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Genetic Divergence among Southeast and East Asian Populations of *Rana limnocharis* (Amphibia: Anura), with Special Reference to Sympatric Cryptic Species in Java

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ABSTRACT—An electrophoretic survey was conducted for the Southeast and East Asian populations of *Rana limnocharis*. Results indicated that specimens from a single locality in Java derived from two genodemes showing complete allelic displacement at eight out of 25 presumptive loci examined. Within each of these genodemes, genotype frequencies at all polymorphic loci were well concordant with the Hardy-Weinberg expectation. These results strongly suggest that those specimens actually represent two biological species. Comprehensive distance analyses suggested that one of the two species from Java is genetically most divergent among all samples examined, whereas the other is most similar to the Laos sample and then to a group consisting of samples from Hongkong, western China, and the southern Ryukyus. Large values of genetic distances obtained for each combination of allopatric samples imply the presence of additional cryptic species in this nominal species.

INTRODUCTION

The Indian rice frog *Rana limnocharis* is a lowland medium-sized ranid with an extraordinarily wide distributional range including East, Southeast, and South Asia (Frost, 1985; Maeda and Matsui, 1989). Recently, several populations in South Asia (Nepal and India), previously assigned to *R. limnocharis*, were described as separate species on the basis of acoustic and slight morphological differences (Dubois, 1975; Dutta, 1997). Moreover, Toda *et al*. (1997), in the allozyme study of *R. limnocharis* in the temperate to subtropical East Asia (i.e., Japan and central eastern and western China), demonstrated a considerable genetic divergence between the southern Ryukyu populations and the remaining genetically remarkably uniform populations, with Nei's (1978) genetic distance values falling within ranges of the interspecific level (Thorpe, 1982). Results of these studies predict that *R. limnocharis* in the remaining, still broad unsurveyed area (Southeast Asia and southern part of East Asia) is actually a composite, with substantial genetic and reproductive divergences. Indeed, several authors also have concerned morphological variation in East and Southeast Asian populations of *R. limnocharis*, but without making any taxonomic changes [see Kuramoto (1968) for the review].

Protein electrophoresis is a powerful tool to detect occurrences of cryptic species within morphologically conservative lineages, e.g., hynobiid salamanders (Matsui, 1987), dicamptodontid salamanders (Good, 1989), and typhlopid snakes (Hedges and Thomas, 1991). In this study, we adopted this approach to assess genetic variation and actual taxonomic diversity in Southeast and southern East Asian populations of *R. limnocharis*. Resultant data, while confirming considerable genetic differentiations among allopatric populations as predicted above, indicated the occurrence of sympatric cryptic species in Java.

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MATERIALS AND METHODS

Specimens examined in this study were collected from New Territories, Hongkong (N = 10), and Malinping, Jawa Barat (western Java), Indonesia (N = 18), and were also obtained from a commercial market in Vientiane, Laos (N = 9) (Fig. 1). Liver and muscles were removed from each specimen, and their extracts were subjected to horizontal starch gel electrophoresis. Twenty-five presumptive loci controlling 19 enzymes were assayed by using six kinds of buffer systems (Table 1). For comparisons, we also incorporated Toda *et al*'s (1997) allozyme data for samples from Wenjiang, western China, and Ishigaki, the southern Ryukyus, into the analyses as representatives of the two divergent lineages of temperate to subtropical East Asia.

In order to estimate overall genetic differentiations among samples, Rogers' (1972) distance and Nei's (1978) unbiased genetic distance (D) were calculated for all pairwise comparisons of samples. The Rogers' distance coefficient was clustered by the Neighbor-Joining (NJ) method (Saitou and Nei, 1987). For the out-group rooting of the NJ network, we incorporated data for *Rana cancrivora* from Toda *et al.* (1997) into the analysis. This species is supposedly closely related to *R. limnocharis* (see Toda *et al.*, 1997). We also calculated the mean expected heterozygosity (*Hexp*) (Nei, 1978) for each sample. The NJ procedure was performed by N. Saitou's original computer program, and the other computations were made by the BIOSYS-1 computer program (Swofford and Selander, 1981).

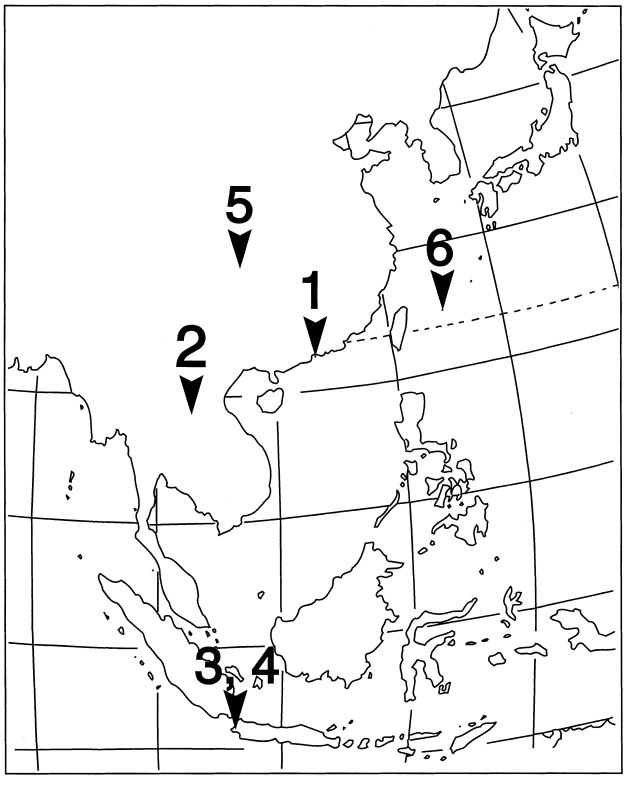

Fig. 1. A map of Southeast and East Asia, showing sampling localities of *Rana limnocharis* analyzed in this study. Numbers denote samples examined: 1 = Hongkong, 2 = Vientiane, 3 = Malinping-A, 4 = Malinping-B, 5 = Wenjiang, 6 = Ishigaki.

RESULTS

Of 25 presumptive loci assayed, all but four (*Ada*, *Ck*, *Gdh* and *Sod*) were polymorphic. Genotype frequencies at these loci were well concordant with the expectation of the Hardy-Weinberg equilibrium in the Hongkong, the Vientiane, and the remaining two East Asian samples. In the Malinping sample, however, the frequencies showed significant deviations at ten loci (*Aat-2*, *Ldh-1*, *Ldh-2*, *Mdh-1*, *Mdh-2*, *Me-1*, *Mpi*, *Pep-lv*, *Pgd*, *Pgm*) due to the deficiency or complete absence of heterozygotes. Based on genotypes at four of these loci (*Aat-2*, *Ldh-2*, *Mdh-2*, *Pgd*) in which heterozygotes were completely lacking, two groups of individuals were recognized in the Malinping sample. One of these groups consisted of eight individuals having genotypes *cc* at *Aat-2*, *aa* at *Ldh-2*, *aa* at *Mdh-2*, and *aa* at *Pgd*, whereas the other group consisted of ten individuals having genotypes *dd*, *bb*, *bb*, and *cc* at respective loci. Moreover, these groups possessed different alleles at other four loci. The former group possessed alleles *c*, *d*, and *e* at *Ldh-1*, *b* and *c* at *Mdh-1*, *b* and *d* at *Pep-lp*, and *b* and *c* at *Pep-lv*, whereas the latter group possessed alleles *a* and *b*, *a* and *d*, *a* and *c*, and only *d* at these loci, respectively. These results strongly suggest that the Malinping sample is actually a mixture of two genetically divergent and reproductively isolated demes. We, therefore, redesignated these two groups of specimens as two separate samples, Malinping-A (N = 8) and Malinping-B (N = 10). With this modification, frequency distributions of genotypes became well concordant with the Hardy-Weinberg expectation at all polymorphic loci in all samples.

Allele frequencies at polymorphic loci examined in this study are given in Table 2. Matrices of Nei's and Rogers' distances among samples are presented in Table 3. The largest value of the Nei's D (0.597) was obtained between the Malinping-B and Ishigaki samples. Each of these two samples was genetically differentiated also from the other samples with large values of Nei's D [0.301-0.458 ($\bar{x}$ = 0.339) between the Malinping-B and the other samples, 0.312-0.440 ($\bar{x}$ = 0.373) between the Ishigaki and the other samples]. On the other hand, D values obtained among the Hongkong, Vientiane, and Malinping-A samples were relatively small [D = 0.170-0.287 ($\bar{x}$ = 0.225)]. The Wenjiang sample, while showing a relatively small D value with the Hongkong sample (D = 0.250), was also considerably differentiated from the remaining samples [D = 0.371-0.414 ($\bar{x}$ = 0.398)].

NJ phenogram based on the Rogers' distance is shown in Fig. 2. In this phenogram, the Malinping-B sample constituted one of the two major clusters by itself. Remaining samples composing the other major cluster were grouped into three sub-clusters: one consisting of the Malinping-A and Vientiane samples, another of the Ishigaki sample alone, and the other of the Hongkong and Wenjiang samples.

Table 1. Enzymes, presumptive loci, tissues and buffer systems used in the analysis of genetic divergence among Southeast and East Asian populations of *Rana limnocharis*

Enzyme	E.C.number	Locus	Tissue	Buffer system
Adenosine deaminase	3.5.4.4	*Ada*	L	TBE8.7, Poulik
Aspartate aminotransferase	2.6.1.1	*Aat-1*	L	CAPM6, LiOH, Poulik
Aspartate aminotransferase	2.6.1.1	*Aat-2*	L	CAPM6, LiOH, Poulik
Creatine kinase	2.7.3.2	*Ck*	M	LiOH
Fumarate hydratase	4.2.1.2	*Fh*	L	LiOH, Pouilk
Glucose-6-phosphate isomerase	5.3.1.9	*Gpi*	M	CAPM6, TBE8.7
Glutamate dehydrogenase	1.4.1.3	*Gdh*	L	Poulik
Glycerate dehydrogenase	1.1.1.29	*Glydh*	L	CAPM6
Glycerol-3-phosphate dehydrogenase	1.1.1.8	*Gpdh*	L	CAPM6
3-Hydroxybuthyrate dehydrogenase	1.1.1.30	*Hbdh*	L	CAPM6
Isocitrate dehydrogenase	1.1.1.42	*Idh-1*	L	CAPM7, TCE7
Isocitrate dehydrogenase	1.1.1.42	*Idh-2*	L,M	CAPM7
L-Lactate dehydrogenase	1.1.1.27	*Ldh-1*	L	CAPM6
L-Lactate dehydrogenase	1.1.1.27	*Ldh-2*	L	CAPM6
Malate dehydrogenase	1.1.1.37	*Mdh-1*	L,M	CAPM6
Malate dehydrogenase	1.1.1.37	*Mdh-2*	L	CAPM6
Malic Enzyme*	1.1.1.40	*Me-1*	L,M	CAPM6, TCE7
Malic Enzyme*	1.1.1.40	*Me-2*	L,M	CAPM6, TCE7
Mannose-6-phosphate isomerase	5.3.1.8	*Mpi*	M	CAPM6, CAPM7
Peptidase (leucyl-leucyl-glycine)	3.4.11.–	*Pep-lgg*	L	CAPM6
Dipeptidase (leucyl-proline)	3.4.11.–	*Pep-lp*	L	CAPM6
Dipeptidase (leucyl-valine)	3.4.11.–	*Pep-lv*	L	CAPM6
Phosphoglucomutase	5.4.2.2	*Pgm*	M	Poulik
Phosphogluconate dehydrogenase	1.1.1.44	*Pgd*	L	CAPM6, CAPM7
Superoxide dismutase	1.15.1.1	*Sod*	L	TBE8.7, TCE7

* NADP dependent malate dehydrogenase
Tissue: L, liver; M, muscle.
Buffer: CAPM6, Citric acid-aminopropylmorpholine, pH 6.0 (Clayton and Tretiak, 1972); CAPM7, Citric acid-aminopropylmorpholine, pH 7.0 (Abersold *et al*., 1987); TCE7, Tris-citric acid-EDTA, pH 7.0 (Ayala *et al*., 1973); TBE8.7, Tris-boric acid-EDTA, pH 8.7 (Boyer *et al*., 1963); LiOH, Lithum hydroxide-boric acid, pH 8.1 (Ridgway *et al*., 1970); Poulik, Discontinuous Tris-citric acid (Poulik, 1957).

DISCUSSION

Present results clearly indicate that specimens from Malinping, though consistently sharing morphological features diagnostic of *R. limnocharis*, actually represent two genetically divergent and reproductively isolated entities. Considering the fact that all specimens of the sample were collected from a limited paddy field area (less than 90 m × 70 m), it is obvious that these "entities" are most appropriately referred as two biological species. This finding, along with the fact that the type locality of *R. limnocharis* is restricted to Java (e.g., Stejneger, 1907), raises a serious taxonomic problem: Because of the close morphological similarity between the two Javanese species, it is expected to be extremely difficult to determine which of these strictly corresponds to the nominal species. Further detailed morphological comparisons among specimens of these cryptic species and the type specimen of *R. limnocharis* are strongly desired.

Result of NJ clustering seems to indicate that the Malinping-B sample is genetically most divergent among all samples analyzed. This suggests that the population was first differentiated from the remaining Southeast and East Asian populations of *R. limnocharis* (*sensu lato*). We therefore consider that this population actually represents one distinct species by itself.

Even among populations other than Malinping-B, levels of genetic differentiations are considerably high. Especially, distances of Wenjiang from Vientiane and Malinping-A (D = 0.371 and 0.414, respectively), and those of Ishigaki from all other samples (D = 0.312-0.440) were large, overlapping with those between the Malinping-B and the other samples (D > 0.301) (Table 3). Furthermore, Nei's D values between the

Table 2. Allele frequencies and mean expected heterozygosities (standard errors in parentheses) in six samples of *Rana limnocharis* from Southeast and East Asia and a sample of *R. cancrivora* (No. 7). Reference numbers of *R. limnocharis* samples (Nos. 1-6) correspond to those in Fig. 1

		Sample						
Locus		1	2	3	4	5	6	7
Aat-1	*a*	1.000	1.000	1.000	.950	1.000	.978	–
	b	–	–	–	.050	–	.022	–
	c	–	–	–	–	–	–	1.000
Aat-2	*a*	.050	–	–	–	–	–	–
	b	–	–	–	–	–	.022	–
	c	.950	1.000	1.000	–	.950	.978	–
	d	–	–	–	1.000	.050	–	1.000
Fh	*a*	.050	–	–	–	–	–	–
	b	.700	.944	1.000	1.000	–	.950	.929
	c	.250	.056	–	–	1.000	.050	.071
Glydh	*a*	–	.286	.063	.100	–	–	–
	b	–	–	–	–	–	–	1.000
	c	1.000	.714	.937	.900	1.000	1.000	–
Gpdh	*a*	–	1.000	–	–	–	1.000	–
	b	1.000	–	1.000	1.000	1.000	–	–
	c	–	–	–	–	–	–	1.000
Gpi	*a*	.900	1.000	1.000	1.000	.950	–	–
	b	.100	–	–	–	.050	.761	–
	c	–	–	–	–	–	–	.929
	d	–	–	–	–	–	.239	–
	e	–	–	–	–	–	–	.071
Hbdh	*a*	–	–	–	–	.250	1.000	–
	b	1.000	.583	1.000	1.000	.063	–	–
	c	–	.417	–	–	.687	–	–
	d	–	–	–	–	–	–	1.000
Idh-1	*a*	–	–	–	–	–	–	1.000
	b	.250	–	–	–	–	–	–
	c	.750	1.000	1.000	1.000	1.000	1.000	–
Idh-2	*a*	.050	–	–	–	–	–	–
	b	.950	.889	1.000	1.000	1.000	1.000	1.000
	c	–	.111	–	–	–	–	–
Ldh-1	*a*	–	–	–	.600	–	–	–
	b	–	–	–	.400	–	–	–
	c	–	.250	.063	–	–	–	–
	d	–	–	.874	–	–	–	–
	e	1.000	.624	.063	–	1.000	1.000	–
	f	–	.063	–	–	–	–	–
	g	–	.063	–	–	–	–	1.000
Ldh-2	*a*	.150	1.000	1.000	–	–	–	1.000
	b	.850	–	–	1.000	1.000	1.000	–
Mdh-1	*a*	.100	.167	–	.600	1.000	1.000	–
	b	–	.056	.313	–	–	–	–
	c	–	.333	.687	–	–	–	–
	d	–	.222	–	.400	–	–	–
	e	.900	.222	–	–	–	–	–
	f	–	–	–	–	–	–	1.000

Table 2 (continued)

Locus		Sample 1	2	3	4	5	6	7
Mdh-2	*a*	1.000	1.000	1.000	–	.800	1.000	–
	b	–	–	–	1.000	.200	–	1.000
Me-1	*a*	–	.056	–	–	–	–	–
	b	1.000	.888	.125	.950	1.000	.043	.071
	c	–	.056	.875	.050	–	.957	.929
Me-2	*a*	–	.056	–	–	–	–	1.000
	b	.100	.056	–	–	–	.043	–
	c	.900	.832	1.000	1.000	.800	.957	–
	d	–	.056	–	–	.200	–	–
Mpi	*a*	–	.167	–	–	–	–	.571
	b	.150	.111	–	.300	1.000	–	–
	c	.850	.555	–	.650	–	1.000	.071
	d	–	.056	–	–	–	–	.358
	e	–	–	1.000	.050	–	–	–
	f	–	.111	–	–	–	–	–
Pep-lgg	*a*	.056	.389	–	–	.050	–	–
	b	.944	.500	1.000	.500	.800	.978	–
	c	–	.111	–	.500	.150	.022	–
	d	–	–	–	–	–	–	.214
	e	–	–	–	–	–	–	.786
Pep-lp	*a*	.200	.100	–	.937	1.000	–	–
	b	.600	.800	.500	–	–	.375	–
	c	–	–	–	.063	–	–	–
	d	.200	.100	.500	–	–	.625	1.000
Pep-lv	*a*	.200	–	–	–	.150	.043	–
	b	.800	.833	.937	–	.850	.935	–
	c	–	.167	.063	–	–	.022	–
	d	–	–	–	1.000	–	–	–
	e	–	–	–	–	–	–	.833
	f	–	–	–	–	–	–	.167
Pgd	*a*	.200	.071	1.000	–	1.000	–	–
	b	–	–	–	–	–	.043	–
	c	.800	.858	–	1.000	–	–	–
	d	–	.071	–	–	–	.957	–
	e	–	–	–	–	–	–	1.000
Pgm	*a*	.950	.111	–	.150	–	–	–
	b	–	.056	–	–	–	–	–
	c	–	.722	1.000	.850	–	–	–
	d	.050	.111	–	–	–	1.000	–
	e	–	–	–	–	–	–	1.000
	f	–	–	–	–	1.000	–	–
Hexp		.146 (.034)	.237 (.052)	.070 (.030)	.113 (.038)	.079 (.030)	.058 (.024)	.067 (.029)

Table 3. Matrices of Rogers' (1972) (above diagonal) and Nei's (1978) genetic distance coefficients (below diagonal) for Southeast and East Asian samples of *Rana limnocharis* and a sample of *Rana cancrivora*

Sample	1	2	3	4	5	6	7
1. Hongkong	----	.250	.303	.322	.277	.321	.752
2. Vientiane	.170	----	.270	.350	.367	.332	.678
3. Malinping-A	.287	.218	----	.390	.370	.366	.682
4. Malinping-B	.301	.371	.458	----	.370	.473	.674
5. Wenjiang	.250	.371	.414	.398	----	.362	.795
6. Ishigaki	.328	.312	.440	.597	.410	----	.713
7. *R.cancrivora*	1.476	1.215	1.167	1.166	1.663	1.286	----

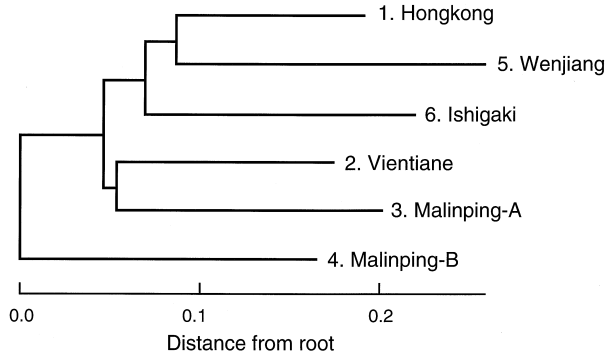

Fig. 2. NJ phenogram for six samples of *Rana limnocharis* from Southeast and East Asia based on Rogers' (1972) distance. The phenogram was rooted at the junction of the out-group (*R. cancrivora*) and the study-group OTUs.

Malinping-A sample and the two representatives of East Asian populations (i.e. Ishigaki and Wenjiang samples: D = 0.440 and 0.414, respectively) were larger than the value obtained between the latter two samples (D = 0.410), which most likely represent two distinct species (Toda *et al.*, 1997). The NJ phenogram also suggests the distant position of the Malinping-A sample in relation to the Ishigaki and Wenjiang samples. These results further suggest that the Malinping-A population has been diverged from both of the two cryptic species of East Asia, as well as from the other species represented by the Malinping-B population. Thus, it is highly likely that there are at least four cryptic species within *R. limnocharis* (*sensu lato*).

Appropriate taxonomic allocations of the remaining populations, (i.e., those from Hongkong and Vientiane) are still unclear. In the NJ phenogram, the Hongkong sample was clustered with the Wenjiang sample, whereas the Vientiane sample was clustered with the Malinping-A sample. Paradoxically, the Hongkong sample seems to be also closely related to the Vientiane sample and *vice versa* with a relatively small D value (0.170). As such, although our results suggest the occurrences of at least four species in the nominal species, *Rana limnocharis*, in Southeast and East Asia, their boundaries remain to be resolved on the basis of additional samples.

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