)) d (0.125)	c (1.000)	c (0.722) d (0.278)	c (1.000)	c (1.000)
SOD-1	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)
SOD-2	c (0.029) d (0.971)	c (0.035) d (0.917)	c (0.010) d (0.990)	d (1.000)	d (1.000)	c (0.083) d (0.917)	d (1.000)	d (1.000)	d (1.000)	d (1.000)	d (1.000)	d (1.000)	d (1.000)

Thon	Pilo (S)	Indi	SriL	BAU (S)	BAU (L)	Chia	Taip	Ping	Hiro	Irio	Okin	Tsuk	Bogo(can)
b (1.000)	b (1.000)	b (1.000)	b (1.000)	a (0.100) b (0.900)	a (0.100) b (0.900)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)
a (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)	a (1.000)
f (1.000)	f (0.5.00) h (0.500)	e (1.000)	c (1.000)	b (0.100) c (0.900)	h (1.000)	h (1.000)	h (1.000)	h (1.000)	h (1.000)	h (1.000)	h (1.000)	h (1.000)	a (1.000)
c (1.000)	a (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	a (1.000)
b (1.000)	c (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)
b (1.000)	c (1.000)	a (1.000)	a (1.000)	a (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)
c (1.000)	c (1.000)	c (1.000)	d (1.000)	d (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	d (1.000)
a (1.000)	B (1.000)	a (1.000)	a (1.000)	a (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)
b (0.500) c (0.500)	b (0.500) c (0.500)	a (1.000)	a (1.000)	a (0.900) c (0.100)	b (0.100) c (0.900)	c (1.000)	b (0.500) c (0.500)	c (1.000)	c (1.000)	b (0.100) c (0.900)	c (1.000)	b (0.333) c (0.667)	d (1.000)
c (1.000)	b (1.000)	a (0.750) b (0.250)	a (1.000)	a (1.000)	b (1.000)	c (1.000)	c (1.000)	c (1.000)	b (1.000)	c (1.000)	c (0.900) d (0.100)	c (0.667) d (0.333)	c (1.000)
b (1.000)	b (1.000)	b (1.000)	b (0.833) c (0.167)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (0.400) c (0.600)	b (1.000)	b (1.000)	b (1.000)	b (1.000)
f (1.000)	e (1.000)	c (0.500) f (0.500)	b (0.167) c (0.833)	c (1.000)	f (1.000)	f (1.000)	f (1.000)	f (1.000)	f (1.000)	f (1.000)	f (1.000)	f (1.000)	d (1.000)
b (1.000)	c (1.000)	b (1.000)	b (1.000)	b (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	b (1.000)
e (1.000)	c (1.000)	a (1.000)	a (0.333) h (0.667)	d (1.000)	b (1.000)	f (1.000)	f (1.000)	f (1.000)	f (1.000)	f (1.000)	f (0.700) l (0.300)	f (1.000)	h (1.000)
f (1.000)	d (0.250) h (0.750)	k (0.750) l (0.250)	k (1.000)	j (0.100) k (0.900)	d (0.100) h (0.900)	a (0.100) c (0.700) e (0.200)	c(1.000)	c(1.000)	c (1.000)	a (0.100) c (0.900)	a (0.200) c (0.800)	a (0.167) c (0.853)	f (1.000)
c (1.000)	c (1.000)	d (1.000)	d (1.000)	d (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	a (1.000)	c (0.900) d (0.100)	c (1.000)	c (1.000)	c (1.000)
e (1.000)	e (1.000)	b (1.000)	a (1.000)	d (0.100) f (0.900)	e (1.000)	e (1.000)	e (1.000)	e (1.000)	e (1.000)	e (1.000)	e (1.000)	e (1.000)	c (1.000)
b (1.000)	b (1.000)	c (1.000)	c (1.000)	c (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	c (1.000)	b (1.000)	b (1.000)	a (1.000)
i (1.000)	f (0.500) l (0.250) n (0.250)	a (0.250) b (0.500) c (0.250)	b (0.167) e (0.167) i (0.667)	b (1.000)	d (1.000)	g (1.000)	g (1.000)	g (1.000)	g (1.000)	i (0.800) m (0.200)	g (1.000)	g (1.000)	f (0.500) l (0.500)
b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	b (1.000)	a (0.100) b (0.900)	b (1.000)	a (0.100) b (0.900)	b (0.667) c (0.333)	b (0.667) c (0.333)
c (1.000)	a (1.000)	a (1.000)	b (1.000)	b (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	d (1.000)
c (1.000)	d (1.000)	d (1.000)	c (1.000)	e (1.000)	c (1.000)	b (0.100) c (0.900)	c (1.000)	b (0.500) c (0.500)	b (0.300) c (0.700)	b (1.000)	b (1.000)	b (1.000)	f (1.000)
c (0.500) d (0.500)	e (1.000)	e (1.000)	a (1.000)	a (0.900) b (0.100)	c (0.100) d (0.800) e (0.100)	c (1.000)	b (0.500) c (0.500)	c (1.000)	b (0.300) c (0.700)	c (0.100) d (0.900)	b (0.200) c (0.800)	c (1.000)	d (1.000)
d (1.000)	e (1.000)	f (1.000)	a (1.000)	b (0.400) f (0.600)	b (0.100) c (0.900)	c (1.000)	d (1.000)	d (1.000)	c (1.000)	d (1.000)	c (1.000)	c (0.833) d (0.167)	g (1.000)
c (1.000)	d (1.000)	c (1.000)	d (1.000)	d (1.000)	c (1.000)	c (1.000)	c (1.000)	c (1.000)	c (0.600) d (0.400)	c (1.000)	c (0.500) d (0.500)	c (1.000)	e (1.000)
a (1.000) d (1.000)	c (1.000) a (1.000)	b (1.000) b (0.750) d (0.250)	c (1.000) a (1.000)	c (1.000) a (1.000)	a (1.000) d (1.000)	a (1.000) d (1.000)	a (1.000) d (1.000)	a (1.000) d (1.000)	a (1.000) d (1.000)	a (1.000) d (1.000)	a (1.000) d (1.000)	a (1.000) d (1.000)	a (1.000) c (1.000)

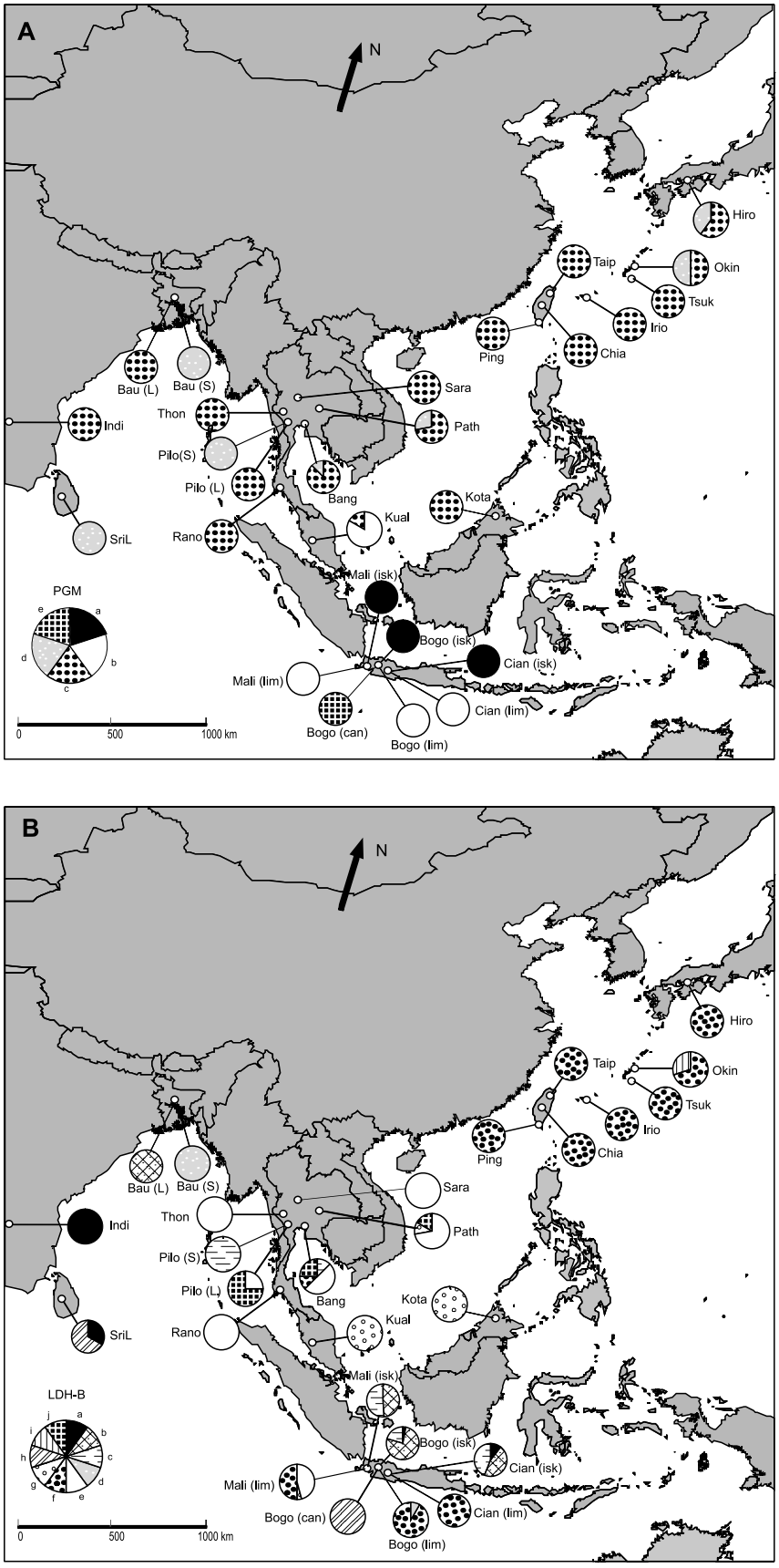

Fig. 2. Geographic distributions of the **(A)** PGM and **(B)** LDH-B alleles among 26 Asian populations of the *Fejervarya limnocharis* complex. See Table 1 for population abbreviations.

the Pilok (Kanchanaburi, Thailand), Mangalore (India), Bentota (Sri Lanka) and Mymensingh (BAU(S), Bangladesh) populations, and the large-type populations were 0.709–1.730, while the D values among the small-type populations were 0.181–1.310.

The NJ tree based on genetic distances revealed that the *F. limnocharis* complex diverged into two groups, the large-type and small-type groups, with BT values of 100%

Table 5. Genetic variabilities at 27 loci in 26 Asian populations of the *F. limnocharis* complex

Species	Country, Locality	Population abbreviation	Sample size	Mean proportion of heterozygous loci per individual (%)	Proportion of polymorphic loci per population (%)	Mean number of alleles per locus
F. iskandari	Indonesia, Bogor	Bogo (isk)	17	8.1 (8.7)	33.3	1.55
	Indonesia, Cianjur	Cian (isk)	57	9.8 (10.2)	48.2	1.67
	Indonesia, Malingping	Mali (isk)	48	11.0 (11.4)	59.3	1.70
F. limnocharis	Indonesia, Bogor	Bogo (lim)	6	9.9 (9.2)	37.0	1.41
complex	Indonesia, Cianjur	Cian (lim)	1	0 (0)	0	1.00
	Indonesia, Malingping	Mali (lim)	12	9.0 (7.5)	22.2	1.30
	Malaysia, Kota Kinabalu	Kota	2	7.4 (5.6)	14.8	1.15
	Malaysia, Kuala Lumpur	Kual	3	9.9 (7.0)	18.5	1.18
	Thailand, Bangkok	Bang	4	13.0 (11.9)	25.9	1.52
	Thailand, Ranong	Rano	2	16.7 (10.7)	22.2	1.30
	Thailand, Pathumthani	Path	9	15.2 (19.0)	63.0	2.00
	Thailand, Saraburi	Sara	2	13.0 (9.7)	25.9	1.26
	Thailand, Pilok	Pilo (L)	2	7.4 (5.5)	14.8	1.15
	Thailand, Thongphaphum	Thon	1	7.4 (3.7)	7.4	1.07
	Thailand, Pilok	Pilo (S)	2	9.3 (7.4)	14.8	1.18
	India, Mangalore	Indi	2	7.4 (8.3)	18.5	1.22
	Sri Lanka, Bentota	SriL	3	6.2 (4.9)	14.8	1.18
	Bangladesh, Mymensingh	BAU (S)	5	5.9 (5.8)	25.9	1.26
	Bangladesh, Mymensingh	BAU (L)	5	3.7 (4.4)	18.5	1.22
	Taiwan, Chiai	Chia	5	3.0 (2.4)	7.4	1.11
	Taiwan, Taipei	Taip	1	7.4 (3.7)	7.4	1.07
	Taiwan, Pingdong	Ping	1	3.7 (1.9)	3.7	1.04
	Japan, Higashihiroshima	Hiro	5	6.7 (7.3)	18.5	1.18
	Japan, Iriomote	Irio	5	4.4 (3.9)	18.5	1.18
	Japan, Okinawa	Okin	5	5.9 (7.1)	22.2	1.22
	Japan, Tsuken	Tsuk	3	9.9 (7.0)	18.5	1.19
	Average		8.0	7.7 (6.8)	21.5	1.23

Parentheses show expected values.

Tabel 6. Nei's (1972) genetic distance (below diagonal) and genetic identity (above diagonal) among 26 Asian populations of the Fejervarya limnocahris complex. See Table 1 for population abbreviations

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1. Bogo (isk)	–	0.988	0.968	0.515	0.487	0.534	0.625	0.551	0.708	0.691	0.752	0.694	0.686	0.689	0.456	0.331	0.281	0.249	0.780	0.693	0.741	0.715	0.687	0.658	0.678	0.654	0.313
2. Cian (isk)	0.012	–	0.973	0.528	0.504	0.524	0.616	0.543	0.691	0.680	0.731	0.674	0.695	0.669	0.492	0.311	0.257	0.224	0.760	0.687	0.730	0.705	0.680	0.643	0.671	0.646	0.290
3. Mali (isk)	0.032	0.027	–	0.498	0.473	0.516	0.617	0.541	0.677	0.662	0.712	0.673	0.668	0.650	0.480	0.318	0.269	0.237	0.748	0.675	0.724	0.697	0.668	0.636	0.660	0.634	0.329
4. Bogo (lim)	0.664	0.638	0.698	–	0.956	0.944	0.646	0.664	0.652	0.724	0.691	0.614	0.619	0.564	0.290	0.281	0.192	0.203	0.598	0.623	0.550	0.597	0.695	0.599	0.655	0.658	0.259
5. Cian (lim)	0.720	0.685	0.749	0.046	–	0.927	0.591	0.615	0.622	0.715	0.668	0.604	0.600	0.566	0.279	0.271	0.209	0.195	0.561	0.604	0.528	0.580	0.670	0.597	0.634	0.647	0.239
6. Mali (lim)	0.628	0.646	0.661	0.058	0.076	–	0.652	0.659	0.689	0.779	0.734	0.659	0.636	0.607	0.244	0.321	0.244	0.249	0.601	0.626	0.548	0.601	0.694	0.595	0.664	0.669	0.289
7. Kota	0.470	0.484	0.483	0.437	0.526	0.428	–	0.866	0.695	0.751	0.750	0.702	0.647	0.680	0.287	0.348	0.248	0.232	0.733	0.671	0.621	0.625	0.703	0.614	0.639	0.631	0.288
8. Kual	0.596	0.612	0.614	0.410	0.487	0.417	0.144	–	0.707	0.674	0.688	0.714	0.626	0.665	0.246	0.311	0.250	0.231	0.674	0.693	0.646	0.640	0.670	0.635	0.679	0.653	0.332
9. Bang	0.345	0.370	0.390	0.427	0.476	0.373	0.363	0.347	–	0.891	0.945	0.945	0.907	0.902	0.345	0.397	0.326	0.293	0.752	0.805	0.817	0.827	0.683	0.735	0.755	0.755	0.403
10. Rano	0.370	0.386	0.412	0.323	0.335	0.250	0.287	0.395	0.115	–	0.930	0.863	0.822	0.844	0.359	0.399	0.318	0.278	0.803	0.779	0.714	0.722	0.735	0.652	0.730	0.731	0.351
11. Path	0.285	0.314	0.340	0.369	0.404	0.310	0.288	0.374	0.056	0.073	–	0.916	0.865	0.890	0.372	0.388	0.330	0.294	0.788	0.767	0.775	0.785	0.728	0.709	0.730	0.731	0.375
12. Sara	0.366	0.394	0.397	0.488	0.504	0.417	0.354	0.337	0.057	0.147	0.088	–	0.865	0.929	0.309	0.377	0.300	0.264	0.725	0.795	0.809	0.816	0.671	0.731	0.743	0.755	0.420
13. Pilo (L)	0.377	0.364	0.404	0.480	0.510	0.453	0.435	0.469	0.097	0.197	0.145	0.145	–	0.874	0.327	0.358	0.277	0.248	0.665	0.758	0.777	0.779	0.635	0.659	0.706	0.715	0.346
14. Thon	0.373	0.403	0.431	0.572	0.569	0.500	0.386	0.408	0.103	0.170	0.116	0.074	0.135	–	0.314	0.384	0.329	0.272	0.689	0.722	0.760	0.743	0.606	0.720	0.674	0.685	0.429
15. Pilo (S)	0.786	0.709	0.733	1.237	1.276	1.410	1.248	1.402	1.065	1.025	0.989	1.175	1.119	1.159	–	0.332	0.270	0.276	0.421	0.351	0.353	0.350	0.348	0.310	0.375	0.346	0.198
16. Indi	1.107	1.167	1.145	1.270	1.306	1.137	1.055	1.168	0.925	0.919	0.946	0.977	1.027	0.956	1.104	–	0.556	0.591	0.317	0.303	0.306	0.303	0.277	0.349	0.287	0.298	0.184
17. SriL	1.269	1.359	1.313	1.652	1.566	1.413	1.396	1.388	1.122	1.145	1.110	1.205	1.284	1.112	1.310	0.588	–	0.835	0.223	0.220	0.226	0.205	0.214	0.251	0.206	0.177	0.245
18. BAU (S)	1.392	1.495	1.442	1.594	1.637	1.392	1.463	1.466	1.227	1.282	1.226	1.332	1.393	1.301	1.289	0.526	0.181	–	0.195	0.197	0.194	0.196	0.189	0.237	0.217	0.187	0.216
19. BAU (L)	0.249	0.274	0.291	0.514	0.579	0.509	0.311	0.394	0.285	0.220	0.239	0.322	0.408	0.372	0.864	1.150	1.502	1.633	–	0.828	0.790	0.780	0.797	0.748	0.789	0.784	0.361
20. Chia	0.367	0.376	0.393	0.473	0.505	0.469	0.399	0.367	0.217	0.250	0.265	0.230	0.277	0.326	1.048	1.193	1.512	1.625	0.188	–	0.940	0.954	0.894	0.827	0.952	0.954	0.369
21. Taip	0.300	0.314	0.324	0.599	0.638	0.602	0.476	0.437	0.202	0.337	0.255	0.212	0.253	0.275	1.041	1.186	1.488	1.638	0.236	0.062	–	0.972	0.847	0.860	0.893	0.913	0.371
22. Ping	0.336	0.350	0.361	0.516	0.546	0.510	0.470	0.447	0.191	0.326	0.242	0.203	0.250	0.297	1.051	1.195	1.587	1.628	0.248	0.048	0.029	–	0.858	0.888	0.935	0.950	0.368
23. Hiro	0.375	0.386	0.404	0.365	0.401	0.365	0.353	0.400	0.382	0.308	0.317	0.399	0.454	0.501	1.056	1.283	1.540	1.666	0.226	0.112	0.166	0.153	–	0.748	0.884	0.875	0.274
24. Irio	0.418	0.442	0.452	0.513	0.516	0.519	0.488	0.455	0.308	0.427	0.344	0.314	0.417	0.329	1.171	1.052	1.384	1.438	0.291	0.190	0.151	0.118	0.290	–	0.847	0.858	0.402
25. Okin	0.388	0.399	0.416	0.423	0.456	0.410	0.449	0.387	0.281	0.315	0.315	0.297	0.348	0.395	0.980	1.248	1.579	1.528	0.237	0.049	0.113	0.067	0.124	0.166	–	0.974	0.372
26. Tsuk	0.425	0.437	0.455	0.418	0.436	0.402	0.461	0.427	0.281	0.313	0.313	0.282	0.336	0.379	1.062	1.212	1.730	1.675	0.243	0.048	0.091	0.052	0.134	0.153	0.027	–	0.361
27. Bogo (can)	1.162	1.238	1.112	1.353	1.432	1.241	1.246	1.102	0.908	1.048	0.981	0.868	1.062	0.846	1.622	1.694	1.407	1.532	1.018	0.997	0.991	1.000	1.294	0.910	0.999	1.020	–

and 72.7%, respectively (Fig. 3). The large-type group was further divided into two subgroups, of which the first subgroup consisted of the *F. iskandari* populations from Indonesia, the Bangladesh population and the Taiwanese-Japanese populations, and the second subgroup consisted of the Thailand populations, the *F. limnocharis* populations and the Malaysian populations. The assemblage clades in these subgroups were not strongly supported, since the BT values were below 50%. The Thailand population showed a paraphyletic assemblage relationship with the Malaysia and *F. limnocharis* populations, and another population showed a monophyletic assemblage relationship. In the small-type group, the Thailand (Pilok) population first diverged from the remainder (BT, 94.2%), followed by the Indian population, leaving the Sri Lanka and Bangladesh populations monophyletic (BT, 97.3%) (Fig. 3).

Crossing experiments

The developmental capacities of the hybrids among 10 populations from five countries and the controls are shown in Tables 7 and 8. In the first crossing experiments involving 36 control and hybrid mating combinations of the *F. limnocharis* complex, except for combinations using females from Thailand, the eggs cleaved normally with frequencies ranging from 43.8–98.5% ($\bar{x}$=81.1%), became normal tail-bud embryos with frequencies ranging from 20.5–81.7% ($\bar{x}$=46.2%), hatched normally with frequencies ranging from 18.1–70.2% ($\bar{x}$=40.6%), became normally feeding tadpoles with frequencies ranging from 11.2–68.2% ($\bar{x}$=32.7%), became normal 30-day-old tadpoles with frequencies ranging from 0–54.5% ($\bar{x}$=16.6%) or underdeveloped with frequencies ranging from 0–55.6% ($\bar{x}$=10.6%), and finally metamorphosed normally with frequencies ranging from 0–49.6% ($\bar{x}$=16.3%). In the second crossing experiments involving 19 control and hybrid mating combinations of the *F. limnocharis* complex, the eggs cleaved normally with frequencies ranging from 34.6–99.2% ($\bar{x}$=89%), became normal tail-bud embryos with frequencies ranging from 27.6–96.8% ($\bar{x}$=69.8%), hatched normally with frequencies ranging from 5.3–93.7% ($\bar{x}$=61.8%), became normally feeding tadpoles with frequencies ranging from 3.5–92.9% ($\bar{x}$=49.5%), became normal 30-day-old tadpoles with frequencies ranging from 0–78.1% ($\bar{x}$=22.0%), and finally metamorphosed normally with frequencies ranging from 0–76.0% ($\bar{x}$=25.5%).

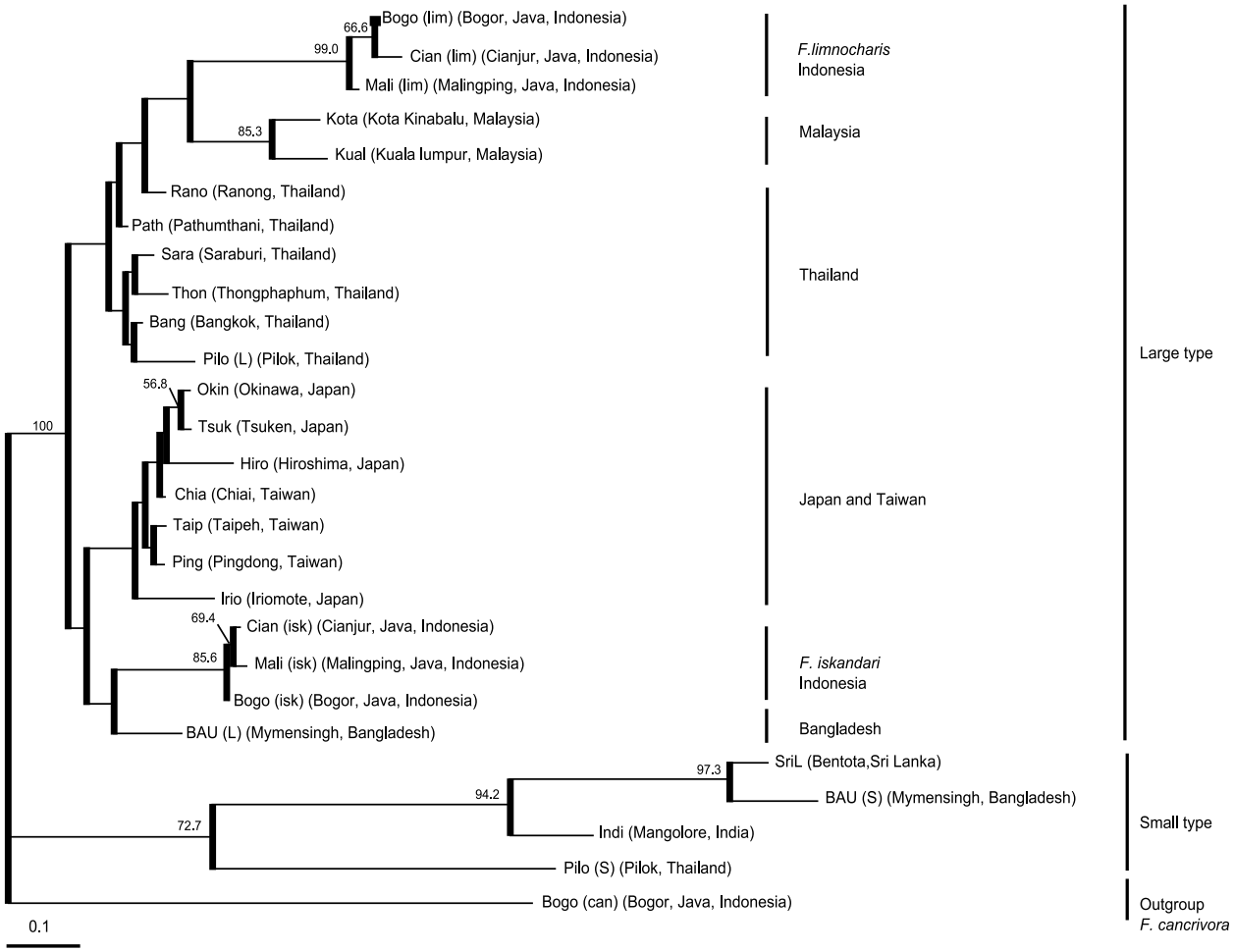

Fig. 3. Neighbor-joining tree among 26 Asian populations of the *Fejervarya limnocharis* complex based on genetic distances as defined by Nei (1972). The value for each branch shows the bootstrap p-value (>50%) for 1,000 replicates. The scale bar represents branch length in terms of Nei's genetic distance.

Table 7. Developmental capacity of the hybrid among 7 Asian populations of the *F. limnocharis* complex and the controls in the first crossing experiments

Parents		No. of eggs	No. of normally cleaved eggs	No. of normal tail-bud embryos	No. of normally hatched tadpoles	No. of normally feeding tadpoles	No. of 30-day-old tadpoles					No. of normally metamorphosed frogs
							Normal	No. of tadpoles examined and chromosome number		Under-developed	No. of tadpoles examined and chromosome number	
Female	Male							2n	3n		2n 3n	
			(%)	(%)	(%)	(%)	(%)			(%)		(%)
Hiroshima-1	Hiroshima-1	61	43 (70.5)	43 (70.5)	39 (63.9)	17 (27.9)	16 (26.2)	4	1	0	0 0	16 (26.2)
Okinawa	Hiroshima-1	196	181 (92.4)	99 (50.5)	55 (28.1)	50 (25.5)	51 (26.0)	–	–	0	0 0	50 (25.5)
Malaysia	Hiroshima-1	435	418 (96.1)	132 (30.3)	114 (26.2)	90 (20.7)	84 (19.3)	–	–	4 (0.9)	– –	84 (19.3)
Thailand-1	Hiroshima-1	121	11 (9.1)	11 (9.1)	11 (9.1)	11 (9.1)	4 (3.3)	0	4	7 (5.8)	5 0	1 (0.8)
Bangladesh-1	Hiroshima-1	247	182 (73.7)	129 (52.2)	123 (49.8)	117 (47.4)	0	0	0	105 (42.5)	– –	9 (3.6)
Bogor (isk)-1	Hiroshima-1	22	15 (68.2)	8 (36.4)	8 (36.4)	8 (36.4)	1 (4.6)	0	1	5 (22.7)	5 0	0
Hiroshima-1	Bogor (lim)	208	198 (95.2)	170 (81.7)	146 (70.2)	103 (49.5)	95 (45.7)	–	–	7 (3.4)	– –	88 (42.3)
Okinawa	Bogor (lim)	299	259 (86.6)	132 (44.2)	122 (40.8)	103 (34.5)	101 (33.8)	–	–	1 (0.3)	– –	102 (34.1)
Malaysia	Bogor (lim)	278	235 (84.5)	102 (36.7)	75 (27.0)	49 (17.6)	46 (16.6)	–	–	2 (0.7)	– –	43 (15.5)
Thailand-1	Bogor (lim)	281	104 (37.0)	104 (37.0)	103 (36.7)	102 (36.3)	0	0	0	100 (35.6)	5 0	85 (30.3)
Bangladesh-1	Bogor (lim)	250	219 (87.6)	70 (28.0)	62 (24.8)	56 (22.4)	0	0	0	48 (19.2)	5 0	11 (4.4)
Bogor (isk)-1	Bogor (lim)	126	118 (93.7)	90 (71.4)	87 (69.1)	86 (68.3)	5 (4.0)	0	5	70 (55.6)	5 0	6 (4.8)
Bogor (isk)-2	Bogor (lim)	89	64 (71.9)	32 (36.0)	19 (21.4)	10 (11.2)	3 (3.4)	0	3	7 (7.8)	5 0	1 (1.1)
Hiroshima-1	Malaysia	196	193 (98.5)	115 (58.7)	97 (49.5)	67 (34.2)	64 (32.7)	–	–	3 (1.5)	– –	63 (32.1)
Okinawa	Malaysia	266	242 (91.0)	167 (62.8)	158 (59.4)	142 (53.4)	138 (51.9)	–	–	4 (1.5)	– –	132 (49.6)
Malaysia	Malaysia	328	289 (88.1)	121 (36.9)	121 (36.9)	82 (25.0)	59 (18.0)	5	0	4 (1.2)	– –	53 (16.2)
Thailand-1	Malaysia	232	114 (49.1)	114 (49.1)	114 (49.1)	110 (47.4)	0	0	0	110 (47.4)	5 0	95 (41.0)
Bangladesh-1	Malaysia	244	211 (86.5)	124 (50.8)	121 (49.6)	82 (33.6)	0	0	0	93 (38.1)	5 0	34 (13.9)
Bogor (isk)-1	Malaysia	88	83 (94.3)	23 (26.1)	23 (26.1)	19 (21.6)	1 (1.1)	0	1	18 (20.5)	5 0	1 (1.1)
Okinawa	Thailand-1	260	245 (94.2)	113 (43.5)	105 (40.4)	95 (36.5)	0	0	0	79 (30.4)	– –	0
Malaysia	Thailand-1	281	179 (63.7)	140 (49.8)	132 (47.0)	101 (35.9)	1 (0.4)	0	1	48 (17.1)	5 0	0
Thailand-1	Thailand-1	182	69 (37.9)	69 (37.9)	66 (36.7)	64 (36.2)	49 (26.9)	5	0	5 (2.8)	– –	40 (22.0)
Bangladesh-1	Thailand-1	226	198 (87.6)	116 (51.3)	109 (48.2)	103 (45.6)	102 (45.1)	–	–	0	0 0	96 (42.5)
Bogor (isk)-1	Thailand-1	83	44 (53.0)	35 (42.2)	35 (42.2)	30 (36.1)	24 (28.9)	–	–	4 (4.8)	– –	19 (22.9)
Hiroshima-1	Bangladesh-1	103	52 (50.5)	52 (50.5)	48 (46.6)	27 (26.2)	7 (6.8)	0	7	16 (15.5)	5 0	3 (2.9)
Okinawa	Bangladesh-1	204	190 (93.1)	67 (32.8)	57 (27.9)	52 (25.5)	2 (1.0)	1	1	18 (8.8)	– –	0
Malaysia	Bangladesh-1	761	747 (98.2)	204 (26.8)	172 (22.6)	116 (15.2)	2 (0.3)	0	2	16 (2.1)	5 0	0
Thailand-1	Bangladesh-1	161	74 (46.0)	38 (23.6)	38 (23.6)	37 (23.0)	38 (23.6)	–	–	0	0 0	38 (23.6)
Bangladesh-1	Bangladesh-1	246	164 (66.7)	164 (66.7)	153 (62.2)	146 (59.4)	134 (54.5)	5	0	0	0 0	114 (46.3)
Bogor (isk)-1	Bangladesh-1	89	39 (43.8)	39 (43.8)	36 (40.5)	35 (39.3)	20 (22.5)	–	–	6 (6.7)	– –	26 (30.3)
Hiroshima-1	Bogor(isk)	164	101 (61.6)	101 (61.6)	92 (56.1)	62 (37.8)	11 (6.7)	0	11	45 (27.4)	5 0	10 (6.1)
Okinawa	Bogor(isk)	201	177 (88.1)	80 (39.8)	68 (33.8)	61 (30.4)	0	0	0	37 (18.4)	– –	1 (0.5)
Malaysia	Bogor(isk)	254	182 (71.7)	72 (28.4)	46 (18.1)	30 (11.8)	0	0	0	16 (6.3)	– –	0
Thailand-1	Bogor(isk)	250	193 (77.2)	52 (20.8)	52 (20.8)	50 (20.0)	45 (18.0)	–	–	5 (2.0)	– –	49 (19.6)
Bangladesh-1	Bogor(isk)	506	454 (89.7)	283 (55.9)	174 (34.4)	160 (31.6)	150 (29.6)	–	–	4 (0.8)	– –	149 (29.5)
Bogor (isk)-1	Bogor(isk)	83	76 (91.6)	17 (20.5)	17 (20.5)	16 (19.3)	16 (19.3)	5	0	0	0 0	16 (19.3)

–, not examined

Table 8. Developmental capacity of the hybrid among 5 Asian populations of the *F. limnocharis* complex and the controls in the second crossing experiments

Parents		No. of Eggs	No. of normally cleaved eggs (%)	No. of normal tail-bud embryos (%)	No. of normally hatched tadpoles (%)	No. of normally feeding tadpoles (%)	No. of 30-day-old tadpoles		No. of normally metamorphosed frogs (%)
Female	Male						Normal (%)	Underdeveloped (%)	
Hiroshima-2	Hiroshima-2	185	168 (90.8)	165 (89.2)	163 (88.1)	130 (70.3)	129 (69.7)	1 (0.5)	120 (64.9)
Thailand-2	Hiroshima-2	294	259 (88.1)	81 (27.6)	63 (21.4)	46 (15.7)	41 (14.0)	3 (1.0)	41 (14.0)
Bangladesh-2	Hiroshima-2	523	490 (93.7)	472 (90.3)	465 (88.9)	457 (87.4)	0	402 (76.9)	23 (4.4)
Bangladesh-3	Hiroshima-2	364	334 (91.8)	311 (85.4)	293 (80.5)	275 (75.6)	3 (0.8)	230 (63.2)	69 (19.0)
Hiroshima-2	Cianjur (lim)	192	157 (81.8)	155 (80.7)	150 (78.1)	147 (76.6)	147 (76.6)	0	146 (76.0)
Thailand-2	Cianjur (lim)	290	232 (80.0)	44 (15.2)	23 (7.9)	9 (3.1)	8 (2.8)	1 (0.3)	8 (2.8)
Bangladesh-2	Cianjur (lim)	344	119 (34.6)	92 (26.7)	88 (25.6)	85 (24.7)	0	76 (22.1)	9 (2.6)
Bangladesh-3	Cianjur (lim)	372	290 (78.0)	274 (73.7)	261 (70.2)	254 (68.3)	1 (0.3)	217 (58.3)	50 (13.4)
Thailand-2	Thailand-2	258	228 (88.4)	143 (55.4)	120 (46.5)	84 (32.6)	58 (22.5)	8 (3.1)	47 (18.2)
Bangladesh-2	Thailand-2	508	495 (97.4)	487 (95.9)	400 (78.7)	388 (76.4)	386 (76.0)	1 (0.2)	375 (73.8)
Banglades-2	Thailand-2	365	355 (97.3)	339 (92.9)	313 (85.8)	288 (78.9)	285 (78.1)	3 (0.8)	274 (75.1)
Hiroshima-2	Bangladesh-2	216	211 (97.7)	197 (91.2)	185 (85.7)	184 (85.2)	0	160 (74.1)	0
Thailand-2	Bangladesh-2	341	303 (88.9)	35 (10.3)	18 (5.3)	12 (3.5)	11 (3.2)	1 (0.3)	11 (3.2)
Bangladesh-2	Bangladesh-2	580	567 (97.8)	457 (78.8)	402 (69.3)	398 (68.6)	274 (47.2)	86 (14.8)	272 (46.9)
Bangladesh-3	Bangladesh-2	402	381 (94.8)	277 (68.9)	268 (66.7)	235 (58.5)	215 (53.5)	11 (2.7)	209 (52.0)
Hiroshima-2	Cianjur (isk)	126	125 (99.2)	122 (96.8)	118 (93.7)	117 (92.9)	0	97 (77.0)	0
Thailand-2	Cianjur (isk)	264	259 (98.1)	168 (63.6)	81 (30.7)	33 (12.5)	33 (12.5)	0	30 (11.4)
Bangladesh-2	Cianjur (isk)	393	379 (96.4)	354 (90.1)	310 (78.9)	35 (8.9)	26 (6.6)	3 (0.8)	26 (6.6)
Bangladesh-3	Cianjur (isk)	395	380 (96.2)	367 (92.9)	287 (72.7)	7 (1.8)	5 (1.3)	1 (0.3)	1 (0.3)

In the first crossing experiments using females from Thailand, the ova appeared to be of low quality, and the developmental capacities of the controls and hybrids were reduced. For the crossing experiments involving females from the Thailand population, the eggs cleaved normally with frequencies ranging from 9.1–77.2% ($\bar{x}$=42.7%), became normal tail-bud embryos with frequencies ranging from 9.1–49.1% ($\bar{x}$=29.6%), hatched normally with frequencies ranging from 9.1–49.1% ($\bar{x}$=29.3%), became normally feeding tadpoles with frequencies ranging from 9.1–47.4% ($\bar{x}$=27.6%), became normal 30-day-old tadpoles with frequencies ranging from 0–26.9% ($\bar{x}$=12%) or underdeveloped with frequencies ranging from 0–47.4% ($\bar{x}$=15.6%), and finally metamorphosed normally with frequencies ranging from 0.8–40.9% ($\bar{x}$=22.9%).

The survival curves of the controls and the hybrids between several Asian populations and topotypic *F. limnocharis* and *F. iskandari* are shown in Fig. 4. The crossing experiments revealed that hybrids between male topotypic *F. limnocharis* and females from the Hiroshima,

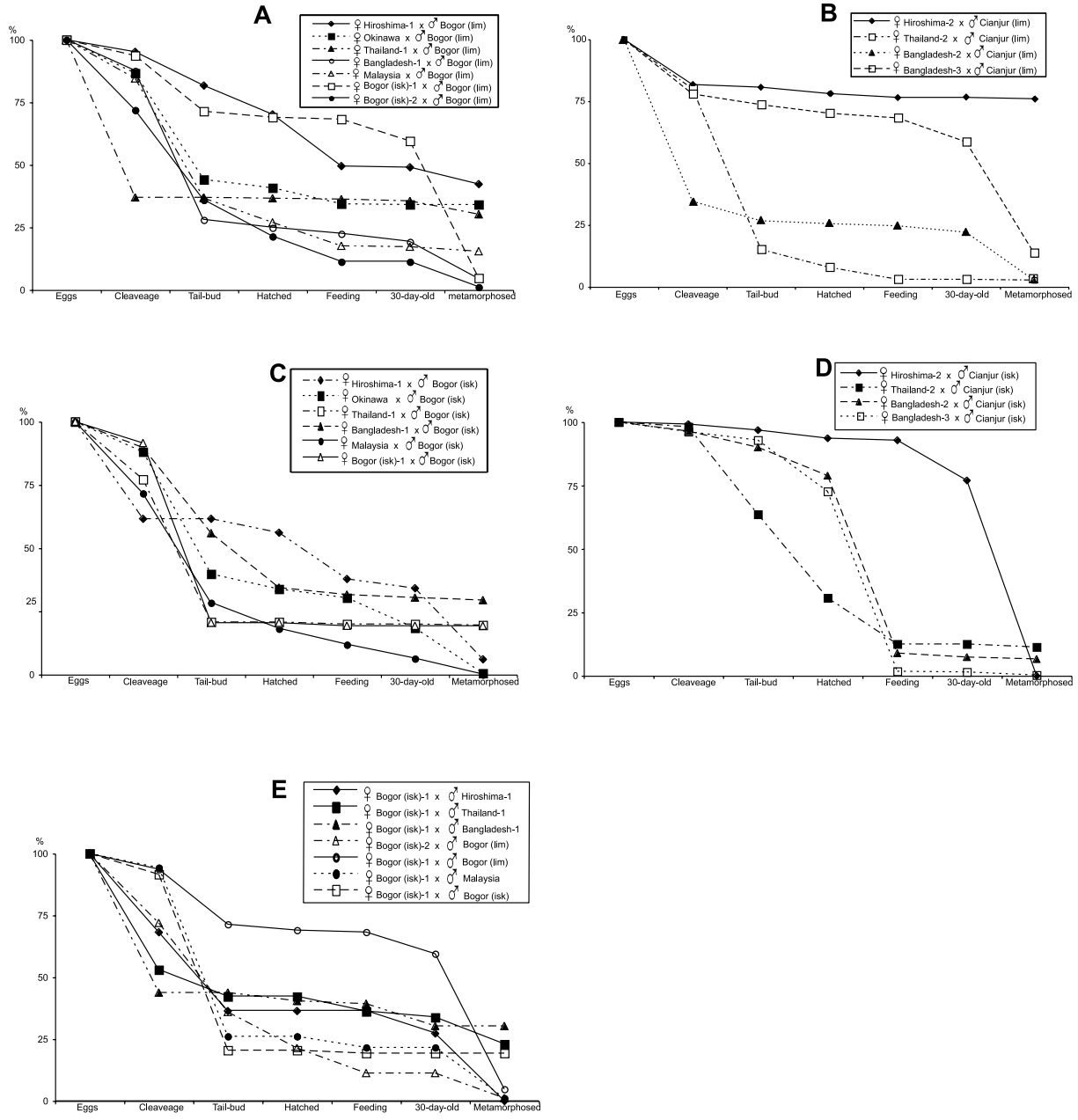

Fig. 4. Survival curves of hybrids between several Asian populations and topotypic *Fejervarya limnocharis* and *F. iskandari* from Java (Indonesia) and the controls. **(A)** First cross between females from several Asian populations and male topotypic *F. limnocharis*. **(B)** Second cross between females from several Asian populations and male topotypic *F. limnocharis*. **(C)** First cross between females from several Asian populations and male topotypic *F. iskandari* and the control. **(D)** Second cross between females from several Asian populations and male topotypic *F. iskandari* and the control. **(E)** First cross between female topotypic *F. iskandari* and males from several Asian populations and the control.

Malaysia and Okinawa populations became normal tadpoles with the capacity for metamorphosis at certain ratios. In contrast, almost all the hybrids between females from Thailand (first cross, Thonphaphum) and Bangladesh (first and second crosses) and males from topotypic *F. limnocharis* populations became underdeveloped (Fig. 5) and died before or just after metamorphosis. A small number of the hybrids between female *F. iskandari* and male *F. limnocharis* became normal tadpoles and underwent metamorphosis. These normal tadpoles were found to be triploid (3n) in their chromosome number (Fig. 4A, B, Table 7). In the second crossing experiments using a female from Pathumthani of Thailand (a local sample different from that used for the first crossing experiments) and males from the Hiroshima, Bangladesh and topotypic *F. iskandari* populations, the hybrids were able to undergo metamorphosis (Table 8).

The crossing experiments between females from the Hiroshima (first and second crosses), Okinawa and Kuala

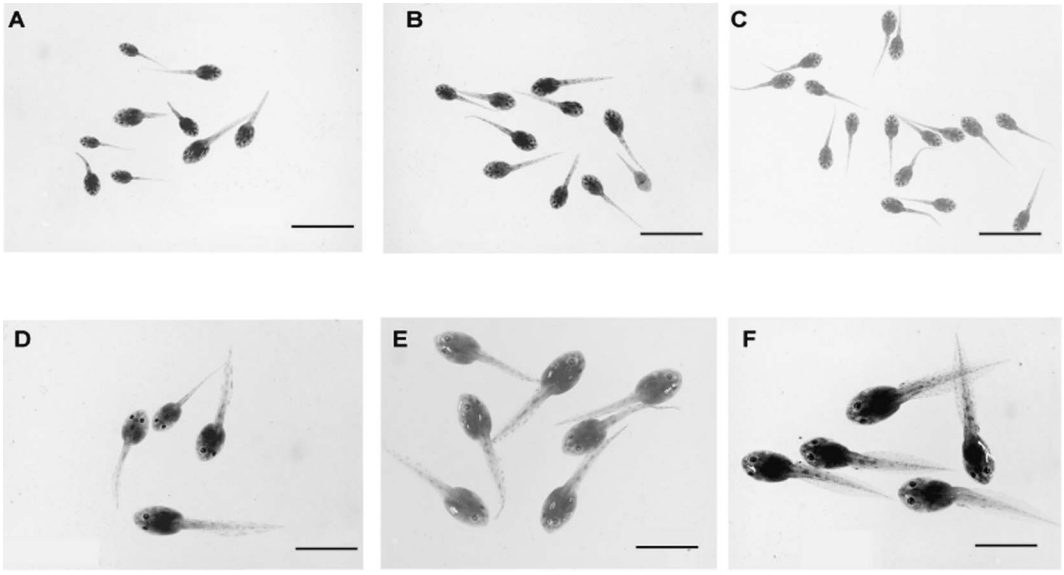

Fig. 5. Thirty-day-old tadpoles of hybrids among the *Fejervarya limnocharis* complex from Asia and the controls. **(A)** Thailand♀×topotypic *F. limnocharis*♂. **(B)** Bangladesh♀×topotypic *F. limnocharis*♂. **(C)** Topotypic *F. iskandari*♀×topotypic *F. limnocharis*♂. **(D)** Thailand control. **(E)** Bangladesh control. **(F)** Topotypic *F. iskandari* control. Scale bar=1.0 cm.

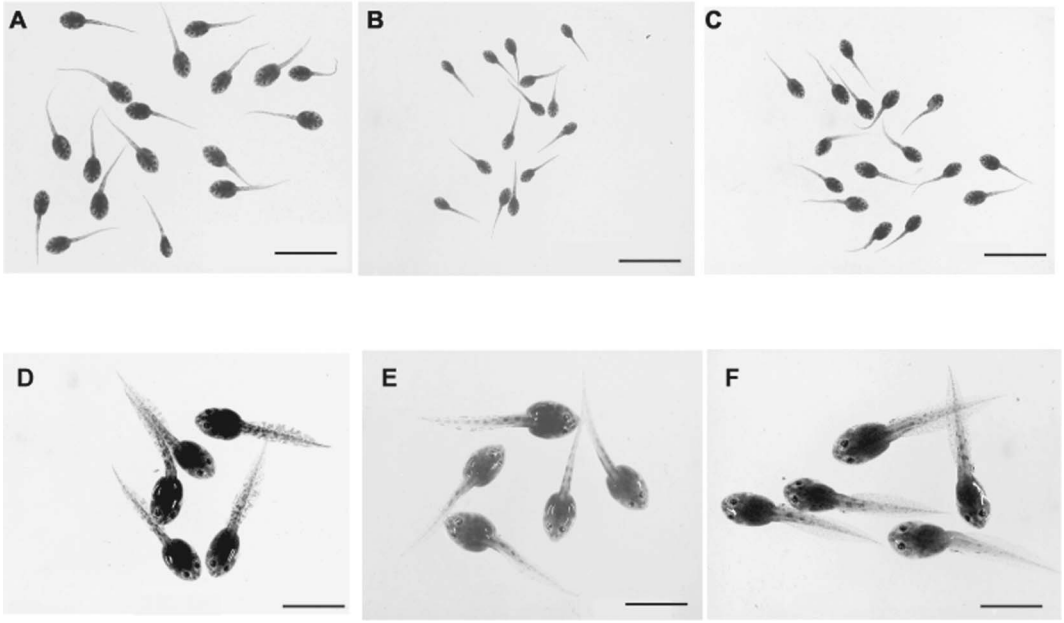

Fig. 6. Thirty-day-old tadpoles of hybrids among the *Fejervarya limnocharis* complex from Asia and the controls. **(A)** Hiroshima♀×topotypic *F. iskandari*♂. **(B)** Malaysia♀×topotypic *F. iskandari*♂. **(C)** Okinawa♀×topotypic *F. iskandari*♂. **(D)** Hiroshima control. **(E)** Malaysia control. **(F)** Topotypic *F. iskandari* control. Scale bar=1.0 cm.

Lumpur (Malaysia) populations and male *F. iskandari* from Java revealed that all the 30-day-old tadpoles became underdeveloped (Fig. 6) and died before metamorphosis, except in the first crosses between a Hiroshima female and a male *F. iskandari*, in which some tadpoles developed normally and reached metamorphosis. Reciprocal crosses between female *F. iskandari* and males of the Hiroshima and Kuala Lumpur (Malaysia) populations also resulted in a large number of underdeveloped tadpoles and only a few normal tadpoles (Fig. 4C–E). By checking the chromosome number, the normal hybrids were found to be triploid (3n), while the underdeveloped hybrids were diploid (2n) (Table 7). The normal hybrids obtained from the crosses between female *F. iskandari* and males from the Thailand and Bangladesh populations and the reciprocal crosses were able to reach metamorphosis.

DISCUSSION

The simple definition of a species is a group of interbreeding natural populations that are reproductively isolated from other such groups (Mayr, 1969). Species are also viewed as genetic systems delimited by isolation mechanisms and genetically based traits that prevent gene exchange (Dobzhansky, 1937; Mayr, 1942). Reproductive isolation is essential for morphological, ecological or genetic divergence between sympatric or allopatric groups (Turelli *et al.*, 2001). Genetic divergence results in reproductive isolation that reflects further accumulation of genetic differentiation at many loci over a long period of time (Ferguson, 2002; Sites and Marshall, 2003, 2004). Wu and Hollocher (1998) argued that if large numbers of genes are responsible for reproductive isolation between taxa and there are gradual changes at all these loci over time, there should be a correlation between genetic divergence and the degree of reproductive isolation. Such correlations between reproductive isolation and genetic divergence have been demonstrated in *Drosophila* (Coyne and Orr, 1989), the salamander *Desmognathus ochrophaeus* (Tilley *et al*., 1990), the salamander *Plethodon* (Highton, 1989, 1990) and some anuran species (Sasa *et al.*, 1998).

Genetic divergence is measured by calculating the genetic distance that represents the degree of dissimilarity between the genetic compositions of taxa, and therefore appears to be an ideal systematic tool (Ayala, 1975; Bullini, 1983). According to Thorpe (1982), allopatric populations with genetic identities above 0.85 should be considered to be conspecific, while those with identities below 0.85 should be considered to be different species. Highton (1989) used Nei's distance of 0.15 to delimit species within the salamanders (*Plethodon*) of North America. Rafinski and Arntzen (1987) found that the genetic distances among *Triturus* species in the Palearctic region were 0.41–2.00. Nishioka and Sumida (1990) reported that the genetic distances between populations and species of 15 anuran species were 0.030–0.241 and 0.301–1.715, respectively. Skibinski *et al.* (1993) reported that the genetic distances among different vertebrate species were above 0.147. Beerli *et al.* (1994) showed that the genetic distances between the water frog *Rana cerigensis* and nine other species in Europe were 0.27–0.95. Sumida and Nishioka (1996) reported that the genetic distances among different populations of 55 amphibian species were 0.007–0.205. Veith *et al.* (2002) found that the mean genetic distance among populations of *Rana temporaria* from Germany was 0.121, while distances between *R. temporaria* and *Rana pyrenaica*, *Rana iberica* and *Rana macrocnemis* were 0.181– 0.667. Zangari *et al*. (2006) found that the genetic distances between *Discoglossus* species in the Mediterranean area were 0.17–1.04. Sasa *et al.* (1998) found positive correlations between the degree of divergence (Nei's (1972) genetic distance) and the degree of postzygotic isolation in anurans, and a lower threshold of genetic distance (0.3) for the evolution of hybrid inviability. Sites and Marshall (2003, 2004) assumed that reproductive isolation is based on divergence across many loci scattered throughout the genome, and that allozyme loci diverge in an approximately clock-like manner, so as to correlate with, and serve as a signature for, the emergence of reproductive isolation. Thus, it may be roughly concluded that allopatric populations with genetic distances above 0.30 can be considered to be different species, and that this value can be considered to represent the threshold of genetic distance for the emergence of reproductive isolation such as hybrid inviability.

Previous studies on the genetic divergence of the *F. limnocharis* complex have been carried out by several research groups. Nishioka and Sumida (1990) reported that the mean genetic distance among seven populations in western Japan was 0.077. The Iriomote population was differentiated from the Hiroshima, Okinawa and Taiwan populations with a mean genetic distance of 0.320. Toda *et al.* (1997) further demonstrated that the mean genetic distance between the southern Ryukyu (including Iriomote Island) and East Asian populations was 0.59. These differences indicate that the absolute divergence values obtained from allozyme analyses are not always consistent with each other due to differences in the number of loci scored, interpretations of electromorphs observed in the genotypes, etc. Toda *et al.* (1998b) reported that *F. limnocharis* in Taiwan had diverged into two groups, the eastern and western groups, with genetic distances of 0.129–0.305. Based on allozyme data, Toda *et al.* (1998a) found a cryptic species from Java with a genetic distance of 0.458 from the syntopic *F. limnocharis*, and Veith *et al.* (2001) described it as a new species, *F. iskandari*, with a genetic distance of 0.316 from the syntopic *F. limnocharis*. Maeda and Matsui (1999) regarded the Sakishima Island populations of *F. limnocharis*, including the Ishigaki and Iriomote Islands in Japan, as a distinct species on the basis of morphological, acoustic and genetic differences. Several investigators have reported conspicuous morphological and genetic divergence of the Sakishima Island populations from other populations of this species (Kuramoto, 1979; Nishioka and Sumida, 1990; Sumida *et al*., 2002; Toda *et al*., 1997; Toda, 1999). On the basis of morphological observations, allozyme data, molecular data and crossing experiments, Sumida *et al*. (2002) suggested that it is reasonable to regard the Sakishima Island populations as a subspecies of *F. limnocharis*.

In the present study based on allozyme data (genetic distances and NJ tree topology) and crossing experiments, the *F. limnocharis* complex from various localities was shown to have largely diverged into two groups, the small-type and large-type groups. The mean genetic distance among the small-type populations was 0.833, while those

between the small-type and large-type populations were 1.160–1.408 (Table 9). In the NJ tree, the small-type group formed one cluster with a BT value of 72.7%, and the mean F_{st} value among the small-type populations was 0.843. In fact, crossing experiments have already revealed that small-type populations are reproductively isolated from large-type populations by complete hybrid inviability at the embryonic stage (Sumida *et al*., unpublished data). This finding clearly indicates that populations belonging to the small- and large-type groups represent different species. Since the large-type group includes *F. limnocharis* sensu stricto and *F. iskandari*, the nomenclature of populations of the small-type group requires consideration. In the small-type group of the *F. limnocharis* complex, the Bangladesh (BAU(S)) population was more closely related to the Sri Lanka population than the Indian and Thailand populations. Specifically, the genetic distances were 0.181 between the Bangladesh (BAU(S)) and Sri Lanka populations, 0.588 between the Indian and Sri Lanka populations, 0.526 between the Indian and Bangladesh (BAU(S)) populations and 1.104–1.310 between the Thailand population and the populations of India, Sri Lanka and Bangladesh (Table 6). Based on mito-

Table 9. Summary of the mean genetic distance (range) among the *F. limnocharis* complex from Asian countries

Species (Population)	Large type						Small type	Outgroup
	F. iskandari	*F. limnocahris* complex						*F. cancrivora*
	Indonesia	Indonesia	Malaysia	Thailand	Bangladesh	Japan Taiwan	India Sri Lanka Bangladesh Thailand	Indonesia
F. iskandari (Indonesia)	0.024 (0.012–0.032)							
F. limnocharis (Indonesia)	0.661 (0.628–0.720)	0.060 (0.046–0.076)						
F. limnocharis (Malaysia)	0.543 (0.470–0.614)	0.451 (0.410–0.526)	0.144					
F. limnocharis (Thailand: Large type)	0.373 (0.285–0.431)	0.431 (0.250–0.572)	0.370 (0.287–0.469)	0.115 (0.056–0.197)				
F. limnocharis (Bangladesh: Large type)	0.271 (0.249–0.291)	0.534 (0.509–0.579)	0.353 (0.311–0.393)	0.308 (0.220–0.408)	–			
F. limnocharis (Japan, Taiwan)	0.387 (0.300–0.455)	0.480 (0.365–0.638)	0.430 (0.353–0.488)	0.309 (0.191–0.501)	0.238 (0.188–0.291)	0.112 (0.027–0.290)		
F. limnocharis (India, Sri Lanka, Bangladesh,Thailand: Small type)	1.160 (0.709–1.495)	1.408 (1.137–1.652)	1.323 (1.055–1.466)	1.126 (0.919–1.393)	1.287 (0.864–1.633)	1.350 (0.980–1.730)	0.833 (0.181– 1.310)	
F. cancrivora (Indonesia)	1.171 (1.162–1.238)	1.342 (1.241–1.432)	1.174 (1.102–1.246)	0.952 (0.846–1.062)	1.018	1.026 (0.910–1.294)	1.564 (1.407–1.694)	–

Parentheses show the range values.

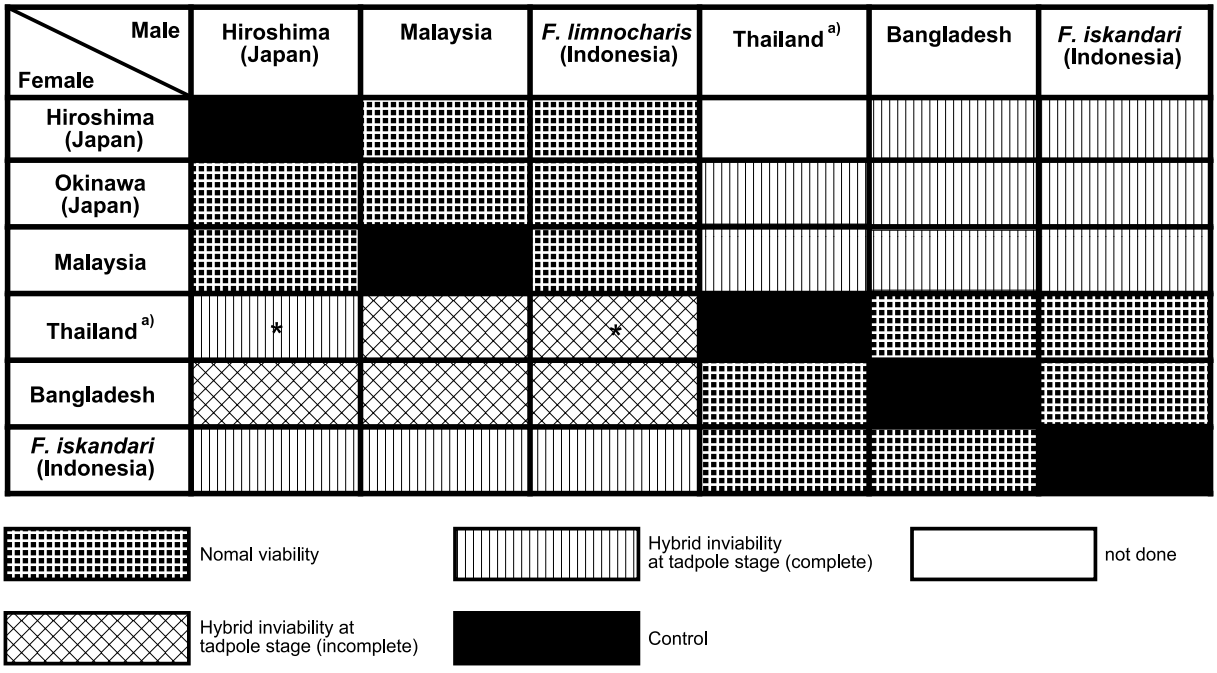

Fig. 7. Reproductive-isolation mechanisms found in various combinations among the *Fejervarya limnocharis* complex from Asia. [a)]Two different populations were used: the first cross involved the Thonphaphum population and the second involved the Pathumthani population. *Normal viability in the second cross.

chondrial 16S rRNA gene sequence data, Sumida *et al.* (unpublished data) also found that the Bangladesh BAU(S), Sri Lanka and *Fejervarya syhadrensis* populations from India made one cluster in a NJ tree, and were distinctly diverged from the Indian and Thailand (Pilok) populations. These data possibly imply that our specimens from the Sri Lanka and Bangladesh (BAU(S)) populations belong to *F. syhadrensis*, and that the Indian and Thailand (Pilok) populations are a different species. The Thailand (Pilok) and Indian populations may possibly be a new species.

In the present study, we used *F. limnocharis* and *F. iskandari* from the type localities on Java, Indonesia. The mean genetic distance between these two species, 0.661 (range, 0.628–0.720), was the highest among the large-type group, whereas that among *F. iskandari* populations from Java was 0.024 (range, 0.012–0.032) and that among *F. limnocharis* populations from Java was 0.060 (range, 0.046–0.076) (Table 9). Furthermore, the results of the crossing experiments between these species revealed that they are isolated by complete hybrid inviability at the tadpole stage (Fig. 7). This result corroborates the conclusions of Toda *et al.* (1998a) and Veith *et al.* (2001) that these sympatric species, although very difficult to distinguish from each other morphologically, can be clearly distinguished by allozyme analyses. In the present study, *F. iskandari* and *F. limnocharis* had diagnostic alleles at 11 loci of 27 presumptive loci examined. Toda *et al*. (1998a) found diagnostic alleles at 10 of 25 loci examined, and Veith *et al.* (2001) found diagnostic alleles at six of 15 presumptive loci analyzed. We found both species in each of three populations from Java, although the percentage compositions of *F. limnocharis* were very low (range, 1.7–26.1%; mean, 15.9%). Moreover, the genetic variability of *F. limnocharis* was lower than that of *F. iskandari* (mean values, H=6.3, P=19.7 and A=1.24 for *F. limnocharis* and H=9.6, P=46.9 and A=1.64 for *F. iskandari*).

Based on the present allozyme data, the large-type group could be divided into two subgroups. One subgroup consisted of the Bangladesh, *F. iskandari* and Japanese-Taiwanese populations, while the other subgroup consisted of the Thailand, Malaysia and *F. limnocharis* populations. These results were contradictory with those of the crossing experiments that showed no reproductive isolation as hybrid inviability among the Japanese-Taiwanese, Malaysia and *F. limnocharis* populations or among the *F. iskandari*, Thailand and Bangladesh populations. Based on mitochondrial 16S rRNA gene sequence data, Sumida *et al*. (unpublished data) found that the *F. iskandari*, Thailand and Bangladesh populations comprise one cluster that is diverged from another cluster consisting of the *F. limnocharis*, Malaysia and Japanese-Taiwanese populations. In addition, the allozyme NJ tree showed no confidence in either of the two large-type subgroups. Therefore, we designated one subgroup including *F. iskandari* and the Thailand and Bangladesh populations as the *F. iskandari* subgroup, and the other subgroup including *F. limnocharis* and the Malaysia and Japanese-Taiwanese populations as the *F. limnocharis* subgroup. The mean genetic distances were 0.271–0.373 among the *F. iskandari* subgroup and 0.430–0.480 among the *F. limnocharis* subgroup (Table 9), and no reproductive-isolation mechanisms existed among each subgroup until the metamorphosis stage. If we apply the genetic distance criteria for allopatric populations defined by Thorpe (1982), Highton (1989) and Skibinski *et al.* (1993) and the correlation between the genetic distance and reproductive isolation (Sasa, 1998; Sites and Marshall, 2003, 2004), as already mentioned in the second paragraph of the Discussion, the *F. limnocharis* complex in the Thailand and Bangladesh populations may be regarded as a subspecies or another species of *F. iskandari* from the type locality of Java, and the *F. limnocharis* complex from the Malaysia and Japanese-Taiwanese populations may be regarded as a subspecies or another species of *F. limnocharis* from the type locality of Java, since the mean genetic distances among the *F. limnocharis* complex from these five countries ranged from 0.238 to 0.661 in the present study. This statement is supported by mean genetic distances of 0.144, 0.112 and 0.115 among the Malaysian populations, Japanese-Taiwanese populations and Thailand populations, respectively (Table 9). Besides, the F_{st} values among subgroup *F. iskandari* and subgroup *F. limnocharis* were 0.604 and 0.818, respectively, and higher than those among *F. iskandari* from Java and *F. limnocharis* from the Java and Thailand populations (0.128, 0.356 and 0.419, respectively). Further studies investigating the fertility or spermatogenesis of mature hybrids, detailed morphological characteristics and molecular divergences will be required to elucidate the exact taxonomic status of the *F. limnocharis* complex from each country in Asia.

ACKNOWLEDGMENTS

We are grateful to Professor Yong Hoi Sen and Mr. Daicus M. Belabut of University of Malaya, Drs. T. Fujii and H. Hasegawa of the University of the Ryukyus, and Dr. M. Katsuren of the Okinawa Prefectural Institute of Health and Environment, for their kindness in making *F. limnocharis* samples from Kuala Lumpur, Okinawa and Iriomote, respectively, available for this study. The first author, Djong Hon Tjong, is grateful to Mr. Yaya Hernawan SP from Soekarno-Hatta Fish Quarantine, Centre for Fish Quarantine, Ministry of Marine Affairs and Fisheries, Indonesia, who permitted the transport of frog samples from Indonesia (No. 03545.TI-210.2010.IV.2005). This work was supported by Technological and Professional Skills Development Sector Project (TPSDP) ADB Loan No. 1792-INO, Directorate General of Higher Education, Department of Education, Indonesia (to Djong Hon Tjong), and by a Grant-in-Aid for Scientific Research (C) (No. 17570082) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to M. Sumida).

REFERENCES

Ayala FJ (1975) Genetic differentiation during the speciation process. Evol Biol 8: 1–78

Beerli P, Hotz H, Tunner HG, Heppich S, Uzzell T (1994) Two new water frog species from the Aegean Island Crete and Karpathos. Acad Nat Sci Philadelphia 470: 1–9

Berg EE, Hamrick JL (1997) Quantification of genetic diversity at allozyme loci. Can J For Res 27: 415–424

Bullini L (1983) Taxonomic and evolutionary inferences from electrophoretic studies of various animal groups. Syst Assoc Special Vol 24: 179–192

Coyne JA, Orr HA (1989) Patterns of speciation in *Drosophila*. Evolution 43: 362–381

Dobzhansky T(1937) Genetic and Origin of Species. Columbia University Press, New York

Dubois A (1975) Un nouveau complexe d'especes jumelles dis-

tinguees par le chan: les grenouilles du Nepal voisnes de *Rana limnocharis* Boie (Amphibiens, Anoures). Acad Sci 281: 1717–1720

Dubois A (1984) Note preliminaire sur le groupe de *Rana limnocharis* Gravenhorst, 1929 (Amphibiens, Anaures). Alytes 3: 143–159

Dubois A (1987) Miscellanea taxomica batrachologica (1). Alytes 5: 7–95

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

Dutta SK (1997) A new species of *Limnonectes* (Anura: Ranidae) from Orissa, India. Hamadryad 22: 1–8

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791

Ferguson JWH (2002) On the use of genetic divergence for identifying species. Biol J Linn Soc 75: 509–516

Harris H, Hopkinson DA (1976) Handbook of Enzyme Electrophoresis in Human Genetics. North-Holland Publishing, Amsterdam

Hartl DL, Clark AG (1989) Principles of population genetics. 2nd ed, Sinauer Associates, Sunderland

Highton R (1989) Biochemical evolution in the slimy salamander of the *Plethodon glutinosus* complex in the Eastern United States. Part I. Geographic protein variation. Illinois Biol Monogr 57: 1–7

Highton R (1990). Taxonomic treatment of genetically differentiated populations. Herpetologica 46: 114–121

Inger RF, Voris HK (2001) Biogeographical relations of the frog and snake of Sundaland. J Biogeogr 28: 863–891

Iskandar DT (1998) The Amphibians of Java and Bali. LIPI, Yayasan Kehati, Bogor

Iskandar DT, Colijn E (2000) Preliminary checklist of Southeast Asian and New Guinean herpetfauna. I. Amphibias. Treubia 31: 1–134

Kawamura T, Nishioka M, Ueda H (1981) Interspecific hybrids among Japanese, Formosan, European and American brown frogs. Sci Rep Lab Amphib Biol Hiroshima Univ 5: 195–323

Kuramoto M (1979) Distribution and isolation in the anurans of the Ryukyu Islands. Jpn Herpetol 8: 8–21

Lewontin RC (1974) The Genetic Basis of Evolutionary Change. Columbia University Press, New York

Maeda N, Matsui M (1999) Frogs and Toads of Japan. Bunichi-Sogo Shuppan, Tokyo

Mayr E (1942) Systematics and the Origin of Species. Columbia University Press, New York

Mayr E (1969) The biological meaning of species. Biol J Linn Soc 1: 311–320

Nei M (1972) Genetic distance between populations. Am Nat 106: 283–292

Nishioka M, Ohtani H, Sumida M (1980) Detection of chromosome bearing the loci for seven kinds of proteins in Japan pond frogs. Sci Rep Lab Amphib Biol Hiroshima Univ 4: 127–184

Nishioka M, Sumida M (1990) Differentiation of *Rana limnocharis* and two allied species elucidated by electrophoretic analyses. Sci Rep Lab Amphib Biol Hiroshima Univ 10: 125–154

Nishioka M, Sumida M, Ohtani H (1992) Differentiation of 70 populations in the *Rana nigromaculata* group by the method of electrophoretic analyses. Sci Rep Lab Amphib Biol Hiroshima Univ 11: 1–70

Rafinski J, Arntzen JW (1987) Biochemical systematics of the old world newts, genus *Triturus*: Allozyme data. Herpetologica 43: 446–457

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic tree. Mol Biol Evol 4: 406–425

Sasa MM, Chippindale PT, Johnson NA (1998) Patters of postzygotic isolation in frogs. Evolution 53: 1811–1820

Sites JW, Marshall JC (2003) Delimitation species: a renaissance issue in systematic biology. Trends Ecol Evol 18: 462–470

Sites JW, Marshall JC (2004) Operational Criteria for delimiting species. Annu Rev Ecol Evol Syst 35: 199–227

Skibinski DO F, Woodwark M, Ward RD (1993) A quantitative test of the neural theory using pooled allozyme data. Genetics 135: 233–248

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

Sumida M, Nishioka M (1996) Genetic variation and population divergence in the mountain brown frog *Rana ornativentris*. Zool Sci 13: 537–549

Thorpe JP (1982) The molecular clock hypothesis: biochemical evolution, genetic differentiation and systematic. Ann Rev Ecol Syst 13: 139–168

Tilley SG, Verrel PA, Arnod SJ (1990) Correspondence between sexual isolation and allozyme differentiation: A test in salamander (*Desmognathus achrophaeus*). Proc Natl Acad Sci USA 87: 2715–2719

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

Toda M, Matsui M, Nishida M, Ota H (1998a) Genetic divergence among Southeast and East Asian population of *Rana limnocharis* (Amphibia: Anura), with species reference to sympatric cryptic species in Java. Zool Sci 14: 607–613

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

Toda M (1999) Historical biogeography of East Asian populations of *Rana limnocharis* (Amphibia: Anura): a review. In Tropical Island Herpetofauna: Origin, Current Diversity, and Conservation Ed by H Ota, Elvier Science, Amsterdam, pp 229–315

Turelli M, Barton NH, Coyne JA (2001) Theory and speciation. Trends Ecol Evol 16: 330–343

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

Veith M, Vences M, Vieites DR, Nieto-roman S, Palanca A (2002) Genetic differentiation and population structure within Spanish common frogs (*Rana temporaria*: Ranidae, Amphibia). Folia Zool 51: 307–318

Wu CI, Hollocher H (1998) Subtle is nature: the genetics of species differentiation and speciation. In “Endless Forms: Species and Speciation” Ed by DJ Howard, SH Brocher, Oxford University Press, New York, pp 339–351

Zangari F, Cimmaruta R, Nascetti G (2006) Genetic relationship of the western Mediterranean painted frogs based on Allozyme and mitochondrial markers: evolutionary and taxonomic inferences (Amphibia, Anura, Discoglossidae). Biol J Linn Soc 87: 515–536

(Received May 30, 2006 / Accepted November 27, 2006)