Genetic Variation and Population Divergence in the Mountain Brown Frog Rana ornativentris

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Source: Zoological Science, 13(4) : 537-549

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.13.537

ZOOLOGICAL SCIENCE 13: 537–549 (1996)

Genetic Variation and Population Divergence in the Mountain Brown Frog *Rana ornativentris*

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ABSTRACT—Fifteen enzymes and two blood proteins encoded by 24 presumptive loci were analyzed using starch-gel electrophoresis in 136 frogs of 16 populations of *Rana ornativentris* and 21 frogs of a sympatric population of *Rana japonica*, in order to elucidate the degree of geographic divergence of *R. ornativentris* in Honshu and its genetic relationships to *R. japonica*. The UPGMA dendrogram constructed from Nei's genetic distances showed that *R. ornativentris* from Honshu was divided into two distinct groups, western and eastern, and that the latter split into three subgroups, southern, central and northern. Genetic divergence was distinct between western and eastern populations of *R. ornativentris* at three loci, *PEP-A*, *SOD-1* and *Hb-1*, with the Fst values of Wright of 0.624, 0.635 and 0.876, respectively. The average value of Fst ($\bar{F}$st), excluding the five invariant loci, was 0.306. Nei's genetic distances among the four western populations of *R. ornativentris* were 0.015~0.061, 0.043 on average. Those among the 12 eastern populations were 0.011~0.179, 0.063 on average, whereas those between the four western and 12 eastern populations were 0.128~0.313, 0.225 on average. The genetic distances between the 16 populations of *R. ornativentris* and one population of *R. japonica* were 0.579~0.956, 0.793 on average. The UPGMA dendrogram showed that *R. ornativentris* was distinctly separated from *R. japonica*.

INTRODUCTION

Rana ornativentris was first described as *Rana japonica* var. *ornativentris* by Werner (1904). Stejneger (1907) placed this taxon in the synonymy of *R. japonica* and Okada and Kawano (1923) described this subspecies as *R. temporaria* var. *montana*. Subsequently the name *R. temporaria ornativentris* was given by Stejneger (1924) and Okada (1931) adopted this combination. Kawamura (1962) elevated this subspecies as a valid species on the basis of its morphology and reproductive isolating mechanism and named it *Rana ornativentris* Werner, which was followed by Okada (1966) and Nakamura and Uéno (1972).

This mountain brown frog usually inhabits mountain regions of Honshu, Shikoku and Kyushu in Japan, and is occasionally found in sympatry with the related brown frog *R. japonica* at lower altitudes. Intraspecific divergence in *R. ornativentris* has not been studied extensively, whereas that of *R. japonica* has been examined reproductively, karyologically and biochemically by Sumida (1981, 1994, 1996) and Sumida and Nishioka (1991, 1994). The reproductive isolating mechanisms between these two species were studied by Kawamura (1950) and Kawamura *et al.* (1981), and a comparative study on the karyotypes of these two species and other related brown frog species was reported by Nishioka *et al.* (1987b).

This study was carried out to investigate the degree of geographic divergence in *Rana ornativentris* and the genetic relationships between *R. ornativentris* and *R. japonica*.

MATERIALS AND METHODS

A total of 136 adult *Rana ornativentris*, consisting of 38 females and 98 males, from 16 geographic locations throughout Honshu and 21 adult *R. japonica*, consisting of five females and 16 males, from Saiki-cho of Hiroshima Prefecture were used in this study (Table 1). Fifteen enzymes and two blood proteins were analyzed with horizontal starch-gel electrophoresis (Table 2). The details of the electrophoretic method have been reported by Nishioka *et al.* (1980). The detection of each enzyme was carried out by means of the agar-overlay method outlined by Harris and Hopkinson (1976). The detection of blood proteins was carried out by the amido-black staining method. Multiple loci except LDH were numbered so that the most anodal was designated "1". The LDH loci were lettered because vertebrate homology was known. The electrophoretic bands corresponding to multiple alleles at each locus were named A, B, C, etc. in the order of mobility from fast to slow with A being fastest, and the alleles were indicated by *a*, *b*, *c*, etc.

The fixation index (Fst) of Wright (1978) was utilized as a standard to indicate the degree of genetic divergence found at a locus among local populations. When multiple alleles existed in a frequency of more than 1% at a locus, this locus was regarded as polymorphic. In order to quantitatively show the genetic variation in local populations, the mean proportions of heterozygous loci per individual, mean proportions of polymorphic loci per population and mean number of alleles per locus were calculated (Lewontin and Hubby, 1966; Lewontin, 1974). The genetic relationships among local populations were evaluated by calculating Nei's genetic distances (D) (Nei, 1972). The phenetic relationships among these local populations were conjectured by seven methods, the unweighted pair-group arithmetic average (UPGMA) clustering method, furthest neighbor method, flexible

Table 1. Specimens used in the present study

Species	Prefecture	Station	No. of frogs			Population	(No.)
			Total	Female	Male		
Rana ornativentris	Aomori	Hirosaki City, Namioka-cho	8	4	4	Hirosaki	(1)
	Akita	Akita City, Toyoiwaishidazaka	3	1	2	Akita	(2)
	Niigata	Nishikanbara-gun, Maki-cho	1	0	1	Maki	(3)
	Niigata	Nakakanbara-gun, Muramatsu-cho	2	1	1	Muramatsu	(4)
	Niigata	Higashikanbara-gun, Kamikawa-mura	2	1	1	Kamikawa	(5)
	Niigata	Itoigawa City, Otokoro	17	0	17	Itoigawa	(6)
	Saitama	Kitakatsushika-gun, Sugito-cho	28	8	20	Sugito	(7)
	Kanagawa	Ashigarakami-gun, Yamakita-cho	8	3	5	Yamakita	(8)
	Nagano	Okaya City	3	2	1	Okaya	(9)
	Nagano	Shiojiri City	1	1	0	Shiojiri	(10)
	Gifu	Yamagata-gun, Takatomi-cho	24	8	16	Takatomi	(11)
	Fukui	Tsuruga City, Shinbo	2	1	1	Tsuruga	(12)
	Hiroshima	Yamagata-gun, Geihoku-cho	3	2	1	Geihoku	(13)
	Hiroshima	Saiki-gun, Saiki-cho, Iinoyama	28	4	24	Saiki	(14)
	Yamaguchi	Yamaguchi City, Sayama	3	1	2	Yamaguchi	(15)
	Shimane	Nima-gun, Nima-cho	3	1	2	Nima	(16)
		Total	136	38	98		
Rana japonica	Hiroshima	Saiki-gun, Saiki-cho, Iinoyama	21	5	16	Saiki	(14')

Table 2. Enzymes and blood proteins analyzed in the present study

Enzyme or blood protein	Abbreviation	E.C.No.	Tissue source	Buffer system
Aspartate transaminase	AAT	2.6.1.1	Skeletal muscle	T-C pH 7.0
Adenosine deaminase	ADA	3.5.4.4	Skeletal muscle	T-C pH 7.0
Adenylate kinase	AK	2.7.4.3	Skeletal muscle	T-C pH 7.0
Creatine kinase	CK	2.7.3.2	Skeletal muscle	T-B-E pH 8.0
Fumarate hydratase	FH	4.2.1.2	Liver	T-B-E pH 8.0
Glycerol-3-phosphate dehydrogenase	GPD	1.1.1.8	Skeletal muscle	T-C pH 6.0
Glucose phosphate isomerase	GPI	5.3.1.9	Skeletal muscle	T-B-E pH 8.0
Isocitrate dehydrogenase	IDH	1.1.1.42	Skeletal muscle	T-C pH 7.0
Lactate dehydrogenase	LDH	1.1.1.27	Skeletal muscle	T-C pH 6.0
Malate dehydrogenase	MDH	1.1.1.37	Skeletal muscle	T-C pH 6.0
Malic enzyme	ME	1.1.1.40	Skeletal muscle	T-C pH 7.0
Mannose phosphate isomerase	MPI	5.3.1.8	Skeletal muscle	T-C pH 7.0
Peptidase	PEP	3.4.11	Liver	T-B-E pH 8.0
Phosphoglucomutase	PGM	2.7.5.1	Skeletal muscle	T-B-E pH 8.0
Superoxide dismutase	SOD	1.15.1.1	Skeletal muscle	T-B-E pH 8.0
Serum albumin	Alb	—	Blood serum	T-B-E pH 8.0
Hemoglobin	Hb	—	Erythrocyte	T-B-E pH 8.6

T-C, Tris-citrate buffer T-B-E, Tris-borate-EDTA buffer

method, centroid method, median method, nearest neighbor method and Ward method (Sneath and Sokal, 1973; Nei, 1975, 1987) on the basis of genetic distances (D).

RESULTS

Electrophoretic patterns and allelomorphs

The electrophoretic patterns showed that the enzymes and blood proteins were controlled by the genes at 24 presumptive loci1 (Table 3, Fig. 1). ADA, MPI, PGM and Alb were monomeric and heterozygotes showed double-banded patterns. AAT, GPD, GPI, IDH, MDH, PEP-A and SOD were dimeric and heterozygotes showed triple-banded patterns. FH, LDH and ME were tetrameric and heterozygotes showed five-banded patterns. Hb also had a tetrameric structure, although one heterozygote showed the pattern of a monomer. AAT, IDH, LDH, MDH, ME, SOD and Hb were each coded by two separate genetic loci. The LDH isozymes of different loci produced several hybrid bands, although some of them were faint or missing. Atypical patterns in heterozygotes were observed at the *IDH-2* and *MPI* loci at the expected relative intensity of bands. Several bands produced from probably post-translational modification were observed at the *MDH-2* and *PGM* loci (Fig.1).

Three of the 24 loci (*AK*, *CK* and *LDH-A*) were invariant. The *MPI* locus was the most polymorphic and 27 phenotypes were produced by 12 alleles. At the other 20 loci, there were two to 13 phenotypes produced by two to six alleles (Table 3).

Table 3. Number and kind of alleles and phenotypes at 24 loci in 16 populations of *Rana ornativentris* and one population of *R. japonica*

Locus	Alleles		Phenotypes	
	No.	Kind	No.	Kind
AAT-1	4	*a~d*	6	AA,BB,CC,AB,AC,BD
AAT-2	2	*a,b*	2	BB,AB
ADA	6	*a~f*	9	BB,CC,EE,FF,AC,BD,BF,CE,DF
AK	1	*a*	1	AA
CK	1	*a*	1	AA
FH	5	*a~e*	6	AA,BB,DD,AB,CD,DE
GPD	2	*a,b*	2	BB,AB
GPI	3	*a~c*	4	AA,BB,CC,AC
IDH-1	4	*a~d*	5	AA,BB,CC,AB,CD
IDH-2	2	*a,b*	2	BB,AB
LDH-A	1	*a*	1	AA
LDH-B	6	*a~f*	13	BB,CC,DD,EE,FF,AB,AD,BC,BD,BF,CD,CF,DF
MDH-1	4	*a~d*	4	BB,DD,AB,BC
MDH-2	2	*a,b*	3	AA,BB,AB
ME-1	5	*a~e*	7	AA,BB,CC,AB,AE,BE,CD
ME-2	3	*a~c*	4	BB,CC,AB,BC
MPI	12	*a~l*	27	BB,CC,EE,FF,GG,HH,AB,AC,BC,BE,BG,BH,BK, BL,CE,CG,CH,DF,EG,EH,EJ,FI,GH,GJ,GK,HJ,HK
PEP-A	4	*a~d*	6	BB,CC,AB,BC,BD,CD
PGM	2	*a,b*	2	BB,AB
SOD-1	2	*a,b*	3	AA,BB,AB
SOD-2	4	*a~d*	8	AA,BB,CC,DD,AB,AC,BC,CD
Alb	6	*a~f*	9	AA,BB,DD,EE,AB,CD,DE,DF,EF
Hb-1	2	*a,b*	3	AA,BB,AB
Hb-2	2	*a,b*	2	AA,BB
Average	3.5		5.4	

Frequencies of phenotypes and alleles

The numbers of individuals exhibiting each scored phenotype are shown in Table 4 as raw data according to Buth (1984). The allele frequencies at all variable loci are presented in Table 5. At four loci (*AAT-2*, *GPD*, *IDH-2* and *PGM*) a single allele predominated in all populations, including the 16 populations of *R. ornativentris* and the one population of *R. japonica*, although another allele was found in low frequencies in several populations (Table 5). At seven loci (*AAT-1*, *ADA*, *FH*, *GPI*, *MDH-1*, *MDH-2* and *Hb-2*) a single allele predominated in the 16 populations of *R. ornativentris*, whereas a different allele predominated in *R. japonica* (Table 5). At five loci (*IDH-1*, *LDH-B*, *ME-1*, *MPI* and *Alb*) the predominant allele was different among several groups of populations of *R. ornativentris*, whereas another allele predominated in *R. japonica* (Table 5, Fig. 2). At the other five loci (*ME-2*, *PEP-A*, *SOD-1*, *SOD-2* and *Hb-1*) the predominant allele was different among several groups of populations of *R. ornativentris*, and one of these alleles also predominated in *R. japonica* (Table 5, Fig. 3).

Genetic variation in R. ornativentris

The fixation index (Fst) was calculated according to Wright (1978) (Table 6). When the allele frequencies at a definite locus are the same in all the 16 populations, the fixation index is zero, whereas it is 1.000 when there is a characteristic allele at a definite locus in one or more populations. The higher the fixation index, the more advanced the divergence in the locus.

The most advanced loci in divergence was the *Hb-1* locus, being 0.876 in Fst. This was followed by the *SOD-1*, *PEP-A*, *SOD-2*, *Alb*, *IDH-1*, *MPI* and *LDH-B* loci, ranging from 0.635 to 0.312 in Fst. At these seven loci, except the *SOD-2* locus, the genetic divergence was distinct between the eastern and western populations, whereas the genetic divergence was clear in the central regions of Honshu at the *SOD-2* locus. The *ME-2*, *ME-1*, *FH*, *IDH-2*, *GPD*, *ADA*, *AAT-1* and *MDH-1* loci were from 0.261 to 0.030 in Fst, and showed various degrees of genetic divergence. The remaining five loci (*AK*, *CK*, *LDH-A*, *MDH-2* and *Hb-2*) were zero in Fst (Table 6). Average value of Fst ($\bar{F}$st) excluding the five invariant loci was 0.306.

The mean proportion of heterozygous loci per individual, mean proportion of polymorphic loci per population and mean number of alleles per locus in the 16 populations of *R. ornativentris* were 4.2%~24.2%, 14.4% on average, 4.2%~66.7%, 32.8% on average, and 1.04~2.21, 1.45 on average, respectively (Table 7). In 11 populations of which the sample size was larger than three, the comparable figures were 12.0%~24.2%, 15.9% on average, 20.8%~66.7%, 39.8% on average, and 1.29~2.21, 1.57 on average, respectively. There were no noticeable differences between these rates and the expected values, except populations consisting of one, two or three samples.

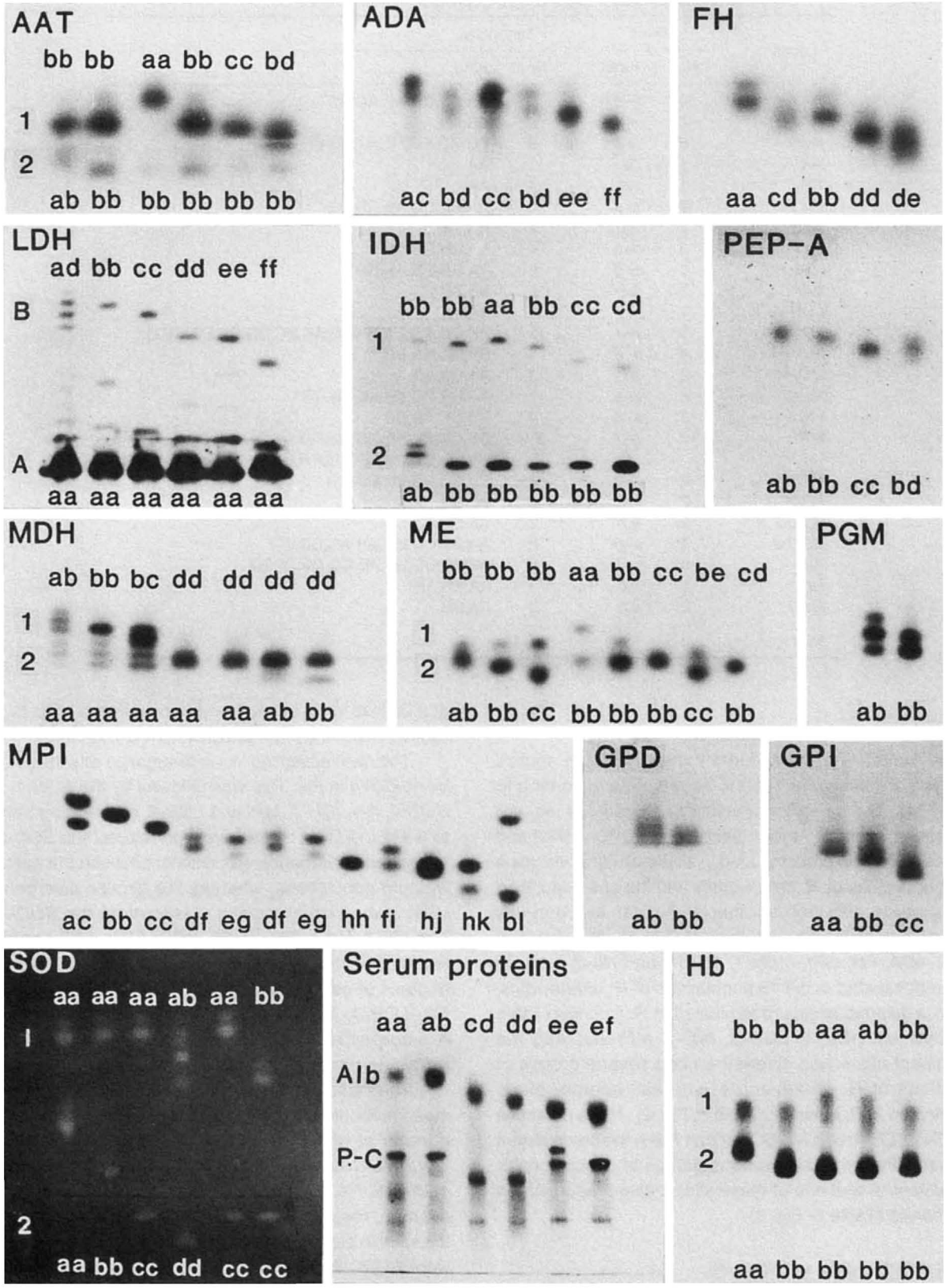

Fig. 1. Electrophoretic patterns of 13 enzymes and two blood proteins in 16 populations of *Rana ornativentris* and one population of *R. japonica*. The genotypes are represented by aa, bb, cc, etc.

Table 4. Frequencies of phenotypes at 24 loci in 16 populations of *Rana ornativentris* and one population of *R. japonica*

Species		*R. orn.*																*R. jap.*
Population		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	14'
Locus	Phenotype	(8)	(3)	(1)	(2)	(2)	(17)	(28)	(8)	(3)	(1)	(24)	(2)	(3)	(28)	(3)	(3)	(21)
AAT-1	AA																	6
	BB	8	3	1	2	2	14	23	7	3	1	22	2	3	26	3	3	
	CC																	5
	AB						1	3				2						
	AC																	10
	BD						2	2	1						2			
AAT-2	BB	8	3	1	2	2	17	28	8	3	1	24	2	3	22	3	3	21
	AB														6			
ADA	BB																	5
	CC	8	3	1	2	2	16	24	8	3	1	24	2	3	22	3	3	
	EE														1			
	FF																	2
	AC							1							2			
	BD																	6
	BF																	6
	CE						1	3							3			
	DF																	2
AK	AA	8	3	1	2	2	17	28	8	3	1	24	2	3	28	3	3	21
CK	AA	8	3	1	2	2	17	28	8	3	1	24	2	3	28	3	3	21
FH	AA																	3
	BB																	7
	DD	8	3	1	2	2	17	26	8	3	1	24	2	1	23	3	2	
	AB																	11
	CD													2	5		1	
	DE							2										
GPD	BB	8	3	1	2	2	17	28	8	3	1	24	2	3	25	3	2	21
	AB														3		1	
GPI	AA	8	3	1	1	2	16	23	8	3	1	7	2	3	27	3	2	
	BB																	21
	CC											3						
	AC				1		1	5				14			1		1	
IDH-1	AA	2	1				2					3		3	28	3	3	
	BB	3		1	1	1	6	23	5	2		7	1					
	CC																	18
	AB	3	2		1	1	9	5	3	1	1	14	1					
	CD																	3
IDH-2	BB	8	3	1	2	2	16	28	5	3	1	24	2	3	28	3	3	21
	AB						1		3									
LDH-A	AA	8	3	1	2	2	17	28	8	3	1	24	2	3	28	3	3	21
LDH-B	BB														1			
	CC						2	3	2			2			2			
	DD	7	3	1	1	2	7	13	1	1		11	2	1	3			
	EE																	21
	FF														1		1	
	AB														1			
	AD														1			
	BC														2			
	BD														3			
	BF														5	1		
	CD	1			1		8	12	5	2	1	11			5			
	CF													1	1	2		
	DF													1	3		2	
MDH-1	BB	8	3	1	2	2	16	28	8	3	1	24	2	3	26	3	3	
	DD																	21
	AB						1											
	BC														2			
MDH-2	AA	8	3	1	2	2	17	28	8	3	1	24	2	3	28	3	3	3
	BB																	10
	AB																	8
ME-1	AA																1	
	BB	6	2	1	2	2	10	19	8	1	1	15	1	3	17	1		
	CC																	17
	AB	1					2	2									7	2	2	
	AE							2									2			
	BE	1	1				5	5		2		9	1				2			
	CD																	4
ME-2	BB	2	2	1	1		10	18	4	2	1	15		2	22	3	1	12
	CC	1						1				3	1				1	
	AB		1		1	2	2		2	1					2			
	BC	5					5	9	2			6	1	1	4		1	9
MPI	BB	1				1	7	10	6	1	1	1						
	CC	1					1	2		1								
	EE											1	1					
	FF																	8
	GG											7			5			
	HH														4	1		
	AB						2	3										
	AC	1					1											
	BC	2	1				4	9		1								
	BE		1			1	2	2				1						
	BG	2			1			1	1			4			1			
	BH														3	1		
	BK														1			
	BL																	
	CE		1														1	
	CG			1				1	1									
	CH														1			
	DF																	8
	EG	1						1										
	EH											10			1			
	EJ																1	
	FI																	5
	GH																1	
	GJ														6			1
	GK														1			
	HJ														1		1	
	HK														2			
PEP-A	BB	8	2	1	2	2	15	28	8	3	1	10			1	1		
	CC												1	2	21	1	2	16
	AB		1															
	BC						2					14	1	1	5	2	1	3
	BD														1			
	CD																	2
PGM	BB	7	3	1	2	2	17	23	8	3	1	24	2	3	27	3	3	21
	AB	1						5							1			
SOD-1	AA	8	3	1	2	2	17	28	8	3	1	17	2		7			
	BB														7	3	1	21
	AB											7		3	14		2	
SOD-2	AA							1				4						
	BB						12	8	5	1	1	9						
	CC	8	3	1	2	2		6		1		2	2	1	13	3	2	21
	DD													1				
	AB						4	5				3						
	AC											2						
	BC						1	8	3	1		4						
	CD													1	15		1	
		(7)	(3)	(1)	(2)	(2)	(17)	(26)	(8)	(3)	(1)	(24)	(2)	(2)	(26)	(3)	(1)	(19)
Alb	AA																	6
	BB																	2
	DD													2	23	2		
	EE	2	1	1	1	1	17	22	6	1		14	1					
	AB																	11
	CD														2	1	1	
	DE	1			1	1				1					1			
	DF													1				
	EF	4	2					4	2	1	1	10						
Hb-1	AA	7	3	1	2	2	14	18	8	3	1							
	BB							4				23	1	2	26	3	1	19
	AB						3	4				1	1					
Hb-2	AA																	19
	BB	7	3	1	2	2	17	26	8	3	1	24	2	2	26	3	1	

Parentheses show the sample size.

Table 5. Allele frequencies at 21 loci in 16 populations of *Rana ornativentris* and one population of *R. japonica*

Species		*Rana ornativentris*																*R. japonica*
Population		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	14'
Locus	Allele	(8)	(3)	(1)	(2)	(2)	(17)	(28)	(8)	(3)	(1)	(24)	(2)	(3)	(28)	(3)	(3)	(21)
AAT-1	*a*						0.029	0.054				0.042						0.524
	b	1.000	1.000	1.000	1.000	1.000	0.912	0.911	0.938	1.000	1.000	0.958	1.000	1.000	0.964	1.000	1.000	
	c																	0.476
	d						0.059	0.036	0.063						0.036			
AAT-2	*a*														0.107			
	b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.893	1.000	1.000	1.000
ADA	*a*							0.018							0.036			
	b																	0.524
	c	1.000	1.000	1.000	1.000	1.000	0.971	0.929	1.000	1.000	1.000	1.000	1.000	1.000	0.875	1.000	1.000	
	d																	0.190
	e						0.029	0.054							0.089			
	f																	0.286
FH	*a*																	0.405
	b																	0.595
	c													0.333	0.089		0.167	
	d	1.000	1.000	1.000	1.000	1.000	1.000	0.964	1.000	1.000	1.000	1.000	1.000	0.667	0.911	1.000	0.833	
	e							0.036										
GPD	*a*														0.054		0.167	
	b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.946	1.000	0.833	1.000
GPI	*a*	1.000	1.000	1.000	0.750	1.000	0.971	0.911	1.000	1.000	1.000	0.583	1.000	1.000	0.982	1.000	0.833	
	b																	1.000
	c				0.250		0.029	0.089				0.417			0.018		0.167	
IDH-1	*a*	0.438	0.667		0.250	0.250	0.382	0.089	0.188	0.167	0.500	0.417	0.250	1.000	1.000	1.000	1.000	
	b	0.563	0.333	1.000	0.750	0.750	0.618	0.911	0.813	0.833	0.500	0.583	0.750					
	c																	0.929
	d																	0.071
IDH-2	*a*						0.029		0.188									
	b	1.000	1.000	1.000	1.000	1.000	0.971	1.000	0.813	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
LDH-B	*a*														0.036			
	b														0.232	0.167		
	c	0.063			0.250		0.353	0.321	0.563	0.333	0.500	0.313		0.167	0.214	0.333		
	d	0.938	1.000	1.000	0.750	1.000	0.647	0.679	0.438	0.667	0.500	0.688	1.000	0.500	0.321		0.333	
	e																	1.000
	f													0.333	0.196	0.500	0.667	
MDH-1	*a*						0.029											
	b	1.000	1.000	1.000	1.000	1.000	0.971	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.964	1.000	1.000	
	c														0.036			
	d																	1.000
MDH-2	*a*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.333
	b																	0.667
ME-1	*a*	0.063					0.059	0.071							0.161	0.333	0.667	
	b	0.875	0.833	1.000	1.000	1.000	0.794	0.804	1.000	0.667	1.000	0.813	0.750	1.000	0.768	0.667	0.333	
	c																	0.905
	d																	0.095
	e	0.063	0.167				0.147	0.125		0.333		0.188	0.250		0.071			
ME-2	*a*		0.167		0.250	0.500	0.059		0.125	0.167					0.036			
	b	0.563	0.833	1.000	0.750	0.500	0.794	0.804	0.750	0.833	1.000	0.750	0.250	0.833	0.893	1.000	0.500	0.786
	c	0.438					0.147	0.196	0.125			0.250	0.750	0.167	0.071		0.500	0.214
MPI	*a*	0.063					0.088	0.054										
	b	0.375	0.333		0.250	0.750	0.647	0.625	0.813	0.500	1.000	0.146	0.250		0.089	0.167		
	c	0.313	0.333	0.500			0.206	0.250	0.063	0.500					0.018			
	d																	0.190
	e	0.063	0.333		0.250	0.250	0.059	0.036				0.271	0.500	0.167	0.036			
	f																	0.690
	g	0.188		0.500	0.500			0.036	0.125			0.583		0.333	0.357		0.167	
	h													0.167	0.393	0.667	0.500	
	i																	0.119
	j													0.167	0.054		0.167	
	k													0.167	0.054	0.167	0.167	
	l												0.250					

Table 5. (continued)

Species		*Rana ornativentris*																*R. japonica*
Population		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	14'
Locus Allele		(8)	(3)	(1)	(2)	(2)	(17)	(28)	(8)	(3)	(1)	(24)	(2)	(3)	(28)	(3)	(3)	(21)
PEP-A	*a*		0.167															
	b	1.000	0.833	1.000	1.000	1.000	0.941	1.000	1.000	1.000	1.000	0.708	0.250	0.167	0.143	0.333	0.167	0.071
	c						0.059					0.292	0.750	0.833	0.839	0.667	0.833	0.881
	d														0.018			0.048
PGM	*a*	0.063						0.089							0.018			
	b	0.938	1.000	1.000	1.000	1.000	1.000	0.911	1.000	1.000	1.000	1.000	1.000	1.000	0.982	1.000	1.000	1.000
SOD-1	*a*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.854	1.000	0.500	0.500		0.333	
	b											0.146		0.500	0.500	1.000	0.667	1.000
SOD-2	*a*						0.118	0.125				0.271						
	b						0.853	0.518	0.813	0.500	1.000	0.521						
	c	1.000	1.000	1.000	1.000	1.000	0.029	0.357	0.188	0.500		0.208	1.000	0.500	0.732	1.000	0.833	1.000
	d													0.500	0.268		0.167	
		(7)	(3)	(1)	(2)	(2)	(17)	(26)	(8)	(3)	(1)	(24)	(2)	(2)	(26)	(3)	(1)	(19)
Alb	*a*																	0.605
	b																	0.395
	c														0.038	0.167	0.500	
	d	0.071			0.250	0.250				0.167			0.250	1.000	0.942	0.833	0.500	
	e	0.643	0.667	1.000	0.750	0.750	1.000	0.923	0.875	0.667	0.500	0.792	0.500		0.019			
	f	0.286	0.333					0.077	0.125	0.167	0.500	0.208	0.250					
Hb-1	*a*	1.000	1.000	1.000	1.000	1.000	0.912	0.769	1.000	1.000	1.000	0.021	0.250					
	b						0.088	0.231				0.979	0.750	1.000	1.000	1.000	1.000	1.000
Hb-2	*a*																	1.000
	b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	

Parentheses show the sample size.

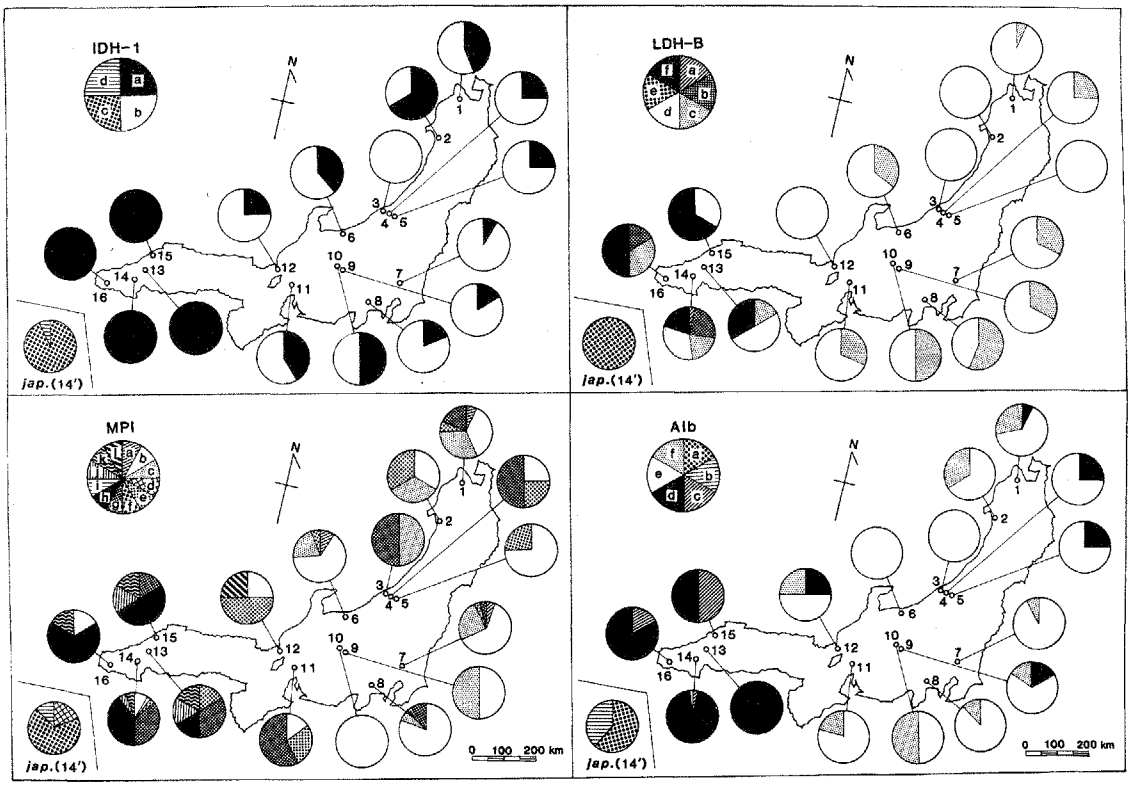

Fig. 2. Geographic distributions of multiple alleles at four loci, the *IDH-1*, *LDH-B*, *MPI* and *Alb* loci, in 16 populations of *Rana ornativentris* and one population of *R. japonica*. Numbers represent populations designated in Table 1.

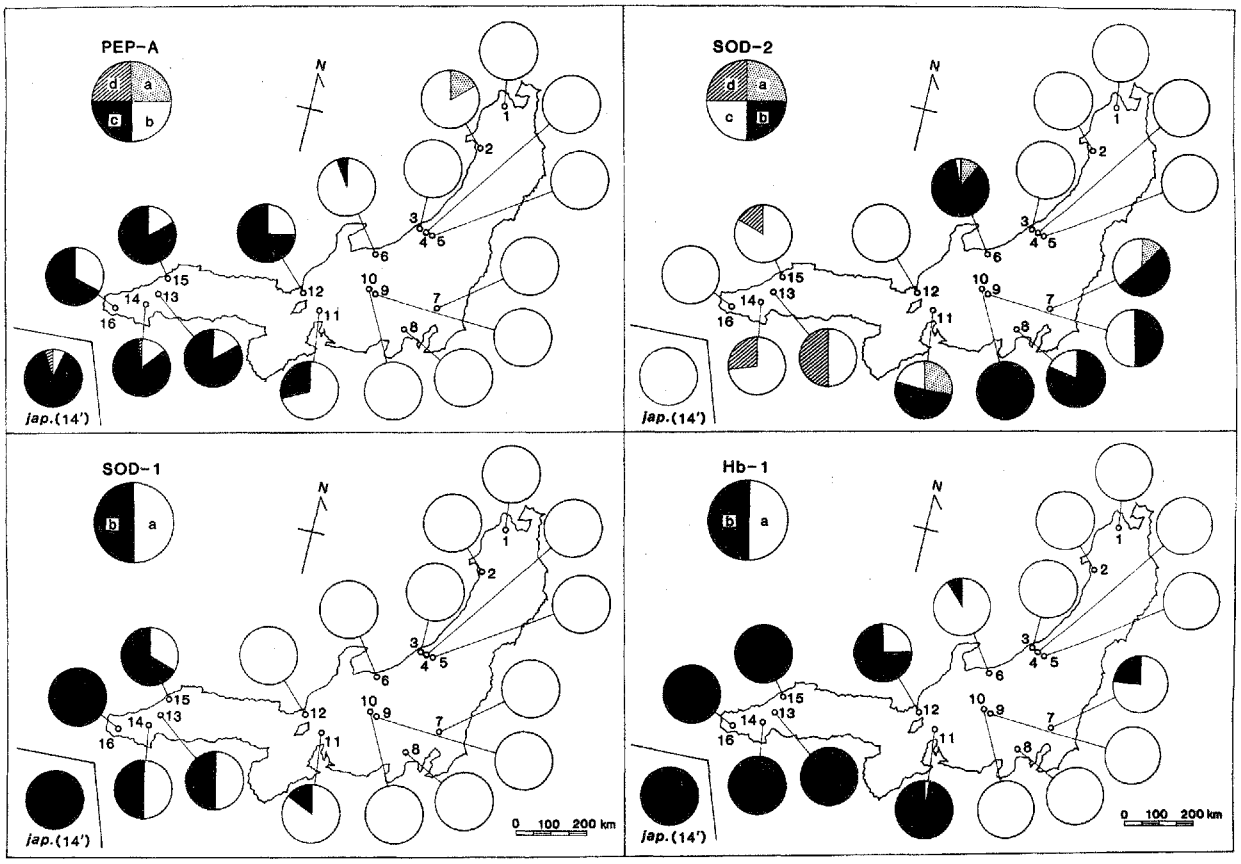

Fig. 3. Geographic distributions of multiple alleles at four loci, the *PEP-A*, *SOD-1*, *SOD-2* and *Hb-1* loci, in 16 populations of *Rana ornativentris* and one population of *R. japonica*. Numbers represent populations designated in Table 1.

Table 6. Fixation index at 24 loci in 16 populations of *Rana ornativentris*

Locus	Fst	Locus	Fst
AAT-1	0.046	*MDH-1*	0.030
AAT-2	0.101	*MDH-2*	0
ADA	0.071	*ME-1*	0.241
AK	0	*ME-2*	0.261
CK	0	*MPI*	0.313
FH	0.208	*PEP-A*	0.624
GPD	0.127	*PGM*	0.062
GPI	0.235	*SOD-1*	0.635
IDH-1	0.466	*SOD-2*	0.578
IDH-2	0.155	*Alb*	0.481
LDH-A	0	*Hb-1*	0.876
LDH-B	0.312	*Hb-2*	0

Genetic distances

The genetic distances among 16 populations of *R. ornativentris* ranged from 0.011 between the Itoigawa and Yamakita populations to 0.313 between the Yamakita and Nima populations, with a mean of 0.127 (Table 8). The genetic distances among the four western populations of *R. ornativentris* ranged from 0.015 between the Geihoku and Saiki populations to 0.061 between the Geihoku and Nima populations, with a mean of 0.043 and those among the 12 eastern populations ranged from 0.011 between the Itoigawa and Yamakita populations to 0.179 between the Shiojiri and Tsuruga populations, with a mean of 0.063 (Table 8). On the other hand, the genetic distances between the four western and 12 eastern populations ranged from 0.128 between the Tsuruga and Geihoku or Saiki populations to 0.313 between the Yamakita and Nima populations, with a mean of 0.225 (Table 8). The genetic distances between the 16 populations of *R. ornativentris* and one population of *R. japonica* ranged from 0.579 between the Yamaguchi population of *R. ornativentris* and the Saiki population of *R. japonica* to 0.956 between the Shiojiri population of *R. ornativentris* and the Saiki population of *R. japonica*, with a mean of 0.793 (Table 8). The genetic distances between the four western populations of *R. ornativentris* and the Saiki population of *R. japonica* were 0.579~0.638, 0.608 on average, whereas those between the 12 eastern populations of *R. ornativentris* and the Saiki population of *R. japonica* were 0.707~0.956, 0.855 on average.

Dendrogram

The phenetic relationships were assumed from a dendrogram drawn by the UPGMA method, which is the most commonly used. The UPGMA dendrogram showed that *R. japonica* is clearly separated from *R. ornativentris*, which constitutes two clusters, the western and eastern (Fig. 4). In

Table 7. Genetic variabilities at 24 loci in 16 populations of *Rana ornativentris*

Population	Sample size (n)	Mean proportion of heterozygous loci per individual (%)	Mean proportion of polymorphic loci per population (%)	Mean number of alleles per locus
Hirosaki	8	12.2 (11.1)	29.2	1.50
Akita	3	13.9 (10.0)	25.0	1.29
Maki	1	4.2 (2.1)	4.2	1.04
Muramatsu	2	14.6 (10.4)	25.0	1.29
Kamikawa	2	10.4 (7.2)	16.7	1.17
Itoigawa	17	14.0 (12.8)	54.2	1.79
Sugito	28	14.0 (14.9)	54.2	1.83
Yamakita	8	12.0 (10.3)	33.3	1.42
Okaya	3	13.9 (12.3)	29.2	1.33
Shiojiri	1	12.5 (6.3)	12.5	1.13
Takatomi	24	19.4 (18.2)	50.0	1.58
Tsuruga	2	14.6 (13.0)	29.2	1.38
Geihoku	3	18.8 (14.1)	29.2	1.46
Saiki	28	18.3 (17.5)	66.7	2.21
Yamaguchi	3	13.9 (9.5)	20.8	1.29
Nima	3	24.2 (18.3)	45.8	1.54
Average	8.5	14.4 (11.8)	32.8	1.45

Parentheses show an expected value calculated from allele frequencies under Hardy-Weinberg Equilibrium condition.

Table 8. Genetic identity (I) and genetic distance (D) among 16 populations of *Rana ornativentris* and one population of *R. japonica*

Species	Population	No.	*R. orn.*																*R. jap.*
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	14'
R. orn.	Hirosaki	1	–	.986	.972	.978	.978	.943	.963	.940	.969	.923	.902	.928	.823	.827	.790	.803	.428
	Akita	2	.014	–	.961	.970	.974	.938	.949	.927	.964	.922	.896	.914	.832	.837	.803	.801	.433
	Maki	3	.028	.039	–	.979	.959	.930	.959	.929	.963	.892	.896	.892	.788	.792	.752	.749	.422
	Muramatsu	4	.023	.030	.021	–	.980	.938	.960	.945	.964	.915	.915	.906	.823	.829	.790	.783	.432
	Kamikawa	5	.023	.026	.041	.020	–	.940	.959	.945	.964	.919	.884	.914	.805	.809	.775	.770	.414
	Itoigawa	6	.058	.064	.072	.064	.061	–	.988	.989	.979	.978	.926	.873	.796	.797	.761	.757	.399
	Sugito	7	.038	.053	.042	.041	.042	.012	–	.985	.987	.963	.937	.902	.799	.803	.769	.766	.424
	Yamakita	8	.061	.076	.073	.057	.057	.011	.016	–	.977	.982	.909	.860	.777	.779	.749	.731	.385
	Okaya	9	.032	.037	.038	.037	.037	.021	.013	.024	–	.961	.909	.890	.800	.808	.777	.764	.415
	Shiojiri	10	.081	.081	.115	.089	.084	.022	.038	.018	.040	–	.897	.836	.789	.791	.761	.738	.385
	Takatomi	11	.104	.110	.110	.089	.124	.077	.065	.096	.096	.109	–	.920	.879	.879	.830	.846	.485
	Tsuruga	12	.075	.090	.115	.099	.090	.136	.104	.151	.116	.179	.084	–	.880	.880	.826	.867	.493
	Geihoku	13	.195	.184	.239	.194	.217	.228	.225	.253	.223	.236	.129	.128	–	.985	.944	.941	.528
	Saiki	14	.190	.178	.233	.188	.212	.227	.220	.250	.214	.235	.129	.128	.015	–	.969	.954	.543
	Yamaguchi	15	.235	.220	.284	.235	.255	.273	.262	.289	.253	.273	.186	.191	.058	.031	–	.957	.561
	Nima	16	.220	.221	.289	.244	.262	.279	.267	.313	.269	.304	.168	.143	.061	.047	.044	–	.549
R. jap.	Saiki	14'	.849	.836	.862	.839	.881	.919	.858	.955	.878	.956	.725	.707	.638	.610	.579	.600	–

Genetic identity (I) is given above the diagonal and genetic distance (D) is given below.

the eastern cluster, the Takatomi and Tsuruga populations constitute a subcluster and split into another 10 populations which form two subclusters, the northern and southern. The other six kinds of dendrograms did not remarkably differ from that drawn by the UPGMA method.

DISCUSSION

The question of how much genetic divergence occurs during the process of speciation is one of the most cardinal problems of evolutionary genetics. The genetic divergence between taxa of various levels of evolutionary divergence has been reviewed in many organisms (Ayala, 1975; Avise, 1976; Avise and Aquadro, 1982). The intraspecific genetic divergence has been estimated by calculating the genetic distances among populations in many amphibian species. The mean genetic distances among different populations were 0.007~0.205 in 55 amphibian species (Sumida, unpublished). It is probable that species having large genetic distances among populations are divided into several local groups which are geographically isolated or diverged long ago.

The present study revealed that the genetic divergence

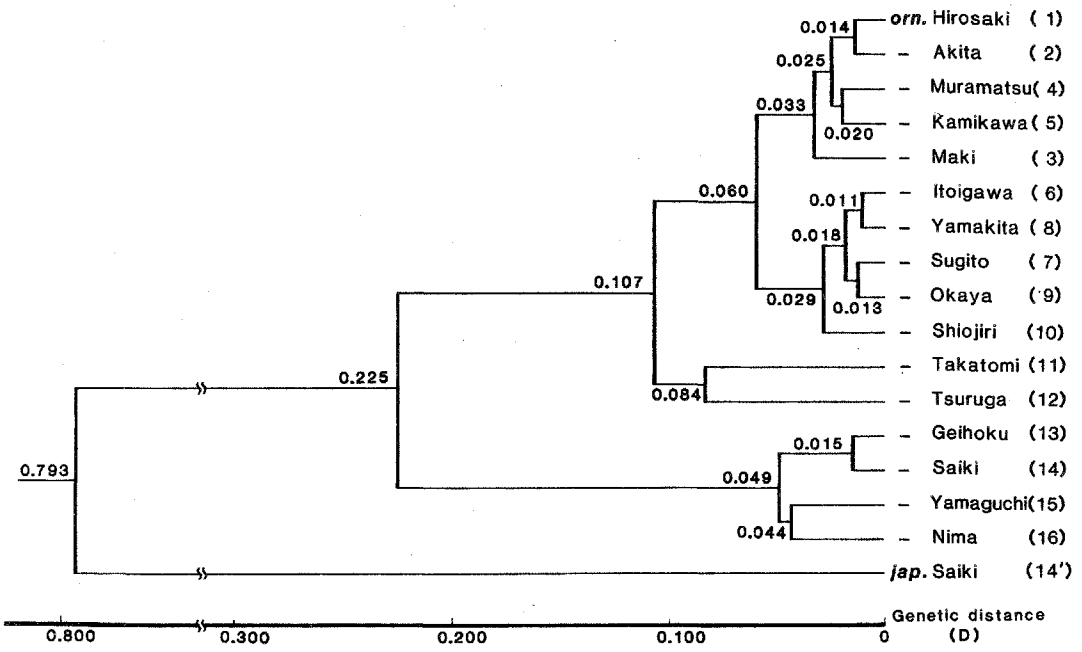

Fig. 4. UPGMA dendrogram for 16 populations of *Rana ornativentris* and one population of *R. japonica*. The horizontal axis is Nei's genetic distance.

between the western and eastern groups of *Rana ornativentris* was distinct. Although no samples from the region between Tsuruga and Nima were examined, it was inferred that the boundary between the eastern and western groups was around the Kinki area on the basis of allelic distribution at several loci. Considerable east-west divergence was evident for seven loci (*IDH-1*, *LDH-B*, *MPI*, *PEP-A*, *SOD-1*, *Alb* and *Hb-1*), 0.312~0.876 in Fst, whereas at the *SOD-2* locus, 0.578 in Fst, the divergence was distinct in the central region of Honshu (Figs. 2 and 3). There were distinct gradients from east to west in the alleles at the *PEP-A*, *SOD-1* and *Hb-1* loci. According to Ueda (Personal communication), the reciprocal hybrids between the Geihoku and Hirosaki populations of *R. ornativentris* are normal both in viability and reproductive capacity. Nei's genetic distances (D) correspond with the divergence time (T) from the common ancestor by the equation of $T = 5 \times 10^6$ D (years) (Nei, 1975, 1987). Applying this equation to the present study, it was speculated that *R. ornativentris* diverged into the eastern and western groups about 1.1 million years ago and then the latter diverged into the three subgroups about 0.3 ~ 0.5 million years ago. Japan was a part of continent up to the end of the Riss glacial stage in the Pleistocene (Minato *et al.*, 1965). It seems probable that after the ancestors of *R. ornativentris* invaded Japan and widened their distribution all over Japan during the Pleistocene, they were isolated into eastern and western groups due to marine transgression during the interglacial stages in the middle Pleistocene (Minato *et al.*, 1965). Before they established a reproductive isolating mechanism, they came into contact with each other during the marine regression in the Riss and Würm glacial stages. Thus the gradients from east to west were presumably formed in the alleles at several loci in the central regions of Honshu.

Studies of biochemical divergence in amphibians distributed in Honshu were carried out using *Bufo japonicus* (Kawamura *et al.*, 1990), *Rana nigromaculata* (Nishioka *et al.*, 1992b), *Rana rugosa* (Nishioka *et al.*, 1993), *Rana japonica* (Sumida and Nishioka, 1991, 1994) and *Cynops pyrrhogaster* (Hayashi and Matsui, 1988, 1990). Biochemical divergence between the western and eastern groups was also observed in *B. japonicus*, *R. rugosa* and *R. japonica*. The divergence of *R. japonica* into the eastern and western groups was evident at the *PEP-A* and *Hb-1* loci, which were 0.722 and 0.781 in Fst, respectively (Sumida and Nishioka, 1994). These two groups were reproductively isolated by incomplete hybrid male sterility (Sumida, 1981, 1994, 1996). In *Bufo japonicus*, there were distinct gradients from east to west in alleles at the *AAT-2*, *IDH-1*, *LDH-B*, *ME-1* and *MPI* loci, which were 0.451, 0.730, 0.346, 0.420 and 0.383 in Fst, respectively. The UPGMA dendrogram showed that the toads distributed in Japan were divided into the eastern and western groups and then each group was divided into several subgroups (Kawamura *et al.*, 1990). Matsui (1984) has reported that *B. japonicus* is divided into eastern and western types on the basis of morphological variation analyses with the central Honshu region as a dividing line, although the eastern and western groups of this species were not reproductively isolated (Kawamura *et al.*, 1980). In *Rana rugosa*, the divergence between the eastern and western groups was distinctly recognizable at the *CK*, *LDH-B*, *MDH-1*, *PEP-A* and *Hb-1* loci, which were 0.781, 0.901, 0.795, 0.979 and 0.863 in Fst, respectively. The UPGMA dendrogram showed that *R. rugosa* was first divided into the western and

eastern groups, and that the latter was divided into three subgroups, northern, intermediate and southern (Nishioka *et al.*, 1993). Levels of population subdivision were estimated by calculating the average values of Fst ($\bar{F}st$) for many amphibian species (Larson, 1980; Larson *et al.*, 1984; Ragghianti and Wake, 1986). The present study revealed that the average value of Fst excluding five invariant loci was 0.306 in the 16 populations of *Rana ornativentris*, whereas that for eight variant loci including *IDH-1*, *LDH-B*, *MPI*, *PEP-A*, *SOD-1*, *SOD-2*, *Alb* and *Hb-1* was 0.537. As is the case for several species mentioned above, *Rana ornativentris* is probably comprised of populations that are isolated from each other.

Genetic variabilities in allopatric populations were reviewed in anurans and urodeles by Nevo *et al.* (1984), Nevo and Beiles (1991) and Shaffer and Breden (1989). According to these researchers, the mean proportions of heterozygous loci per individual and polymorphic loci per population were 7.3±0.4% and 25.5±1.1%, respectively, in 188 amphibian species (two order and 13 families) including 123 urodeles (five families) and 65 anurans (eight families) (Nevo and Beiles, 1991). These two parameters were 8.7±0.5% and 26.4±1.4%, respectively, in 102 species from 19 genera and six families of urodeles (Shaffer and Breden, 1989). In the genus *Rana*, mean genetic indices for all 22 species were 7.5±1.3% and 23.3±2.9%, respectively (Nevo and Beiles, 1991). The two parameters for four Japanese *Rana* species, *R. limnocharis*, *R. nigromaculata*, *R. porosa* and *R. rugosa*, are 6.1~9.9% and 23.1~31.1%, respectively (Nishioka and Sumida, 1990; Nishioka *et al.*, 1992b, 1993). These four species generally inhabit constant environments such as plains and lowlands, especially around rice fields, although *Rana rugosa* is also found in low mountains near water. On the other hand, in three Japanese *Rana* species, *R. tagoi*, *R. japonica* and *R. ornativentris*, the two parameters were high, 11.3~16.1% and 39.8~55.2%, respectively (Nishioka *et al.*, 1987a; Sumida and Nishioka, 1994; present study). These three brown frog species chiefly inhabit variable and narrow habitats such as hillsides and mountain districts. *Rana ornativentris* is usually abundant in mountain regions up to 1900 m (Maeda and Matsui, 1989). Dessauer *et al.* (1975) and Nevo (1978) have suggested that the amounts of genetic polymorphism and heterozygosity are correlated with ecological heterogeneity, and may be regarded as an adaptive strategy for increasing population fitness in an ecologically variable environment. This interpretation may be applicable not only to *R. tagoi* and *R. japonica* but also to *R. ornativentris* in this study.

The genetic distances have been estimated among various species of the genus *Rana*. The genetic distances between *Rana boylei* and *R. muscosa* were 0.68~0.77 (Case, 1978). Those among five species endemic to western North America, *R. boylii* (probably *R. boylei*), *R. muscosa*, *R. aurora*, *R. cascadae* and *R. pretiosa*, were 0.171~0.733, whereas those between these five species and three related species, *R. sylvatica*, *R. temporaria* and *R. dybowskii*, were 0.506~1.122 (Green, 1986). He found that the 24 chromosome species, *R. dybowskii*, was very distant genetically from all other species examined and had a Nei's genetic distance of over 0.940, on average, from all other samples. Fanglin et al. (1989) reported that the genetic distances among three Eurasian brown frog species, *R. amurensis*, *R. chaochiaoensis* and *R. japonica*, were 0.500~0.876. Nishioka *et al.* (1992a) presented the genetic divergence among 30 populations of 12 brown frog species distributed in the Palearctic region. Among seven species having 26 chromosomes, *R. japonica*, *R. tsushimensis*, *R. okinavana*, *R. longicrus*, *R. temporaria*, *R. asiatica* and *R. amurensis*, the genetic distances were 0.294~1.396, whereas those among four species having 24 chromosomes, *R. ornativentris*, *R. pirica* (Matsui, 1991; Matsui *et al.*, 1993), *R. dybowskii* and *R. arvalis*, were 0.474~1.018. Those between the seven species with 26 chromosomes and the four species with 24 chromosomes were 0.410~1.715. Green and Borkin (1993) reported that the genetic distances among 11 Eurasian brown frog species including *R. chensinensis*, *R. dybowskii*, *R. ornativentris*, *R. arvalis*, *R. japonica*, *R. tagoi*, *R. amurensis*, *R. temporaria*, *R. dalmatina*, *R. camerani* and *R. macrocnemis* were 0.232~1.127.

The present study revealed that the genetic distances between *R. ornativentris* and sympatric *R. japonica*, which have a diploid chromosome number of 24 and 26, respectively, were relatively large, 0.579~0.956, 0.793 on average. The genetic distances between *R. japonica* and *R. ornativentris* were also reported by Matsui (1991), Nishioka *et al.* (1992a), Matsui and Wilkinson (1992) and Green and Borkin (1993). Applying Nei's equation to these studies, it was speculated that the lineage of two species diverged about 3~5 million years ago. *Rana ornativentris* and *R. japonica* are occasionally found sympatrically, but are completely reproductively isolated from each other by hybrid sterility or gametic isolation (Kawamura, 1950; Kawamura *et al.*, 1981). The hybrids between female *R. japonica* and male *R. ornativentris* developed normally but became sterile males, and no eggs of *R. ornativentris* cleaved by insemination with the sperm of *R. japonica*. According to Nishioka *et al.* (1992a) and Green and Borkin (1993), it seems likely that *R. ornativentris* is more closely related to *R. dybowskii* and *R. chensinensis* (sic), which have 24 chromosomes than to *R. japonica*, which has 26 chromosomes. Tanaka *et al.* (1994) investigated phylogenetic relationships among five Japanese brown frog species by the analysis of molecular sequences in the cytochrome b gene of mtDNA, and revealed that *R. ornativentris* and *R. pirica* having 24 chromosomes formed a subcluster and split from *R. japonica* having 26 chromosomes. It is probable that after the two brown frog lineages having 24 and 26 chromosomes entered Japan, they diverged into several valid species having 24 and 26 chromosomes, respectively.

ACKNOWLEDGMENTS

The authors are grateful to Dr. N. Yoshizaki, Gifu University, Dr. H. Iwao, Yamaguchi University, Dr. M. Ryuzaki, Kitasato University, Mr. K. Kinebuchi, Niigata Senior High School, Dr. I. Miura and Mr. H. Hanada, Hiroshima University, for collecting and providing us valuable specimens, and to Dr. K. Sekiya, Niigata University, Dr. T. Fujii,

Hiroshima Women's University, Messrs. T. Hongo and S. Nakagawa, Araya Senior High School, for their kind aid in collecting specimens. This study was supported in part by a Grant-in-Aid (No. 07640931) for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan to M. Sumida.

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(Received February 5, 1996 / Accepted April 19, 1996)