Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. 10: 53–91 (1990)

Genetic Relationships among 13 *Bufo* Species and Subspecies Elucidated by the Method of Electrophoretic Analyses

By

Midori NISHIOKA[1], Masayuki SUMIDA[1], Hiroaki UEDA[1]
and Zheng'an WU[2]

[1]*Laboratory for Amphibian Biology, Faculty of Science,*
Hiroshima University, Hiroshima 730, Japan
and
[2]*Institute of Developmental Biology, Academia Sinica,*
Beijing, People's Republic of China

ABSTRACT

Thirteen enzymes extracted from the skeletal muscles and two blood proteins were analyzed by the horizontal electrophoretic method in 312 toads belonging to 23 populations of 13 *Bufo* species and subspecies. These populations included one of *B. j. montanus,* seven of *B. j. japonicus,* one of *B. j. yakushimensis,* one of *B. j. torrenticola,* one of *B. j. miyakonis,* two of *B. j. gargarizans,* one of *B. j. bankorensis,* two of *B. b. bufo,* one of *B. melanostictus,* one of *B. viridis,* one of *B. raddei,* three of *B. marinus* and one of *B. americanus.* The enzymes and blood proteins were controlled by genes at 21 loci. There were 12.2 phenotypes produced by 8.7 alleles at each locus, on the average.

The genetic distances among different populations of the same subspecies or species were as follows: 0.006∼0.014 among the three populations of *B. marinus,* 0.168 between the two populations of *B. j. gargarizans,* 0.187 between the two populations of *B. b. bufo,* and 0.016∼0.241 among the seven populations of *B. j. japonicus.* When these seven populations were divided into four eastern and three western groups, the genetic distances among the eastern group were 0.024∼0.070, while those among the western group were 0.016∼0.031. The genetic distances between *B. j. montanus* and the eastern populations of *B. j. japonicus* were 0.008∼0.037, and those between *B. j. yakushimensis* and the western populations of *B. j. japonicus* were 0.009∼0.024. Those between *B. j. torrenticola* and the western populations of *B. j. japonicus* were 0.060∼0.094.

Bufo j. miyakonis fairly differed from *montanus, japonicus, yakushimensis* and *torrenticola* in genetic distance, being 0.329∼0.438. The genetic distance between *miyakonis* and *gargarizans* from China was 0.112, while that between *miyakonis* and *gargarizans* from Taiwan was 0.366. The genetic distances among seven species were 0.895∼2.294. It was found that *B. japonicus* is genetically closer to *B. bufo* than to the other species.

A dendrogram for the 23 populations belonging to seven species and six subspecies of *Bufo* was drawn by the method of UPGMA on the basis of genetic distances. It was found that *B. japonicus* in the Far East was divided into two groups, one of which became two subspecies, *B. j. gargarizans* in China and Taiwan and *B. j. miyakonis* in Miyako Isl. of Japan, while the other group became four subspecies in the mainland and Yaku Isl. of Japan.

INTRODUCTION

KAWAMURA, NISHIOKA, SUMIDA and RYUZAKI (1990) have clarified the genetic relationships among 40 populations of *Bufo japonicus* distributed in Japan by electrophoretic analyses of enzymes and blood proteins. The 40 populations included five subspecies, *B. j. japonicus* SCHLEGEL, *B. j. montanus* OKADA, *B. j. torrenticola* MATSUI, *B. j. yakushimensis* OKADA and *B. j. miyakonis* OKADA. It was remarkable that *B. j. miyakonis* distinctly differed from the others in genetic distance, while the other four subspecies were closely allied with each other in these respects. This finding seems to agree with the classification of Japanese toads by NAKAMURA and UENO (1963) who combined three subspecies, *japonicus, montanus* and *yakushimensis,* into *japonicus,* and placed *miyakonis* as a synonym of *B. bufo gargarizans* distributed widely in China. As *B. j. japonicus* was named by SCHLEGEL as *B. vulgaris japonicus* in 1838, earlier than *B. gargarizans* CANTOR which was named in 1842, KAWAMURA, NISHIOKA and UEDA (1980) placed the latter in the status of a subspecies of *B. japonicus* and called it *B. japonicus gargarizans* CANTOR. In fact, female *B. j. gargarizans* behaved like female *B. j. japonicus* or *B. j. miyakonis* in the crossings with male hybrids obtained from *miyakonis* ♀ × *japonicus* ♂, and *miyakonis* ♀ × *torrenticola* ♂, as confirmed by their crossing experiments (1980).

In the northern and southern districts of China, *B. raddei* and *B. melanostictus* are widely distributed, respectively, in addition to *B. j. gargarizans.* In Taiwan, there are *B. j. gargarizans, B. j. bankorensis* and *B. melanostictus.* Of these toads, *B. j. bankorensis* is often placed as a synonym of *B. j. gargarizans.*

In the present study, five subspecies of *B. japonicus, japonicus, montanus, torrenticola, yakushimensis* and *miyakonis,* were genetically compared with *B. j. gargarizans* distributed in China and Taiwan as well as several populations of *Bufo* species such as *B. raddei, B. bufo, B. viridis, B. melanostictus, B. marinus* and *B. americanus,* by the method of starch-gel protein electrophoresis. On the basis of the results of electrophoretic analyses, the phylogenetic relationships of *B. japonicus* within the genus *Bufo* having similar appearances will be discussed.

MATERIALS AND METHODS

The electrophoretic analyses were made in 127 females and 185 males of 23 populations belonging to 13 *Bufo* species and subspecies. These toads were collected during the 10 year period from 1976 to 1985 (Table 1; Fig. 1).

Eleven populations including the Zao population of *B. japonicus montanus,* the Namioka, Mito, Zama, Neo, Arashi (Arashiyama), Yoshiwa and Kagoshima populations of *B. j. japonicus,* the Yaku (Yaku Isl.) population of *B. j. yakushimensis,* the Odai (Odaigahara) population of *B. j. torrenticola* and the Miyako (Miyako Isl.) population of *B. j. miyakonis* were the same as those used in another study (KAWAMURA, NISHIOKA, SUMIDA and RYUZAKI, 1990). Of the two populations of *B. j. gargarizans,* the China population was collected from Beijing and given to us

TABLE 1
Collecting stations and the number of the toads examined in the present study

Species or subspecies	Station	No. of toads		
		Total	Male	Female
B. japonicus montanus	Zao	17	13	4
B. j. japonicus	Namioka	7	6	1
〃	Mito	42	29	13
〃	Zama	26	19	7
〃	Neo	15	5	10
〃	Arashiyama	9	6	3
〃	Yoshiwa	19	14	5
〃	Kagoshima	15	11	4
B. j. yakushimensis	Yaku Isl.	5	2	3
B. j. torrenticola	Odaigahara	10	6	4
B. j. miyakonis	Miyako Isl.	6	2	4
B. j. gargarizans	China	23	14	9
〃	Taiwan	22	13	9
B. j. bankorensis	Taiwan	6	2	4
B. bufo bufo	Portugal	4	2	2
〃	France	1	1	0
B. melanostictus	Taiwan	17	6	11
B. viridis	Turkey	2	1	1
B. raddei	China	17	9	8
B. marinus	New Guinea	9	5	4
〃	Ogasawara	15	7	8
〃	Philippine	22	10	12
B. americanus	America	3	2	1
Total		312	185	127

on September 26, 1979 by Prof. Chih-Ye Chang, Institute of Zoology, Academia Sinica. The Taiwan population of this subspecies was collected in November, 1981 by Prof. M. Kuramoto, Fukuoka University of Education, from Nan-tou (1100 m above the sea-level), Luku, Shi-tou, Taiwan and given to us. The Taiwan population of *B. j. bankorensis* was collected in January, 1981 by Mr. C. S. Wang and Mr. P. S. Lin, National Taiwan University, from Kuantzuling (800 m above the sea-level), Tainan and sent to us. The Portugal population of *B. b. bufo* was collected in Minho, Portugal and sent to us in January, 1976 by Dr. E. Crespo, while the France population of the same species was collected in the forest of Chize and sent to us in October, 1976 by Prof. B. Lanza. The Taiwan population of *B. melanostictus* was collected in August, 1982 by Prof. M. Kuramoto from Taipei City and given to us. The Turkey population of *B. viridis* was given to us in April, 1977 by Prof. M. Kuramoto. The China population of *B. raddei*

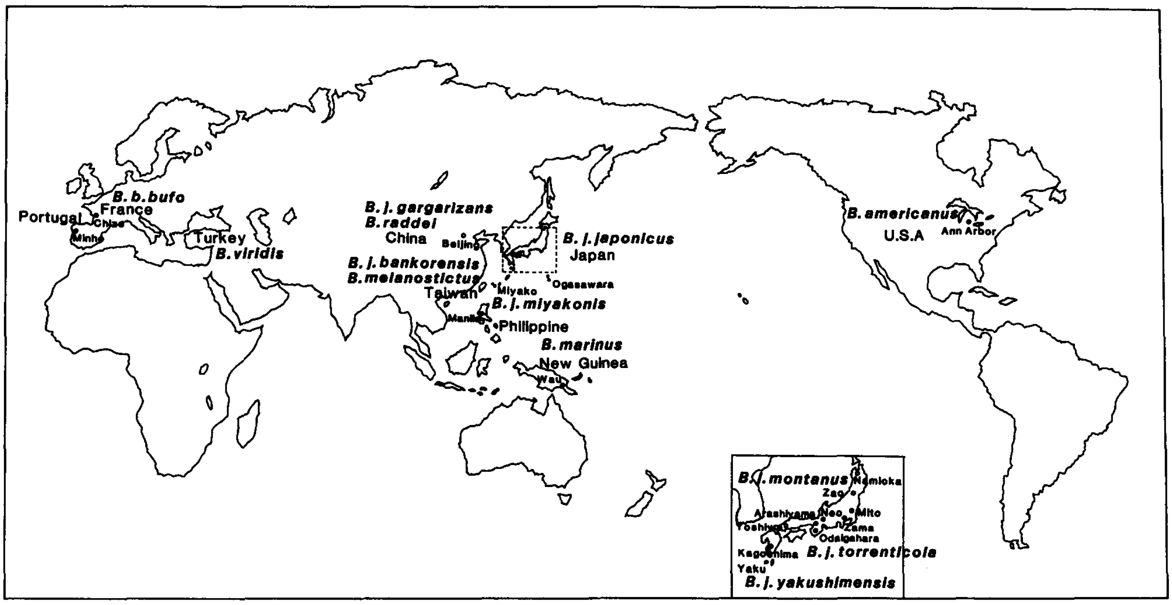


Fig. 1. Map showing localities of 23 populations of *Bufo* species and subspecies.

TABLE 2
Enzymes and blood proteins analyzed in the present study

Enzyme or blood protein	Abbreviation	E. C. No.	Sample	Buffer system
Aspartate aminotransferase	AAT	2.6.1.1	Skeletal muscle	T-C pH 7.0
Adenosine deaminase	ADA	3.5.4.4	〃	〃
Adenylate kinase	AK	2.7.4.3	〃	〃
Creatine kinase	CK	2.7.3.2	〃	T-B-E pH 8.0
α–Glycerophosphate dehydrogenase	α–GDH	1.1.1.8	〃	T-C pH 6.0
Glucose phosphate isomerase	GPI	5.3.1.9	〃	T-B-E pH 8.0
Isocitrate dehydrogenase	IDH	1.1.1.42	〃	T-C pH 7.0
Lactate dehydrogenase	LDH	1.1.1.27	〃	T-C pH 6.0
Malate dehydrogenase	MDH	1.1.1.37	〃	〃
Malic enzyme	ME	1.1.1.40	〃	〃
Mannose phosphate isomerase	MPI	5.3.1.8	〃	T-C pH 7.0
Phosphoglucomutase	PGM	2.7.5.1	〃	T-B-E pH 8.0
Superoxide dismutase	SOD	1.15.1.1	〃	〃
Serum albumin	Ab	—	Blood serum	〃
Hemoglobin	Hb	—	Erythrocyte	T-B-E pH 8.6

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

was collected in October, 1980 from Beijing by Dr. Zheng'an Wu. Of *B. marinus*, the New Guinea population was collected in January, 1985 from Wau by Dr. M. Sumida, the Ogasawara population was collected in August, 1982 by the staff of Tokyo TV Company and given to us, and the Philippine population was collected in December, 1980 from Manila by Prof. M. Nishioka. The American population of *B. americanus* was collected in August, 1976 from Ann Arbor, Michigan by Prof. M. Nishioka.

Thirteen enzymes extracted from the skeletal muscles and two blood proteins of each toad were analyzed by the horizontal starch-gel electrophoretic method. The enzymes and blood proteins analyzed and their abbreviations, E. C. No., samples used for electrophoresis and buffer systems are shown in Table 2. The method of electrophoresis was the same as described by NISHIOKA, OHTANI and SUMIDA (1980). Each enzyme was detected according to BREWER (1970) and HARRIS and HOPKINSON (1976) by a slight modification of the agar overlay method. Blood proteins were detected by amido-black staining.

When each of multiple alleles exists in a frequency of more than 1% at a locus, this locus is considered to be polymorphic. Genetic variations among various populations were shown by the mean proportions of polymorphic loci per population and by the mean proportions of heterozygous loci per individual (LEWONTIN and HUBBY, 1966; LEWONTIN, 1974). The genetic relationships among species, subspecies and populations were evaluated by calculating the genetic distances (D) by the method of NEI (1975). A dendrogram for these species, subspecies and populations was drawn on the basis of the genetic distances by the unweighted pair-group arithmetic average (UPGMA) clustering method (SNEATH and SOKAL, 1973; NEI, 1975).

OBSERVATION

I. Electrophoretic patterns and allelomorphs

The electrophoretic patterns of 13 enzymes and two blood proteins were examined in 312 toads of 23 populations belonging to seven species and six subspecies of *Bufo*. The results showed that 21 loci participate in controlling these enzymes and blood proteins. The kind and number of phenotypes and the alleles at each locus are shown in Table 3. The electrophoretic bands corresponding to multiple alleles at each locus were generally named A, B, C, ··· in the order of mobility from fast to slow, and the alleles were shown by *a, b, c,* ··· (Figs. 2 and 3).

At the AAT–A and AK of the 21 loci, there were four phenotypes produced by three alleles (*a~c*). At the IDH–A locus, six phenotypes produced by four alleles (*a~d*) were observed. At the SOD–A locus, five phenotypes produced by five alleles (*a~e*) were observed. At the ME–A locus, there were nine phenotypes produced by seven alleles (*a~g*). At each of five loci, the AAT–B, CK, LDH–A, PGM and Hb, there were nine to 12 phenotypes produced by eight alleles (*a~h*). At each of three loci, the ADA, MDH–A and SOD–B, 10 to 16 phenotypes produced by nine alleles (*a~i*) were observed. At each of four loci, the GPI, IDH–B, MPI and Ab, 14 to 19 phenotypes produced by 10 alleles (*a~j*) were observed. At the MDH–B locus, there were 12 phenotypes produced by 11 alleles (*a~k*). At the α–GDH and LDH–B loci, there were 14 and 16 phenotypes, respectively, produced by 12 alleles (*a~l*). At the ME–B locus which was the most polymorphic, there were 35 phenotypes produced by 19 alleles (*a~s*) (Table 3).

TABLE 3

Number of alleles and phenotypes at each of 21 loci in 23 populations of *Bufo* species and subspecies

Locus	Alleles		Phenotypes	
	No.	Kind	No.	Kind
AAT–A	3	*a~c*	4	AA,BB,CC,AC
AAT–B	8	*a~h*	9	DD,EE,FF,GG,AD,BD,CD,DF,DH
ADA	9	*a~i*	16	AA,CC,DD,EE,GG,HH,II,AB,CD,CE,DF,DG,DH,DI,FH,GI
AK	3	*a~c*	4	BB,CC,AB,BC,
CK	8	*a~h*	9	CC,DD,EE,FF,GG,HH,AF,BD,CF
α–GDH	12	*a~l*	14	BB,EE,FF,GG,HH,II,JJ,LL,AB,CE,DI,FI,GI,IK
GPI	10	*a~j*	15	BB,DD,EE,GG,HH,II,JJ,AB,BD,CE,DH,EH,FI,GJ,HJ
IDH–A	4	*a~d*	6	BB,CC,DD,AC,BD,CD
IDH–B	10	*a~j*	14	AA,BB,CC,EE,GG,HH,AB,AC,DG,EG,FH,GH,GI,HJ
LDH–A	8	*a~h*	10	BB,CC,DD,EE,FF,HH,AC,CG,EH,FG
LDH–B	12	*a~l*	16	AA,CC,DD,EE,FF,GG,HH,II,JJ,LL,BG,CG,GH,GJ,HL,IK
MDH–A	9	*a~i*	10	AA,BB,DD,EE,GG,HH,CH,EH,FH,HI
MDH–B	11	*a~k*	12	AA,DD,EE,FF,GG,HH,II,JJ,KK,BE,CJ,EF
ME–A	7	*a~g*	9	AA,BB,CC,DD,AB,BD,DE,DF,DG
ME–B	19	*a~s*	35	BB,CC,DD,EE,FF,GG,HH,II,JJ,KK,LL,MM,NN,PP,QQ,RR,AC,BG BI,BL,BR,CD,EJ,FI,GI,GL,IL,IR,LN,LP,LR,LS,OQ,PR,PS
MPI	10	*a~j*	19	CC,EE,FF,GG,HH,II,AC,AF,AG,AH,BG,CF,CH,DF,DG,EF,FH,FI,FJ
PGM	8	*a~h*	9	CC,DD,EE,GG,HH,AE,BC,DF,EG
SOD–A	5	*a~e*	5	AA,BB,CC,DD,EE
SOD–B	9	*a~i*	13	AA,BB,CC,DD,EE,GG,HH,II,AH,BG,DI,EI,FH
Ab	10	*a~j*	16	AA,BB,CC,DD,EE,FF,GG,HH,II,AB,BC,DF,DG,EG,EI,GJ
Hb	8	*a~h*	12	AA,BB,CC,DD,EE,FF,GG,HH,AD,BD,CD,EH
Average	8.7		12.2	

At the foregoing 21 loci, there were 12.2 phenotypes produced by 8.7 alleles on the average.

II. Gene frequency

1. AAT–A locus

At the AAT–A locus, four phenotypes produced by three alleles, *a, b* and *c,* were observed in the 312 toads of the 23 populations. The Namioka population of *B. j. japonicus,* the Taiwan population of *B. melanostictus* and the China population of *B. raddei* had only allele *a.* The two populations of *B. b. bufo,* the Turkey population of *B. viridis* and the three populations of *B. marinus* had only allele *b.* The American population of *B. americanus,* the Yoshiwa and Kagoshima populations of *B. j. japonicus,* the Yaku population of *B. j. yakushimensis,* the Odai population of *B. j. torrenticola,* the Miyako population of *B. j. miyakonis,* and the two Taiwan populations of *B. j. gargarizans* and *B. j. bankorensis* had only allele *c.*

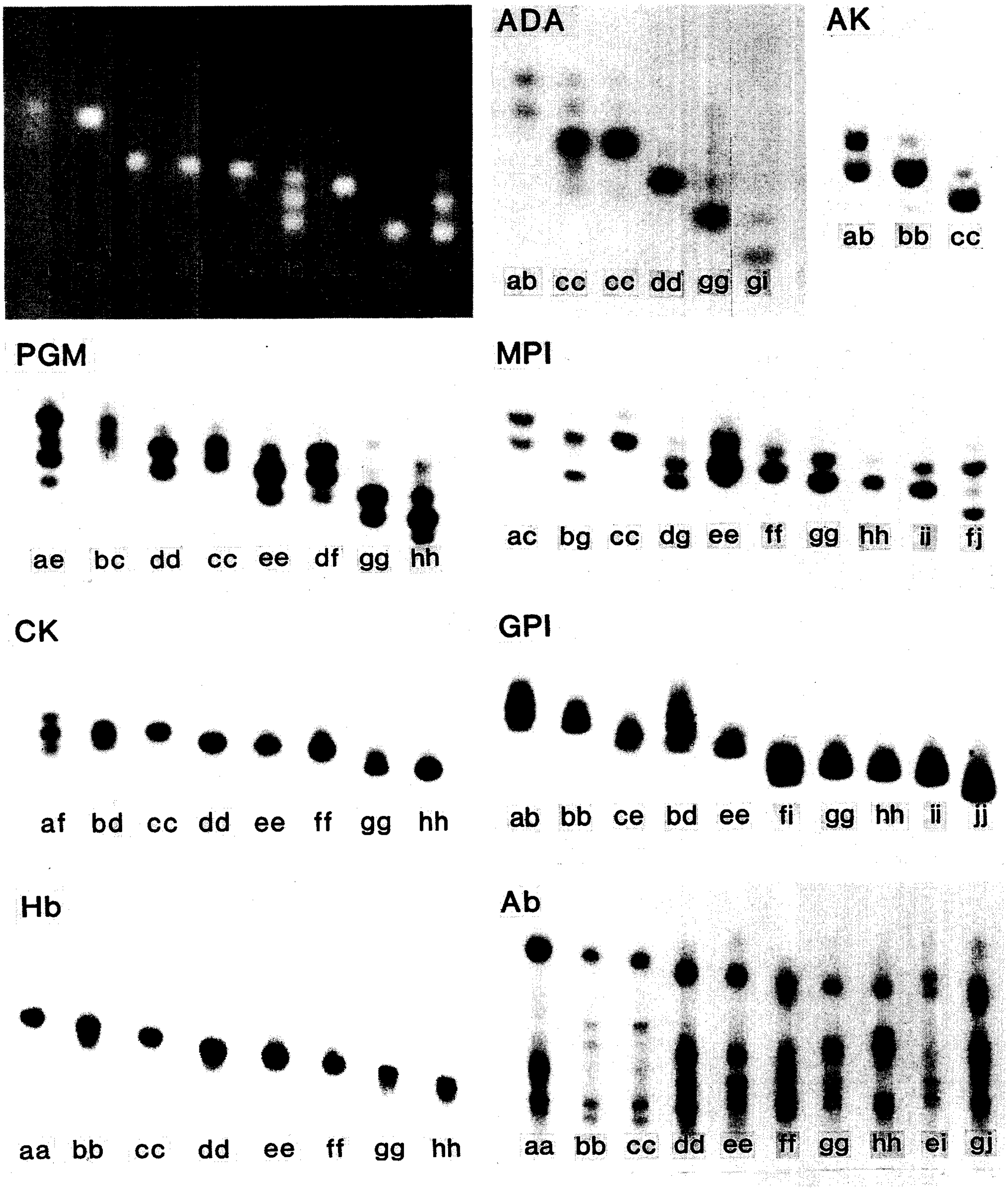


Fig. 2. Electrophoretic patterns of seven enzymes, SOD–B, ADA, AK, PGM, MPI, CK and GPI, and two blood proteins, Hb and Ab, in 23 populations of *Bufo* species and subspecies.

In the Zama, Neo and Arashi populations of *B. j. japonicus* and the China population of *B. j. gargarizans,* allele *c* was 0.615~0.935 in frequency, while allele *a* was 0.065~0.385. In contrast, in the Mito population of *B. j. japonicus* and the Zao population of *B. j. montanus,* allele *a* was 0.571 and 0.618 in frequency, respectively, while allele *c* was 0.429 and 0.382, respectively (Table 4; Fig. 4).

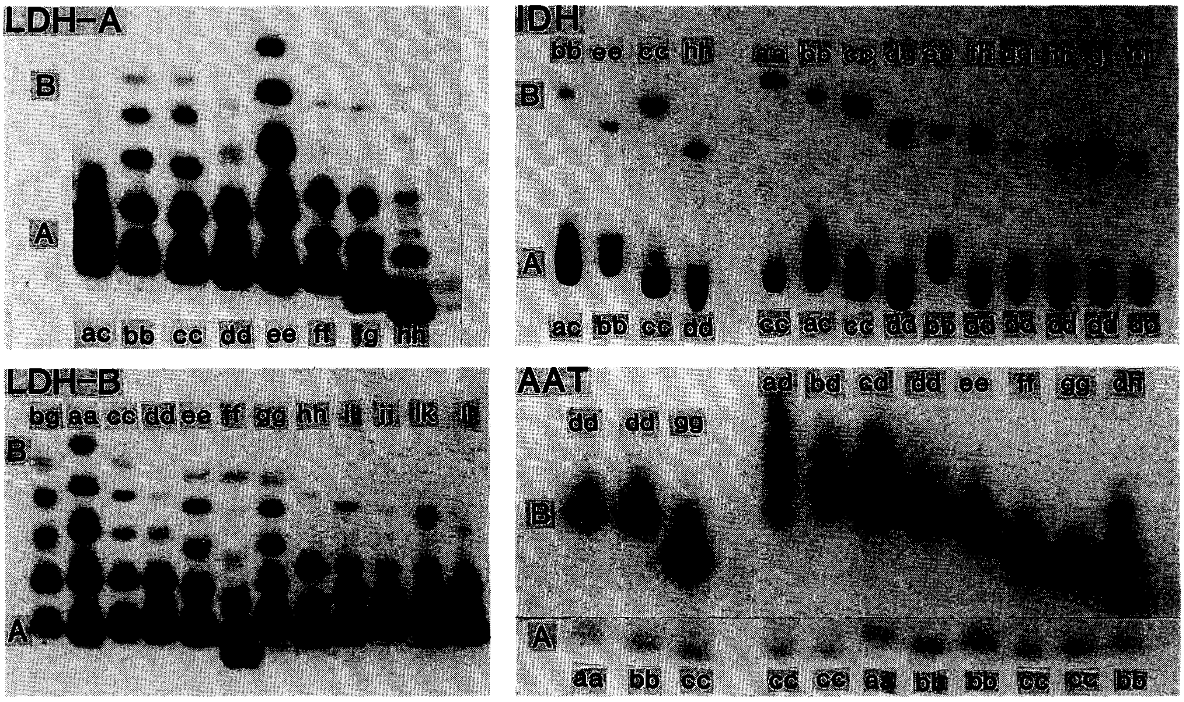


Fig. 3. Electrophoretic patterns of three enzymes, LDH, IDH and AAT, in 23 populations of *Bufo* species and subspecies.

TABLE 4

Gene frequencies at 19 loci in 23 populations

Species	Population	Sample size	AAT-A			AAT-B								ADA								
			a	*b*	*c*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
B. j. mon.	Zao	17	0.618		0.382				0.971		0.029					0.118	0.853					0.029
B. j. jap.	Namioka	7	1.000					0.071	0.929								1.000					
〃	Mito	42	0.571		0.429			0.071	0.917				0.012			0.036	0.952		0.012			
〃	Zama	26	0.135		0.865			0.077	0.923								0.942		0.058			
〃	Neo	15	0.385		0.615				0.692		0.308					0.033	0.967					
〃	Arashi	9	0.167		0.833				0.944		0.056						1.000					
〃	Yoshiwa	19			1.000	0.026			0.632		0.342						1.000					
〃	Kagoshima	15			1.000				0.700		0.300					0.067	0.933					
B. j. yak.	Yaku	5			1.000				1.000								1.000					
B. j. tor.	Odai	10			1.000				1.000							0.050	0.950					
B. j. miy.	Miyako	6			1.000				1.000													1.000
B. j. gar.	China	23	0.065		0.935		0.043		0.957								0.522			0.109		0.370
〃	Taiwan	22			1.000		0.023		0.977								1.000					
B. j. bank.	Taiwan	6			1.000		0.083		0.917								1.000					
B. b. bufo	Portugal	4		1.000					0.875				0.125				0.375		0.125		0.500	
〃	France	1		1.000					1.000								0.500		0.500			
B. mela.	Taiwan	17	1.000						1.000							0.735		0.265				
B. vir.	Turkey	2		1.000						1.000										1.000		
B. rad.	China	17	1.000						1.000								0.176			0.824		
B. mar.	New Guinea	9		1.000					1.000								1.000					
〃	Ogasawara	15		1.000					1.000								1.000					
〃	Philippine	22		1.000					1.000								1.000					
B. amer.	America	3			1.000							1.000		0.833	0.167							

2. AAT–B locus

At the AAT–B locus, there were nine phenotypes produced by eight alleles (*a*~*h*) in the 312 toads of the 23 populations. The Turkey population of *B. viridis* and the American population of *B. americanus* had only alleles *e* and *g*, respectively. In the other 21 of the 23 populations, allele *d* was high in frequency, being 0.632~1.000. Of these populations, the Yaku population of *B. j. yakushimensis*, the Odai population of *B. j. torrenticola*, the Miyako population of *B. j. miyakonis*, the France population of *B. b. bufo*, the Taiwan population of *B. melanostictus*, the China population of *B. raddei* and the three populations of *B. marinus* had only allele *d*, while in the Zao population of *B. j. montanus* and the Neo, Arashi and Kagoshima populations of *B. j. japonicus*, there was allele *f* which was 0.029~0.308 in frequency, in addition to allele *d*. In the Namioka and Zama populations of *B. j. japonicus*, allele *c* was 0.071 and 0.077 in frequency, respectively, in addition to allele *d*. In the Mito population of *B. j. japonicus*, there were alleles *c* and *h* which were 0.071 and 0.012 in frequency, respectively, in addition to allele *d*. In the Yoshiwa population of the same subspecies, there were alleles *a* and *f* which were 0.026 and 0.342 in frequency, respectively, in addition to allele *d*. In the two populations of *B. j. gargarizans* and the Taiwan population of *B. j. bankorensis*, there was allele *b* which was 0.023~0.083 in frequency, in addition to allele *d*. In the Portugal population of *B. b. bufo*, allele *h* was found to be 0.125 in frequency in

of *Bufo* species and subspecies

AK			CK								α–GDH											
a	*b*	*c*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*
	1.000							1.000						0.029					0.971			
	1.000							1.000						0.071					0.929			
	1.000							1.000						0.024					0.976			
	0.981	0.019						1.000											1.000			
	0.933	0.067						1.000						0.033					0.967			
	0.944	0.056	0.111					0.889											1.000			
	1.000							1.000											0.974		0.026	
	1.000							1.000											1.000			
	1.000							1.000											1.000			
	1.000							1.000											1.000			
	1.000							1.000								0.500			0.500			
	1.000							1.000									1.000					
	1.000				0.364			0.636									0.977		0.023			
	1.000							1.000									0.917		0.083			
	1.000									1.000	0.125	0.875										
	1.000									1.000		1.000										
0.029	0.971						1.000													1.000		
	1.000						1.000								1.000							
0.250	0.750								1.000									1.000				
	0.056	0.944						1.000					0.056		0.944							
		1.000						1.000							1.000							
	0.205	0.795						1.000					0.045		0.955							
0.167	0.833			0.333		0.667																1.000

TABLE 4 Continued-2

Species	Population	Sample size	GPI										IDH-A				IDH-B					
			a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*a*	*b*	*c*	*d*	*a*	*b*	*c*	*d*	*e*	*f*
B. j. mon.	Zao	17								0.941		0.059				1.000					0.824	
B. j. jap.	Namioka	7				0.143				0.857						1.000					1.000	
〃	Mito	42				0.012				0.833		0.155		0.024		0.976					0.805	
〃	Zama	26				0.135				0.865						1.000					0.865	
〃	Neo	15				0.033				0.967						1.000					1.000	
〃	Arashi	9				0.056				0.944						1.000					0.278	
〃	Yoshiwa	19				0.079				0.921						1.000						
〃	Kagoshima	15								1.000						1.000						
B. j. yak.	Yaku	5								1.000						1.000						
B. j. tor.	Odai	10								0.950		0.050				1.000				0.250		
B. j. miy.	Miyako	6				0.167				0.833				1.000							1.000	
B. j. gar.	China	23					0.109			0.891				1.000							1.000	
〃	Taiwan	22								1.000						1.000					1.000	
B. j. bank.	Taiwan	6					0.167			0.833						1.000					1.000	
B. b. bufo	Portugal	4							0.125			0.875				1.000						0.125
〃	France	1							1.000							1.000						
B. mela.	Taiwan	17	0.176	0.824											1.000		1.000					
B. vir.	Turkey	2						0.250			0.750				1.000		0.750		0.250			
B. rad.	China	17					1.000						0.059		0.941		0.147	0.853				
B. mar.	New Guinea	9		0.111		0.889									1.000				1.000			
〃	Ogasawara	15		0.133		0.867									1.000				1.000			
〃	Philippine	22		0.114		0.886									1.000				1.000			
B. amer.	America	3			0.333		0.667								0.667	0.333			1.000			

TABLE 4 Continued-3

Species	Population	Sample size	MDH-A									MDH-B										
			a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*
B. j. mon.	Zao	17								1.000						1.000						
B. j. jap.	Namioka	7					0.071			0.929						1.000						
〃	Mito	42								1.000						1.000						
〃	Zama	26								1.000		0.038				0.962						
〃	Neo	15								1.000						1.000						
〃	Arashi	9								1.000						1.000						
〃	Yoshiwa	19								1.000						1.000						
〃	Kagoshima	15								1.000						1.000						
B. j. yak.	Yaku	5								1.000						1.000						
B. j. tor.	Odai	10								1.000						0.900		0.100				
B. j. miy.	Miyako	6			0.083					0.917			0.167			0.833						
B. j. gar.	China	23								1.000						1.000						
〃	Taiwan	22						0.136		0.864						1.000						
B. j. bank.	Taiwan	6						0.167		0.833						1.000						
B. b. bufo	Portugal	4								0.875	0.125				1.000							
〃	France	1								1.000					1.000							
B. mela.	Taiwan	17				1.000								0.029							0.971	
B. vir.	Turkey	2							1.000							0.250	0.750					
B. rad.	China	17					1.000															1.000
B. mar.	New Guinea	9		1.000																1.000		
〃	Ogasawara	15		1.000																1.000		
〃	Philippine	22		1.000																1.000		
B. amer.	America	3	1.000																1.000			

				LDH-A								LDH-B											
g	*h*	*i*	*j*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*
0.176						1.000								0.406				0.594					
						1.000								0.286				0.714					
0.195				0.012		0.988								0.512				0.488					
0.135						1.000								0.212				0.788					
						1.000							0.033					0.967					
0.556		0.167				0.944				0.056				0.056				0.944					
0.974	0.026					1.000								0.053				0.868	0.079				
1.000						1.000												1.000					
1.000						1.000												1.000					
0.750						1.000												1.000					
					1.000													1.000					
					1.000													1.000					
					1.000													0.386			0.614		
					1.000													0.333			0.667		
	0.875								0.875	0.125									0.750				0.250
	0.500		0.500						1.000														1.000
						1.000										1.000							
								0.250			0.750						1.000						
								1.000				1.000											
							1.000								1.000								
							1.000								1.000								
							1.000								1.000								
								1.000												0.833		0.167	

ME-A							ME-B																		
a	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*	*m*	*n*	*o*	*p*	*q*	*r*	*s*
			0.971			0.029									0.029			0.824				0.029		0.118	
			1.000															1.000							
			0.976			0.024												0.714				0.083		0.155	0.048
			1.000															0.558						0.442	
			1.000												0.033			0.733						0.233	
			1.000					0.444					0.222		0.222									0.111	
			1.000					0.526					0.316		0.105			0.053							
			1.000					0.967					0.033												
			1.000					0.900					0.100												
			1.000															0.100		0.900					
			1.000							1.000															
			1.000						0.100	0.900															
	0.477		0.523				0.250		0.750																
	0.083		0.917				0.300		0.700																
			1.000								0.625					0.375									
			1.000								1.000														
	0.382		0.618																1.000						
			0.750	0.250								0.750			0.250										
			0.853		0.147												1.000								
0.667	0.333													1.000											
0.800	0.200													1.000											
0.386	0.614													1.000											
		1.000																			0.333		0.667		

TABLE 4 Continued-4

Species	Population	Sample size	MPI *a*	MPI *b*	MPI *c*	MPI *d*	MPI *e*	MPI *f*	MPI *g*	MPI *h*	MPI *i*	MPI *j*	PGM *a*	PGM *b*	PGM *c*	PGM *d*	PGM *e*	PGM *f*	PGM *g*	PGM *h*
B. j. mon.	Zao	17			0.118			0.882									1.000			
B. j. jap.	Namioka	7						0.857		0.143							0.929		0.071	
〃	Mito	42			0.155			0.560		0.286							1.000			
〃	Zama	26			0.250			0.385		0.365							1.000			
〃	Neo	15			0.233			0.767					0.067				0.900		0.033	
〃	Arashi	9			0.722			0.278									0.833		0.167	
〃	Yoshiwa	19	0.026		0.553			0.421									1.000			
〃	Kagoshima	15	0.233		0.733			0.033									1.000			
B. j. yak.	Yaku	5			1.000												1.000			
B. j. tor.	Odai	10			0.100	0.050		0.850									1.000			
B. j. miy.	Miyako	6		0.083		0.083			0.833								0.667		0.333	
B. j. gar.	China	23	0.043		0.065			0.739	0.109	0.043							1.000			
〃	Taiwan	22			0.023			0.818				0.159					1.000			
B. j. bank.	Taiwan	6						0.833				0.167					1.000			
B. b. bufo	Portugal	4			1.000												1.000			
〃	France	1			1.000												1.000			
B. mela.	Taiwan	17					0.559	0.441									1.000			
B. vir.	Turkey	2									1.000					0.750		0.250		
B. rad.	China	17								1.000				0.029	0.971					
B. mar.	New Guinea	9					0.722	0.278												1.000
〃	Ogasawara	15					0.500	0.500												1.000
〃	Philippine	22					0.659	0.341												1.000
B. amer.	America	3						0.667			0.333								1.000	

addition to allele *d* (Table 4; Fig. 4).

3. ADA locus

At the ADA locus, 16 phenotypes produced by nine alleles (*a*~*i*) were observed in the 312 toads of the 23 populations. In 15 populations including the Zao population of *B. j. montanus,* the seven populations of *B. j. japonicus,* the Yaku population of *B. j. yakushimensis,* the Odai population of *B. j. torrenticola,* the Taiwan populations of *B. j. gargarizans* and *B. j. bankorensis,* and the three populations of *B. marinus,* the allele *d* was overwhelmingly high in frequency, being 0.853~1.000. Of these 15 populations, nine including the Namioka, Arashi and Yoshiwa populations of *B. j. japonicus,* the Yaku population of *B. j. yakushimensis,* the Taiwan populations of *B. j. gargarizans* and *B. j. bankorensis,* and the three populations of *B. marinus* had only allele *d,* while three populations including the Neo population of *B. j. japonicus,* the Kagoshima population of *B. j. japonicus* and the Odai population of *B. j. torrenticola* had allele *c* which was 0.033~0.067 in frequency, the Zama population of *B. j. japonicus* had allele *f* in frequency of 0.058, the Zao population of *B. j. montanus* had alleles *c* and *i* in frequencies of 0.118 and 0.029, respectively, and the Mito population of *B. j. japonicus* had alleles *c* and *f* in frequencies of 0.036 and 0.012, respectively, in addition to allele *d.* Three populations including the China population of *B. j. gargarizans* and the France and Portugal populations of *B. b. bufo* had allele *d* in frequencies of 0.522, 0.500 and 0.375, respectively. In addition to allele *d,* the China population of *B. j. gargarizans* had alleles *g* and *i* in frequencies of 0.109 and 0.370, respectively. The France population of *B. b. bufo* had allele *f*

SOD-A					SOD-B								
a	*b*	*c*	*d*	*e*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
				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						0.079		0.921	
				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							
			1.000				1.000						
	1.000					0.088					0.912		
	1.000								0.750				0.250
		1.000						0.971					0.029
1.000									0.333				0.667
1.000									0.133				0.867
1.000									0.091				0.909
			1.000		0.667							0.333	

in frequency of 0.500, while the Portugal population had alleles *f* and *h* in frequencies of 0.125 and 0.500, respectively. The Miyako population of *B. j. miyakonis* had only allele *i*, while the Turkey population of *B. viridis* had only allele *g*. The China population of *B. raddei* had alleles *g* and *d* in frequencies of 0.824 and 0.176, respectively. The Taiwan population of *B. melanostictus* had alleles *c* and *e* in frequencies of 0.735 and 0.265, respectively. In the American population of *B. americanus*, alleles *a* and *b* were 0.833 and 0.167 in frequency, respectively (Table 4; Fig. 4).

4. AK locus

At the AK locus, four phenotypes produced by three alleles (*a*~*c*) were observed. In the 20 populations of six species and six subspecies other than the three populations of *B. marinus*, allele *b* was overwhelmingly high in frequency, being 0.750~1.000. In the Zama, Neo and Arashi populations of *B. j. japonicus*, there was allele *c* in frequencies of 0.019, 0.067 and 0.056, respectively, in addition to allele *b*. In three populations including the Taiwan population of *B. melanostictus*, the China population of *B. raddei* and the American population of *B. americanus*, there was allele *a* in frequencies of 0.029, 0.250 and 0.167, respectively, in addition to allele *b*. The remaining 14 populations had only allele *b*. Of the three populations of *B. marinus*, the Ogasawara population had only allele *c*, while the New Guinea and Philippine populations had allele *c* in frequencies of 0.944 and 0.795, respectively, together with allele *b* in frequencies of 0.056 and 0.205, respectively (Table 4; Fig. 4).

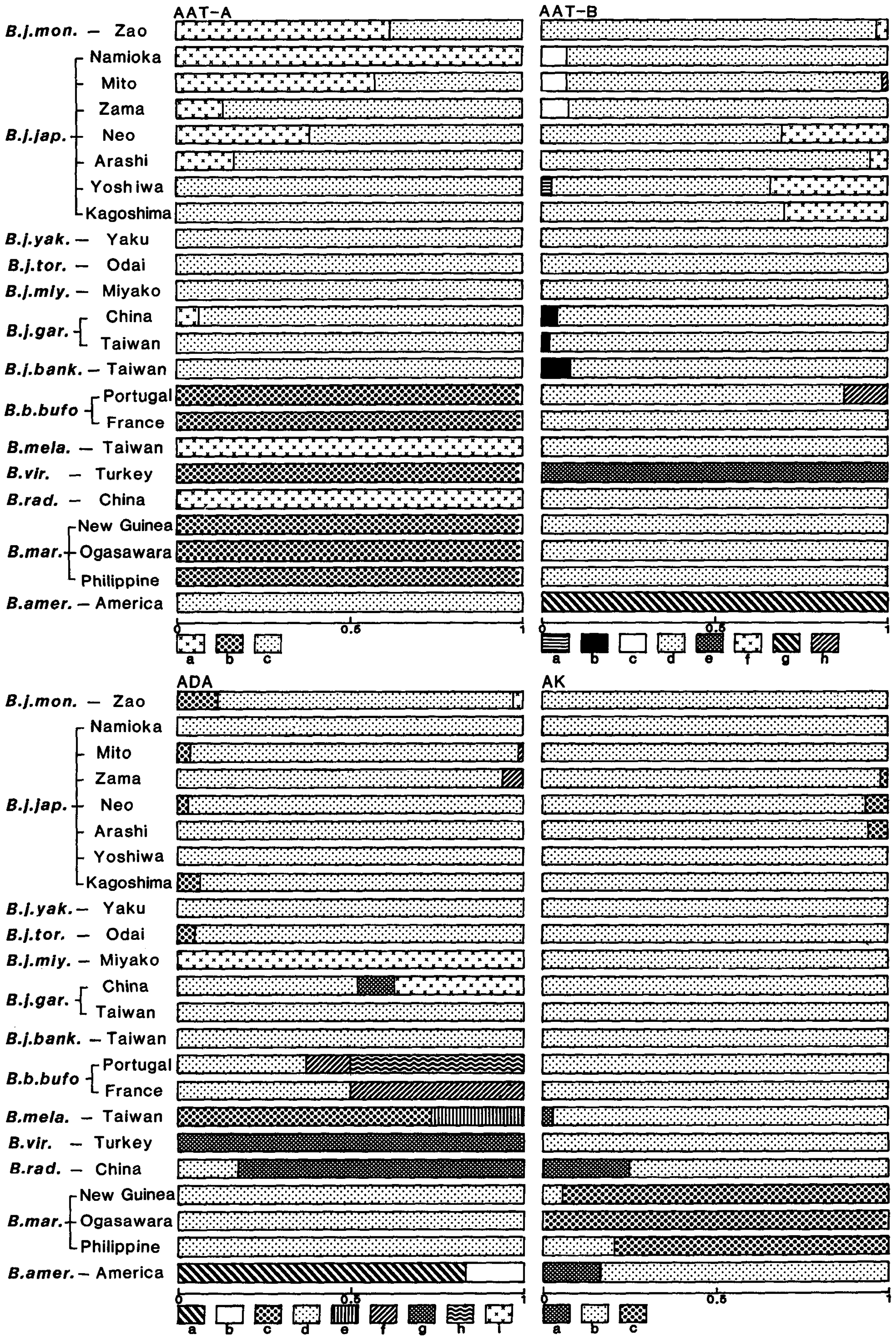


Fig. 4. Gene frequencies at four loci, AAT-A, AAT-B, ADA and AK, in 23 populations of *Bufo* species and subspecies.

5. CK locus

At the CK locus, nine phenotypes produced by eight alleles (*a*~*h*) were observed. Fifteen populations including 12 populations of *B. japonicus* other than the Arashi population of *B. j. japonicus* and the Taiwan population of *B. j. gargarizans* and the three populations of *B. marinus* had only allele *f*. While in the Arashi population of *B. j. japonicus* and the Taiwan population of *B. j. gargarizans,* allele *f* was high in frequency, being 0.889 and 0.636, respectively. In addition to allele *f,* the Arashi population had allele *a* in frequency of 0.111 and the Taiwan population of *B. j. gargarizans* had allele *c* in frequency of 0.364. The Taiwan population of *B. melanostictus* and the Turkey population of *B. viridis* had only allele *e*. The two populations of *B. b. bufo* and the China population of *B. raddei* had only alleles *h* and *g,* respectively. The American population of *B. americanus* had alleles *d* and *b* in frequencies of 0.667 and 0.333, respectively (Table 4; Fig. 5).

6. α-GDH locus

At the α-GDH locus, there were 14 phenotypes produced by 12 alleles (*a*~*l*). Allele *e* was very high in frequency both in the Turkey population of *B. viridis* and in the three populations of *B. marinus.* Each of the other alleles was peculiar to each species or subspecies. In 10 populations including the Zao population of *B. j. montanus,* the seven populations of *B. j. japonicus,* the Yaku population of *B. j. yakushimensis* and the Odai population of *B. j. torrenticola,* allele *i* was overwhelmingly high in frequency, being 0.929~1.000. Of these populations, the Zao population of *B. j. montanus* and the Namioka, Mito and Neo populations of *B. j. japonicus* had allele *d* in frequencies of 0.024~0.071 in addition to allele *i*. In the Yoshiwa population of *B. j. japonicus,* there was allele *k* in frequency of 0.026. All the remaining five of the 10 populations had only allele *i*. In the Miyako population of *B. j. miyakonis,* alleles *f* and *i* were both 0.500 in frequency.

In the two populations of *B. j. gargarizans* and the Taiwan population of *B. j. bankorensis,* allele *g* was overwhelmingly high in frequency, being 0.917~1.000. The China population of *B. j. gargarizans* had only allele *g,* while the Taiwan populations of *B. j. gargarizans* and *B. j. bankorensis* had allele *i* in frequencies of 0.023 and 0.083, respectively, in addition to allele *g*. In the two populations of *B. b. bufo,* allele *b* was overwhelmingly high in frequency, being 0.875 and 1.000, respectively. In the Portugal population of *B. b. bufo,* allele *a* was contained in frequency of 0.125 in addition to allele *b*. The Taiwan population of *B. melanostictus,* the China population of *B. raddei* and the American population of *B. americanus* had only alleles *j, h* and *l,* respectively. In the Turkey population of *B. viridis* and the three populations of *B. marinus,* allele *e* was overwhelmingly high in frequency, being 0.944~1.000. While the Turkey population of *B. viridis* and the Ogasawara population of *B. marinus* had only allele *e,* the New Guinea and Philippine populations of *B. marinus* had allele *c* in frequencies of 0.056 and 0.045, respectively, in addition to allele *e* (Table 4; Fig. 5).

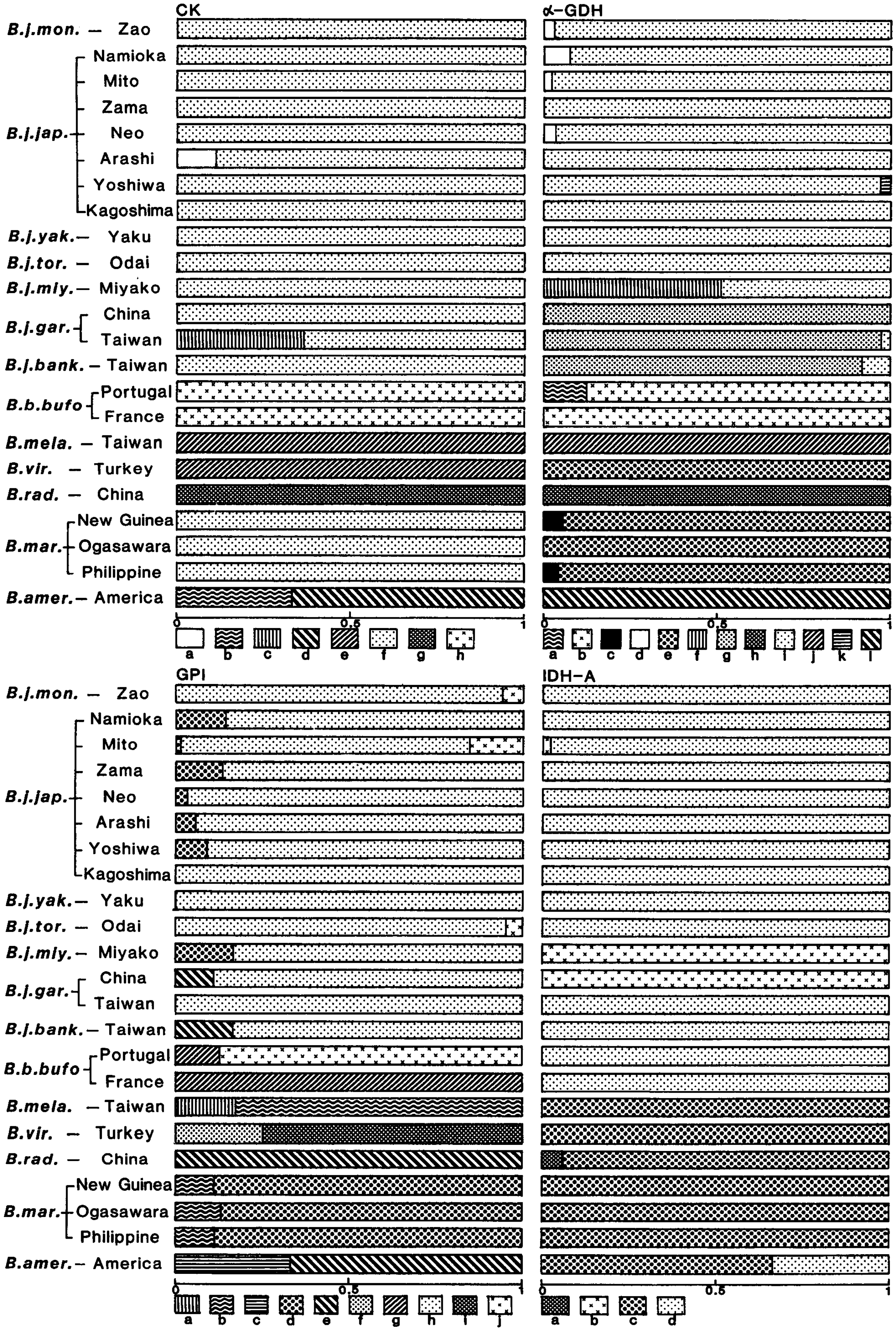


Fig. 5. Gene frequencies at four loci, CK, α–GDH, GPI and IDH–A, in 23 populations of *Bufo* species and subspecies.

7. GPI locus

At the GPI locus, 15 phenotypes produced by 10 alleles (*a~j*) were observed. In the 14 populations belonging to seven subspecies of *B. japonicus,* allele *h* was overwhelmingly high in frequency, being 0.833~1.000. Of these populations, the Kagoshima population of *B. j. japonicus,* the Yaku population of *B. j. yakushimensis* and the Taiwan population of *B. j. gargarizans* had only allele *h.* The Namioka, Zama, Neo, Arashi and Yoshiwa populations of *B. j. japonicus* and the Miyako population of *B. j. miyakonis* had allele *d* in frequencies of 0.033~0.167, in addition to allele *h.* The Zao population of *B. j. montanus* and the Odai population of *B. j. torrenticola* had allele *j* in frequencies of 0.059 and 0.050, respectively. The Mito population of *B. j. japonicus* had alleles *d* and *j* in frequencies of 0.012 and 0.155, respectively. In the China population of *B. j. gargarizans* and the Taiwan population of *B. j. bankorensis,* allele *e* was found in the frequencies of 0.109 and 0.167, respectively.

The one toad of the France population of *B. b. bufo* had only allele *g,* while the Portugal population of the same species had alleles *j* and *g* in frequencies of 0.875 and 0.125, respectively. In the Taiwan population of *B. melanostictus,* there were alleles *b* and *a* in frequencies of 0.824 and 0.176, respectively. In the Turkey population of *B. viridis,* there were alleles *i* and *f* in frequencies of 0.750 and 0.250, respectively. The China population of *B. raddei* had only allele *e.* In the three populations of *B. marinus,* there were alleles *d* and *b.* Allele *d* was overwhelmingly high in frequency, being 0.867~0.889, while allele *b* was 0.111~0.133. In the American population of *B. americanus,* there were alleles *e* and *c* in frequencies of 0.667 and 0.333, respectively (Table 4; Fig. 5).

8. IDH–A locus

At the IDH–A locus, six phenotypes produced by four alleles (*a~d*) were observed. Thirteen populations including the Zao population of *B. j. montanus,* the six populations of *B. j. japonicus* other than the Mito population, the Yaku population of *B. j. yakushimensis,* the Odai population of *B. j. torrenticola,* the Taiwan populations of *B. j. gargarizans* and *B. j. bankorensis* and the two populations of *B. b. bufo* had only allele *d.* In the Mito population of *B. j. japonicus,* allele *d* was overwhelmingly high in frequency, being 0.976, while there was allele *b* in frequency of 0.024.

The Miyako population of *B. j. miyakonis* and the China population of *B. j. gargarizans* had only allele *b.* The Taiwan population of *B. melanostictus,* the Turkey population of *B. viridis* and the three populations of *B. marinus* had only allele *c.* In the China population of *B. raddei,* alleles *c* and *a* were 0.941 and 0.059 in frequency, respectively. In the American population of *B. americanus,* alleles *c* and *d* were 0.667 and 0.333 in frequency, respectively (Table 4; Fig. 5).

9. IDH–B locus

At the IDH–B locus, 14 phenotypes produced by 10 alleles (*a~j*) were observed.

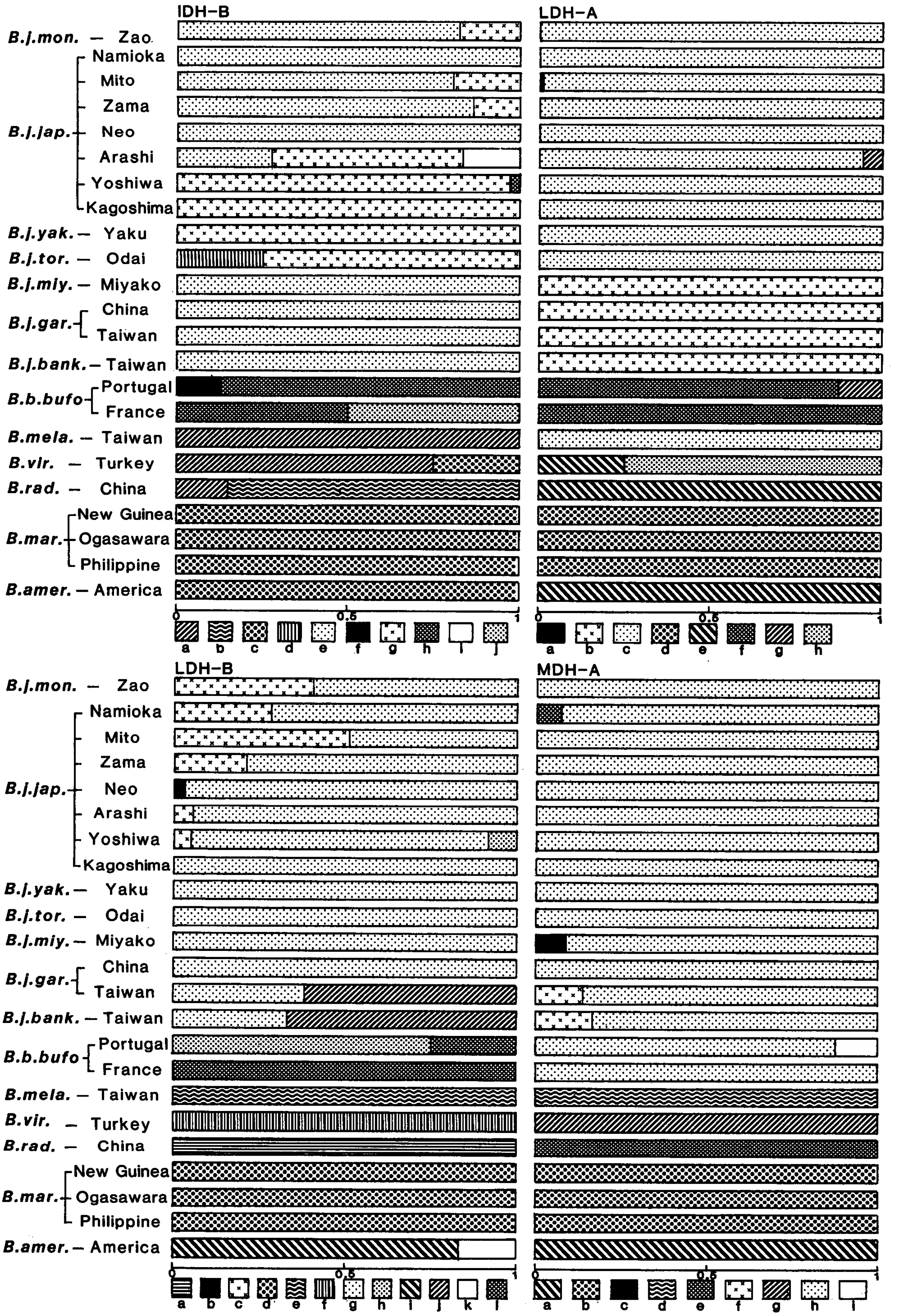


Fig. 6. Gene frequencies at four loci, IDH-B, LDH-A, LDH-B and MDH-A, in 23 populations of *Bufo* species and subspecies.

Six populations including the Namioka and Neo populations of *B. j. japonicus,* the Miyako population of *B. j. miyakonis,* the two populations of *B. j. gargarizans* and the Taiwan population of *B. j. bankorensis* had only allele *e.* In the Zao population of *B. j. montanus* and the Mito and Zama populations of *B. j. japonicus,* there were alleles *e* and *g* in frequencies of 0.805~0.865 and 0.135~0.195, respectively. The Kagoshima population of *B. j. japonicus* and the Yaku population of *B. j. yakushimensis* had only allele *g.* The Yoshiwa population of *B. j. japonicus* had alleles *g* and *h* in frequencies of 0.974 and 0.026, respectively. The Odai population of *B. j. torrenticola* had alleles *g* and *d* in frequencies of 0.750 and 0.250, respectively. In the Arashi population of *B. j. japonicus,* there were alleles *e, g* and *i* in frequencies of 0.278, 0.556 and 0.167, respectively. The Portugal population of *B. b. bufo* had alleles *h* and *f* in frequencies of 0.875 and 0.125, respectively, while the France population of the same species had alleles *h* and *j* in frequencies of 0.500 and 0.500, respectively. The Taiwan population of *B. melanostictus* had only allele *a.* All the three populations of *B. marinus* and the American population of *B. americanus* had only allele *c.* The Turkey population of *B. viridis* had alleles *a* and *c* in frequencies of 0.750 and 0.250, respectively. The China population of *B. raddei* had alleles *b* and *a* in frequencies of 0.853 and 0.147, respectively (Table 4; Fig. 6).

10. LDH-A locus

At the LDH-A locus, 10 phenotypes produced by eight alleles (*a~h*) were observed. Eleven populations including the Zao population of *B. j. montanus,* the seven populations of *B. j. japonicus,* the Yaku population of *B. j. yakushimensis,* the Odai population of *B. j. torrenticola* and the Taiwan population of *B. melanostictus* had only allele *c,* except that the Mito and Arashi populations of *B. j. japonicus* had alleles *a* and *g* in frequencies of 0.012 and 0.056, respectively, in addition to allele *c.* Four populations including the Miyako population of *B. j. miyakonis,* the two populations of *B. j. gargarizans* and the Taiwan population of *B. j. bankorensis* had only allele *b.* In the two populations of *B. b. bufo,* allele *f* was overwhelmingly high in frequency. The France population had only allele *f,* while the Portugal population had allele *g* in frequency of 0.125 in addition to allele *f.* In the Turkey population of *B. viridis,* there were alleles *h* and *e* in frequencies of 0.750 and 0.250, respectively. The China population of *B. raddei* and the American population of *B. americanus* had only allele *e,* while the three populations of *B. marinus* had only allele *d* (Table 4; Fig. 6).

11. LDH-B locus

At the LDH-B locus, 16 phenotypes produced by 12 alleles (*a~l*) were observed. It was found that each of the seven *Bufo* species had a specific allele. The 14 populations of *B. japonicus* had allele *g* in frequencies of 0.333~1.000. Of these populations, five including the Kagoshima population of *B. j. japonicus,* the Yaku population of *B. j. yakushimensis,* the Odai population of *B. j. torrenticola,* the Miyako population of *B. j. miyakonis* and the China population of *B. j. gargarizans* had only allele *g*, while five others including the Zao population of *B. j. montanus* and the Namioka, Mito, Zama and Arashi populations of *B. j. japonicus* had allele *c* in frequencies of 0.056~0.512 in addition to allele *g*. In addition to allele *g*, the Neo population of *B. j. japonicus* had allele *b* in frequency of 0.033, the Yoshiwa population had alleles *c* and *h* in frequencies of 0.053 and 0.079, respectively, and the Taiwan populations of *B. j. gargarizans* and *B. j. bankorensis* had allele *j* in frequencies of 0.614 and 0.667, respectively.

The single toad of the France population of *B. b. bufo* had only allele *l*. In the Portugal population of the same species, there were alleles *h* and *l* in frequencies of 0.750 and 0.250, respectively. The Taiwan population of *B. melanostictus*, the Turkey population of *B. viridis*, the China population of *B. raddei* and the three populations of *B. marinus* had only alleles *e, f, a* and *d*, respectively. In the American population of *B. americanus*, there were alleles *i* and *k* in frequencies of 0.833 and 0.167, respectively (Table 4; Fig. 6).

12. MDH-A locus

At the MDH-A locus, 10 phenotypes produced by nine alleles (*a~i*) were observed. In the 14 populations of *B. japonicus* and the two populations of *B. bufo*, allele *h* was very high in frequency, being 0.833~1.000. In addition to allele *h*, the Namioka population of *B. j. japonicus* had allele *e* in frequency of 0.071, the Miyako population of *B. j. miyakonis* had allele *c* in frequency of 0.083, and the Taiwan populations of *B. j. gargarizans* and *B. j. bankorensis* had allele *f* in frequencies of 0.136 and 0.167, respectively. In the Portugal population of *B. b. bufo*, allele *i* was observed in frequency of 0.125 in addition to allele *h*. The remaining 10 populations of *B. japonicus* and *B. bufo* had only allele *h*. Each of the other five species had a specific allele. The Taiwan population of *B. melanostictus*, the Turkey population of *B. viridis*, the China population of *B. raddei*, the three populations of *B. marinus* and the American population of *B. americanus* had only alleles *d, g, e, b* and *a*, respectively (Table 4; Fig. 6).

13. MDH-B locus

At the MDH-B locus, 12 phenotypes produced by 11 alleles (*a~k*) were observed. In the 14 populations of *B. japonicus*, allele *e* was mostly high in frequency, being 0.833~1.000. In addition to allele *e*, the Zama population of *B. j. japonicus* had allele *a* in frequency of 0.038, the Odai population of *B. j. torrenticola* had allele *g* in frequency of 0.100 and the Miyako population of *B. j. miyakonis* had allele *b* in frequency of 0.167. All the remaining 11 populations of *B. japonicus* had only allele *e*.

Each of the six *Bufo* species other than *B. japonicus* had a specific allele. The two populations of *B. b. bufo* had only allele *d*. The Taiwan population of *B. melanostictus* had alleles *j* and *c* in frequencies of 0.971 and 0.029, respectively. In the Turkey population of *B. viridis*, there were alleles *f* and *e* in frequencies of 0.750 and 0.250, respectively. The China population of *B. raddei*, the three populations of *B. marinus* and the American population of *B. americanus* had only alleles *k, i* and

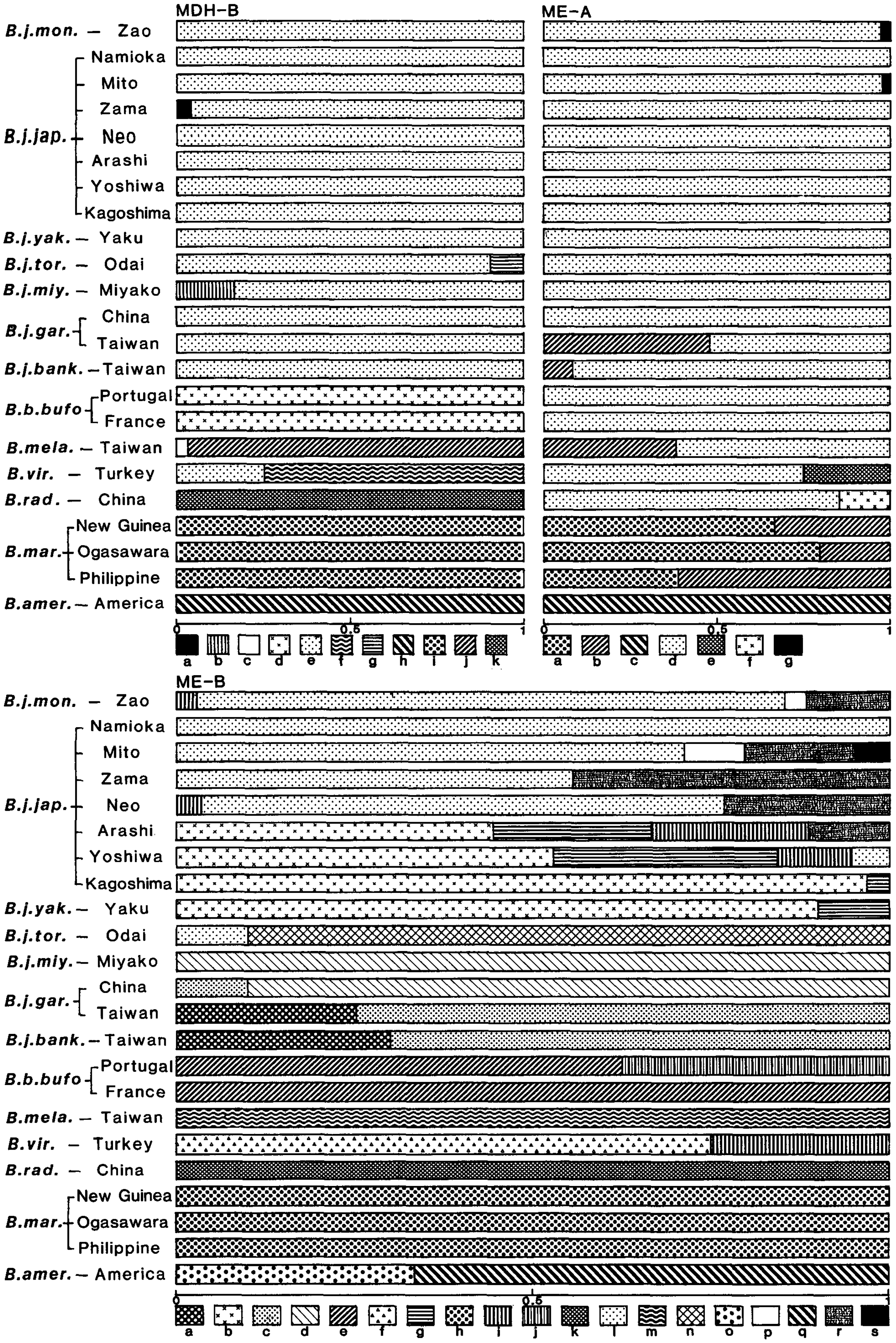


Fig. 7. Gene frequencies at three loci, MDH-B, ME-A and ME-B, in 23 populations of *Bufo* species and subspecies.

h, respectively (Table 4; Fig. 7).

14. ME–A locus

At the ME–A locus, nine phenotypes produced by seven alleles (*a*~*g*) were observed. In the 19 populations other than the three populations of *B. marinus* and the American population of *B. americanus*, allele *d* was the highest in frequency, being 0.523~1.000. In addition to allele *d*, the Zao population of *B. j. montanus* and the Mito population of *B. j. japonicus* had allele *g* in frequencies of 0.029 and 0.024, respectively. In the Taiwan populations of *B. j. gargarizans*, *B. j. bankorensis* and *B. melanostictus*, there was allele *b* in frequencies of 0.477, 0.083 and 0.382, respectively, in addition to allele *d*. In the Turkey population of *B. viridis* and the China population of *B. raddei*, there were alleles *e* and *f* in frequencies of 0.250 and 0.147, respectively, in addition to allele *d*. The remaining 12 populations had only allele *d*. In the three populations of *B. marinus*, alleles *a* and *b* were 0.386~0.800 and 0.200~0.614 in frequency, respectively. The American population of *B. americanus* had only allele *c* (Table 4; Fig. 7).

15. ME–B locus

At the ME–B locus, 35 phenotypes produced by 19 alleles (*a*~*s*) were observed. In the Zao population of *B. j. montanus* and the four eastern populations of *B. j. japonicus*, the Namioka, Mito, Zama and Neo populations, allele *l* was mostly high in frequency, being 0.558~1.000. While the Namioka population of these five populations had only allele *l*, the other populations had some other alleles in addition to allele *l*. The Zao population had alleles *i*, *p* and *r* in frequencies of 0.029, 0.029 and 0.118, respectively. The Mito population had alleles *p*, *r* and *s* in frequencies of 0.083, 0.155 and 0.048, respectively. The Zama population had allele *r* in frequency of 0.442. The Neo population had alleles *i* and *r* in frequencies of 0.033 and 0.233, respectively. In the three western populations of *B. j. japonicus*, the Arashi, Yoshiwa and Kagoshima populations, and the Yaku population of *B. j. yakushimensis*, allele *b* was high in frequency, being 0.444~0.967. In addition to allele *b*, the Arashi population had alleles *g*, *i* and *r* in frequencies of 0.222, 0.222 and 0.111, respectively. The Yoshiwa population had alleles *g*, *i* and *l* in frequencies of 0.316, 0.105 and 0.053, respectively. The Kagoshima and Yaku populations had allele *g* in frequencies of 0.033 and 0.100, respectively. The Odai population of *B. j. torrenticola* had alleles *n* and *l* in frequencies of 0.900 and 0.100, respectively. The Miyako population of *B. j. miyakonis* had only allele *d*. The China population of *B. j. gargarizans* had alleles *d* and *c* in frequencies of 0.900 and 0.100, respectively, while the Taiwan populations of *B. j. gargarizans* and *B. j. bankorensis* had allele *c* in frequencies of 0.750 and 0.700, respectively, and allele *a* in frequencies of 0.250 and 0.300, respectively. The France population of *B. b. bufo* had only allele *e*, while the Portugal population of the same species had alleles *e* and *j* in frequencies of 0.625 and 0.375, respectively. The Taiwan population of *B. melanostictus*, the China population of *B. raddei* and the three populations of *B. marinus* had only alleles *m*, *k* and *h*, respectively. The Turkey population of *B. viridis* had alleles *f* and *i* in frequencies of 0.750 and 0.250, respectively. The American population of *B. americanus* had alleles *o* and *q* in frequencies of 0.333 and 0.667, respectively (Table 4; Fig. 7).

16. MPI locus

At the MPI locus, 19 phenotypes produced by 10 alleles (*a*~*j*) were observed. In nine populations including the Zao population of *B. j. montanus,* the Namioka, Mito and Neo populations of *B. j. japonicus,* the Odai population of *B. j. torrenticola,* the two populations of *B. j. gargarizans,* the Taiwan population of *B. j. bankorensis* and the American population of *B. americanus,* allele *f* was high in frequency, being 0.560~0.882. In addition to allele *f,* the Zao and Neo populations had allele *c* in frequencies of 0.118 and 0.233, respectively, the Namioka population had allele *h* in frequency of 0.143, and the Mito population had alleles *h* and *c* in frequencies of 0.286 and 0.155, respectively. The Odai population had alleles *c* and *d* in frequencies of 0.100 and 0.050, respectively, the China population of *B. j. gargarizans* had alleles *a, c, g* and *h* in frequencies of 0.043, 0.065, 0.109 and 0.043, respectively, and the Taiwan population of the same subspecies had alleles *c* and *j* in frequencies of 0.023 and 0.159, respectively, in addition to allele *f.* The Taiwan population of *B. j. bankorensis* had allele *j* in frequency of 0.167, and the American population of *B. americanus* had allele *i* in frequency of 0.333 in addition to allele *f.*

In five populations including the Yaku population of *B. j. yakushimensis,* the Arashi and Kagoshima populations of *B. j. japonicus,* and the two populations of *B. b. bufo,* the allele *c* was overwhelmingly high in frequency, being 0.722~1.000. In addition to allele *c,* the Arashi population had allele *f* in frequency of 0.278, and the Kagoshima population had alleles *a* and *f* in frequencies of 0.233 and 0.033, respectively. The remaining three of the five populations had only allele *c.* In the Zama population of *B. j. japonicus,* there were alleles *f, h* and *c* in frequencies of 0.385, 0.365 and 0.250, respectively, while in the Yoshiwa population of the same subspecies, there were alleles *c, f* and *a* in frequencies of 0.553, 0.421 and 0.026, respectively. In the Miyako population of *B. j. miyakonis,* there were alleles *g, b* and *d* in frequencies of 0.833, 0.083 and 0.083, respectively.

The Taiwan population of *B. melanostictus* had alleles *e* and *f* in frequencies of 0.559 and 0.441, respectively. In the three populations of *B. marinus,* alleles *e* and *f* were 0.500~0.722 and 0.278~0.500 in frequency, respectively. The Turkey population of *B. viridis* had only allele *i,* and the China population of *B. raddei* had only allele *h* (Table 4; Fig. 8).

17. PGM locus

At the PGM locus, nine phenotypes produced by eight alleles (*a*~*h*) were observed. In 17 populations including the 14 populations of *B. japonicus,* the two populations of *B. b. bufo* and the Taiwan population of *B. melanostictus,* allele *e* was the highest in frequency, being 0.667~1.000. In addition to allele *e,* the Namioka and Arashi populations of *B. j. japonicus* and the Miyako population of *B. j.*

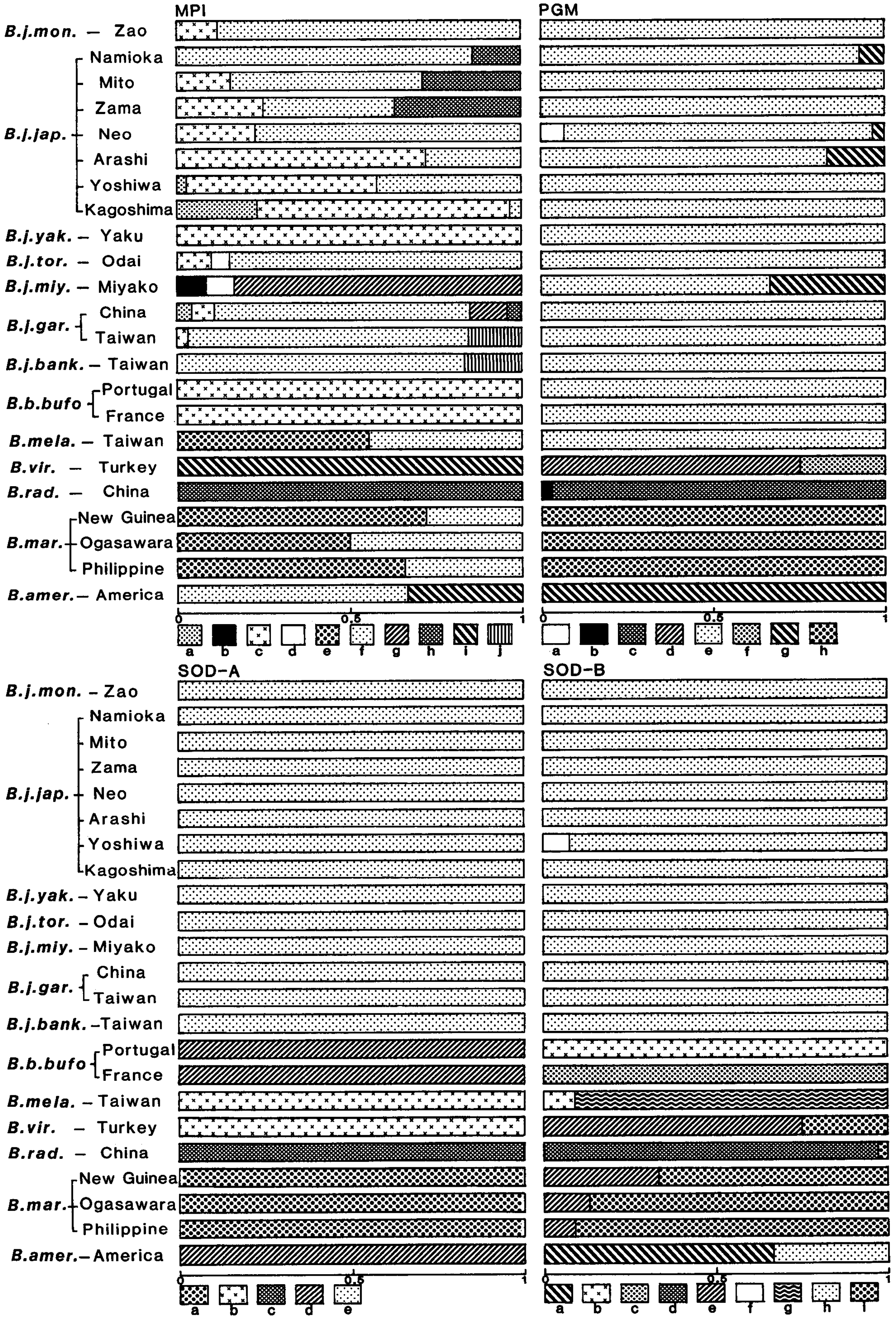


Fig. 8. Gene frequencies at four loci, MPI, PGM, SOD-A and SOD-B, in 23 populations of *Bufo* species and subspecies.

miyakonis had allele *g* in frequencies of 0.071~0.333, and the Neo population of *B. j. japonicus* had alleles *a* and *g* in frequencies of 0.067 and 0.033, respectively. All the remaining 13 of the 17 populations had only allele *e*. In the Turkey population of *B. viridis*, there were alleles *d* and *f* in frequencies of 0.750 and 0.250, respectively. In the China population of *B. raddei*, there were alleles *c* and *b* in frequencies of 0.971 and 0.029, respectively. The three populations of *B. marinus* and the American population of *B. americanus* had only alleles *h* and *g*, respectively (Table 4; Fig. 8).

18. SOD–A locus

At the SOD–A locus, five phenotypes produced by five alleles (*a*~*e*) were observed. All the 14 populations of *B. japonicus* had only allele *e*. While the two populations of *B. b. bufo* and the American population of *B. americanus* had only allele *d*, the Taiwan population of *B. melanostictus* and the Turkey population of *B. viridis* had only allele *b*. The three populations of *B. marinus* had only allele *a* and the China population of *B. raddei* had only allele *c* (Table 4; Fig. 8).

19. SOD–B locus

At the SOD–B locus, 13 phenotypes produced by nine alleles (*a*~*i*) were observed. All the 14 populations of *B. japonicus* had only allele *h*, except that the Yoshiwa population had allele *f* in frequency of 0.079 in addition to allele *h*. The Portugal population of *B. b. bufo* had only allele *b*, while the only toad of the France population of the same species had allele *c*. In the Taiwan population of *B. melanostictus*, there were alleles *g* and *b* in frequencies of 0.912 and 0.088, respectively. In the Turkey population of *B. viridis*, there were alleles *e* and *i* in frequencies of 0.750 and 0.250, respectively. In the China population of *B. raddei*, there were alleles *d* and *i* in frequencies of 0.971 and 0.029, respectively. In the three populations of *B. marinus*, allele *i* was high in frequency, being 0.667~0.909, while there was allele *e* in frequencies of 0.091~0.333 in addition. In the American population of *B. americanus*, there were alleles *a* and *h* in frequencies of 0.667 and 0.333, respectively (Table 4; Fig. 8).

20. Ab locus

At the Ab locus, 16 phenotypes produced by 10 alleles (*a*~*j*) were observed. In the Zao population of *B. j. montanus* and the Mito and Yoshiwa populations of *B. j. japonicus*, allele *g* was high in frequency, being 0.735~0.972. In addition to allele *g*, the Mito population had allele *e* in frequency of 0.028, the Zao population had alleles *e* and *j* in frequencies of 0.206 and 0.059, respectively, and the Yoshiwa population had alleles *d* and *f* in frequencies of 0.143 and 0.071, respectively. In the Zama and Neo populations of *B. j. japonicus* and the Odai population of *B. j. torrenticola*, alleles *e* and *g* were 0.500~0.688 and 0.313~0.500 in frequency, respectively. In addition to these alleles, the Odai population had allele *i* in frequency of 0.056. In five populations including the Kagoshima population of *B. j. japonicus*, the Yaku population of *B. j. yakushimensis*, the Miyako population of *B.*

j. miyakonis, the China population of *B. j. gargarizans* and the Portugal population of *B. b bufo,* allele *d* was high in frequency, being 0.667~1.000. Of these populations, the Miyako and China populations had only allele *d,* while in the other three populations, there was allele *f* in frequencies of 0.125~0.333 in addition to allele *d.* The Taiwan populations of *B. j. gargarizans* and *B. j. bankorensis* had only allele *h.* The single toad of the France population of *B. b. bufo* had allele *i.* In the Taiwan population of *B. melanostictus,* there were alleles *b* and *c* in frequencies of 0.667 and 0.333, respectively. The Turkey population of *B. viridis* and the three populations of *B. marinus* had only allele *a.* The China population of *B. raddei* had only allele *f.* In the American population of *B. americanus,* there were alleles *b* and *a* in frequencies of 0.833 and 0.167, respectively (Table 5; Fig. 9).

21. Hb locus

At the Hb locus, 12 phenotypes produced by eight alleles (*a*~*h*) were observed. Four populations including the Mito, Zama and Kagoshima populations of *B. j. japonicus* and the Yaku population of *B. j. yakushimensis* had only allele *b.* In the Zao population of *B. j. montanus* and the Neo population of *B. j. japonicus,* allele *b* was high in frequency, being 0.735 and 0.933, respectively, while there was allele *d* in frequencies of 0.265 and 0.067, respectively, in addition to allele *b.* In the Yoshiwa population of *B. j. japonicus,* there were alleles *b* and *d* in frequencies of 0.417 and 0.583, respectively. In the Odai population of *B. j. torrenticola,* there

TABLE 5

Gene frequencies at two loci, Ab and Hb, in 21 populations of *Bufo* species and subspecies

Species	Population	Sample size	Ab										Hb							
			a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*
B. j. mon.	Zao	17					0.206		0.735			0.059		0.735		0.265				
B. j. jap.	Namioka	—																		
〃	Mito	36					0.028		0.972					1.000						
〃	Zama	25					0.688		0.313					1.000						
〃	Neo	15					0.500		0.500					0.933		0.067				
〃	Arashi	—																		
〃	Yoshiwa	7				0.143		0.071	0.786					0.417		0.583				
〃	Kagoshima	15				0.700		0.300						1.000						
B. j. yak.	Yaku	3				0.667		0.333						1.000						
B. j. tor.	Odai	9					0.500		0.444		0.056				0.944	0.056				
B. j. miy.	Miyako	6				1.000							1.000							
B. j. gar.	China	23				1.000							1.000							
〃	Taiwan	22								1.000			0.932			0.068				
B. j. bank.	Taiwan	6								1.000			0.917			0.083				
B. b. bufo	Portugal	4				0.875		0.125									0.750			0.250
〃	France	1									1.000									1.000
B. mela.	Taiwan	9		0.667	0.333														1.000	
B. vir.	Turkey	2	1.000												0.250	0.750				
B. rad.	China	16						1.000									1.000			
B. mar.	New Guinea	9	1.000															1.000		
〃	Ogasawara	15	1.000															1.000		
〃	Philippine	22	1.000															1.000		
B. amer.	America	3	0.167	0.833													1.000			

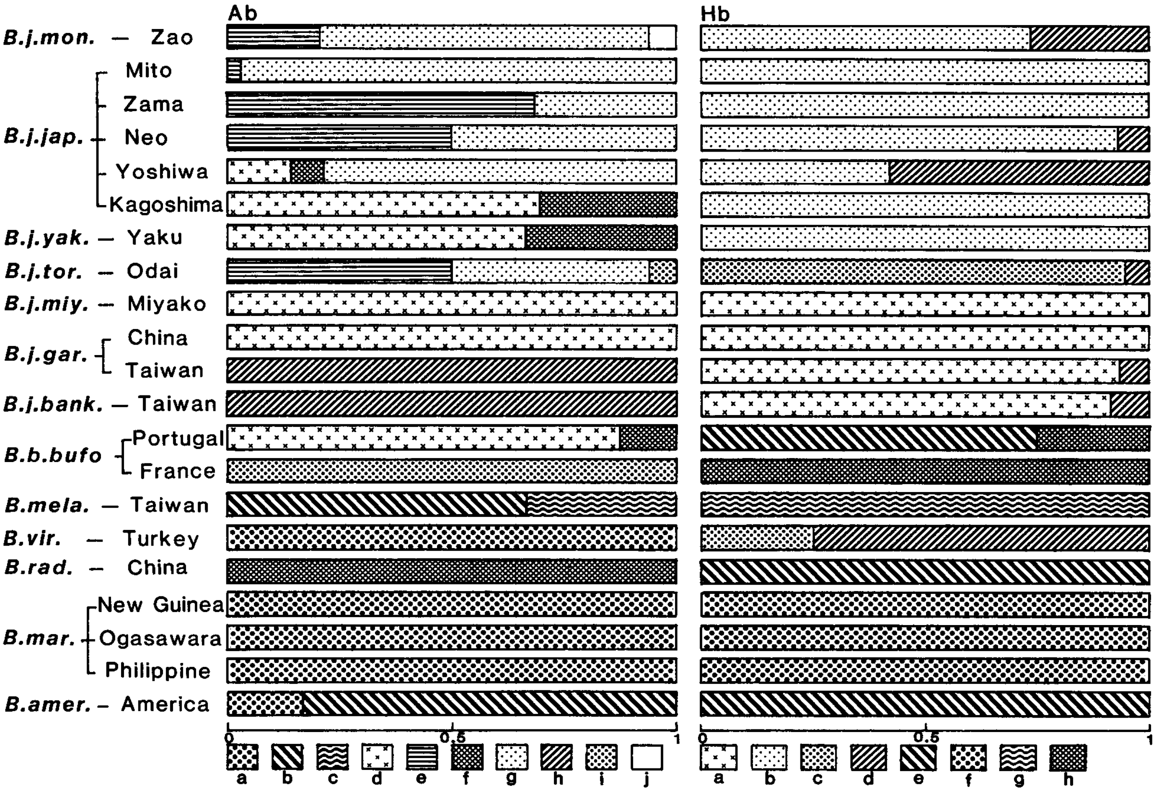


Fig. 9. Gene frequencies at two loci, Ab and Hb, in 21 populations of *Bufo* species and subspecies.

were alleles *c* and *d* in frequencies of 0.944 and 0.056, respectively. In the Miyako population of *B. j. miyakonis,* the two populations of *B. j. gargarizans,* and the Taiwan population of *B. j. bankorensis,* allele *a* was overwhelmingly high in frequency, being 0.917~1.000. Of these populations, the Taiwan populations of *gargarizans* and *bankorensis* had allele *d* in frequencies of 0.068 and 0.083, respectively, in addition to allele *a.* In the Portugal population of *B. b. bufo,* there were alleles *e* and *h* in frequencies of 0.750 and 0.250, respectively. The single toad of the France population of the same subspecies had allele *h.* In the Turkey population of *B. viridis,* there were alleles *d* and *c* in frequencies of 0.750 and 0.250, respectively. The China population of *B. raddei* and the American population of *B. americanus* had only allele *e,* while the three populations of *B. marinus* had only allele *f* (Table 5; Fig. 9).

III. Genetic variation

1. Proportion of heterozygous loci

When the mean proportion of heterozygous loci per individual was estimated at the 19 loci controlling the 13 enzymes in each of the 23 populations belonging to seven species and six subspecies of *Bufo,* it was 1.1~26.3%, 11.2% on the average. In the Yaku population of *B. j. yakushimensis,* it was the smallest, being 1.1%. This was followed by three populations including the Namioka population of *B. j. japonicus,* the Odai population of *B. j. torrenticola* and the Kagoshima population of

B. j. japonicus, being 6.0~6.7%. In the three populations of *B. marinus,* they were 7.6~9.9%, while in four populations including the China populations of *B. j. gargarizans* and *B. raddei,* the Yoshiwa population of *B. j. japonicus* and the Miyako population of *B. j. miyakonis,* they were 7.8~9.6%. In five populations including the France population of *B. b. bufo,* the Taiwan population of *B. b. bankorensis,* the Neo population of *B. j. japonicus,* the Zao population of *B. j. montanus* and the Taiwan population of *B. melanostictus,* they were 10.5~10.9%. The Zama population of *B. j. japonicus,* the Taiwan population of *B. j. gargarizans,* the Portugal population of *B. b. bufo,* and the Mito and Arashi populations of *B. j. japonicus* were gradually larger in the mean proportion of heterozygous loci, being 13.2~17.0%. In the Turkey population of *B. viridis,* it was 21.1%, while in the American population of *B. americanus,* it was 26.3% which was the largest.

The foregoing values estimated in the 23 populations were compared with expected values obtained by the method of LEWONTIN and HUBBY (1966). It was found that the largest difference between the actual and expected values was 7.9% in the American population of *B. americanus.* The second largest difference was 5.3% in the Turkey population of *B. viridis,* followed by 5.2% in the France population of *B. b. bufo.* However, it should be noted that these three populations were extremely small in sample size, as they contained only one to three toads. Although in the Yoshiwa population of *B. j. japonicus,* the difference of the mean proportion of heterozygous loci from the expected value was comparatively large, being 2.7%, the differences were 0~1.6% in the remaining 19 populations (Table 6).

2. Proportion of polymorphic loci

In each of the 23 populations of the seven species and six subspecies of *Bufo,* the mean proportion of polymorphic loci in which each of the alleles was contained at the rate of more than 1% was estimated at the 19 loci controlling the 13 enzymes. The mean proportions of polymorphic loci in 23 populations were 5.3~63.2%, 37.8% on the average. Of these populations, the Yaku population of *B. j. yakushimensis* was the smallest in proportion, being 5.3%, followed by the France population of *B. b. bufo,* being 10.5%. They were 21.1% in each of the Ogasawara population of *B. marinus* and the Kagoshima population of *B. j. japonicus,* 31.6% in each of five populations including the Odai population of *B. j. torrenticola,* the Miyako population of *B. j. miyakonis,* the China population of *B. j. gargarizans* and the New Guinea and Philippine populations of *B. marinus,* 36.8% in each of three populations including the Namioka population of *B. j. japonicus,* the Taiwan population of *B. melanostictus* and the China population of *B. raddei,* 42.1% in each of four populations including the Yoshiwa population of *B. j. japonicus,* the Taiwan populations of *B. j. gargarizans* and *bankorensis* and the Turkey population of *B. viridis,* 47.4% in each of the Portugal population of *B. b. bufo* and the American population of *B. americanus,* and 52.6% in each of three populations including the Zao population of *B. j. montanus* and the Zama and Neo populations of *B. j. japonicus.* The mean proportion of polymorphic loci in the Arashi population of *B.*

TABLE 6

Genetic variabilities at 19 loci in 23 populations of *Bufo* species and subspecies

Species or subspecies	Population	Sample size	Mean proportion of heterozygous loci per individual (%)	Mean proportion of polymorphic loci per population (%)	Mean number of alleles per locus
B. j. mon.	Zao	17	10.9 (12.1)	52.6	1.68
B. j. jap.	Namioka	7	6.0 (7.5)	36.8	1.37
〃	Mito	42	15.4 (16.0)	63.2	1.95
〃	Zama	26	13.2 (13.4)	52.6	1.58
〃	Neo	15	10.7 (11.7)	52.6	1.63
〃	Arashi	9	17.0 (15.6)	57.9	1.74
〃	Yoshiwa	19	9.1 (11.8)	42.1	1.68
〃	Kagoshima	15	6.7 (5.3)	21.1	1.26
B. j. yak.	Yaku	5	1.1 (0.9)	5.3	1.05
B. j. tor.	Odai	10	6.3 (6.3)	31.6	1.37
B. j. miy.	Miyako	6	9.6 (10.2)	31.6	1.37
B. j. gar.	China	23	7.8 (8.4)	31.6	1.53
〃	Taiwan	22	14.4 (12.8)	42.1	1.47
B. j. bank.	Taiwan	6	10.6 (11.3)	42.1	1.42
B. b. bufo	Portugal	4	14.5 (14.5)	47.4	1.53
〃	France	1	10.5 (5.3)	10.5	1.11
B. mela.	Taiwan	17	10.9 (10.1)	36.8	1.37
B. vir.	Turkey	2	21.1 (15.8)	42.1	1.42
B. rad.	China	17	8.1 (7.3)	36.8	1.37
B. mar.	New Guinea	9	9.9 (8.9)	31.6	1.32
〃	Ogasawara	15	7.6 (6.7)	21.1	1.21
〃	Philippine	22	8.8 (9.0)	31.6	1.32
B. amer.	America	3	26.3 (18.4)	47.4	1.47
Average (Total)		13.6 (312)	11.2 (10.4)	37.8	1.44

Parentheses show an expected value.

j. japonicus was 57.9%. It was the largest in the Mito population of the same species, being 63.2% (Table 6).

3. Mean number of alleles per locus

The mean number of alleles at 19 loci controlling 13 enzymes in the 23 populations was 1.05~1.95, 1.44 on the average. The population which was the smallest in the mean number of alleles per locus was the Yaku population of *B. j. yakushimensis,* being 1.05. The France population of *B. b. bufo* was the next, being 1.11. The three populations of *B. marinus* were 1.21~1.32, while the Kagoshima population of *B. j. japonicus* was 1.26. Each of the five populations including the Namioka population of *B. j. japonicus,* the Odai population of *B. j. torrenticola,* the

Miyako population of *B. j. miyakonis,* the Taiwan population of *B. melanostictus* and the China population of *B. raddei* was 1.37. Each of the Taiwan population of *B. j. bankorensis* and the Turkey population of *B. viridis* was 1.42. Each of the American population of *B. americanus* and the Taiwan population of *B. j. gargarizans* was 1.47, while each of the China population of *B. j. gargarizans* and the Portugal population of *B. b. bufo* was 1.53. The Zama, Neo and Yoshiwa populations of *B. j. japonicus* were 1.58, 1.63 and 1.68, respectively. The Zao population of *B. j. montanus* was 1.68, while the Arashi and Mito populations of the same species were 1.74 and 1.95, respectively (Table 6).

IV. Genetic distance and dendrogram

1. Genetic distance

On the basis of the gene frequencies at the 19 loci controlling 13 enzymes in the 312 toads of the 23 populations belonging to the seven species and six subspecies of *Bufo,* genetic distances (D) between different populations, subspecies and species were estimated according to NEI's method (1975).

a. Populational difference

The smallest genetic distances between different populations were found among the three populations (20~22) of *B. marinus.* They were 0.006~0.014 in value. The genetic distance between the two populations (12, 13) of *B. j. gargarizans* was 0.168, and that between the two populations (15, 16) of *B. b. bufo* was 0.187. Those among the seven populations (2~8) of *B. j. japonicus* were 0.016~0.241. When these seven populations of *B. j. japonicus* were divided into the eastern group including the Namioka, Mito, Zama and Neo populations (2~5) and the western group including the Arashi, Yoshiwa and Kagoshima populations (6~8), the genetic distances among the former populations were 0.024~0.070, while those among the latter populations were 0.016~0.031. On the other hand, the genetic distances between the Arashi population (6) and the four eastern populations (2~5) were 0.057~0.147, those between the Yoshiwa population (7) and the four eastern populations (2~5) were 0.089~0.195, and those between the Kagoshima population (8) and the four eastern populations (2~5) were 0.116~0.241 (Table 7).

b. Subspecific difference

The genetic distances among the seven subspecies were 0.008~0.438. Those between the Zao population (1) of *B. j. montanus* and the four eastern populations (2~5) of *B. j. japonicus* including the Namioka, Mito, Zama and Neo populations were 0.008~0.037, while those between the Zao population (1) and the three western populations (6~8) of *B. j. japonicus* including the Arashi, Yoshiwa and Kagoshima populations were 0.094~0.169. The genetic distances between the Yaku population (9) of *B. j. yakushimensis* and the three western populations (6~8) of *B. j. japonicus* were 0.009~0.024, while those between the Yaku population (9)

TABLE 7

Genetic identity(I) and genetic distance(D) among 23 populations of *Bufo* species and subsecies

Species	Population		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
B. j. mon.	Zao	1	—	0.984	0.992	0.964	0.978	0.910	0.882	0.845	0.845	0.897	0.679	0.743	0.778	0.803	0.375	0.374	0.334	0.121	0.193	0.184	0.190	0.196	0.153
B. j. jap.	Namioka	2	0.016	—	0.975	0.933	0.962	0.863	0.823	0.786	0.786	0.843	0.645	0.712	0.742	0.768	0.351	0.353	0.336	0.119	0.223	0.192	0.197	0.204	0.130
〃	Mito	3	0.008	0.025	—	0.976	0.970	0.918	0.889	0.857	0.859	0.893	0.681	0.737	0.773	0.801	0.390	0.384	0.322	0.124	0.210	0.186	0.188	0.197	0.146
〃	Zama	4	0.037	0.070	0.024	—	0.976	0.945	0.915	0.890	0.893	0.914	0.719	0.761	0.791	0.817	0.384	0.387	0.286	0.122	0.187	0.188	0.187	0.198	0.163
〃	Neo	5	0.022	0.039	0.031	0.024	—	0.925	0.897	0.865	0.858	0.900	0.701	0.761	0.787	0.812	0.358	0.359	0.287	0.119	0.163	0.177	0.182	0.188	0.161
〃	Arashi	6	0.094	0.147	0.086	0.057	0.078	—	0.978	0.970	0.977	0.935	0.695	0.730	0.757	0.779	0.408	0.409	0.275	0.125	0.169	0.184	0.183	0.193	0.167
〃	Yoshiwa	7	0.126	0.195	0.118	0.089	0.108	0.022	—	0.984	0.977	0.942	0.657	0.704	0.732	0.758	0.390	0.386	0.260	0.124	0.140	0.169	0.169	0.179	0.171
〃	Kagoshima	8	0.169	0.241	0.154	0.116	0.145	0.031	0.016	—	0.991	0.910	0.654	0.682	0.701	0.724	0.384	0.383	0.248	0.118	0.139	0.153	0.148	0.162	0.151
B. j. yak.	Yaku	9	0.169	0.241	0.152	0.113	0.153	0.023	0.024	0.009	—	0.909	0.656	0.683	0.704	0.725	0.407	0.407	0.256	0.115	0.153	0.169	0.164	0.178	0.146
B. j. tor.	Odai	10	0.108	0.170	0.113	0.090	0.105	0.067	0.060	0.094	0.095	—	0.667	0.725	0.752	0.776	0.367	0.367	0.287	0.117	0.156	0.185	0.190	0.196	0.185
B. j. miy.	Miyako	11	0.387	0.438	0.384	0.329	0.355	0.364	0.420	0.425	0.422	0.405	—	0.894	0.694	0.718	0.261	0.262	0.191	0.119	0.150	0.128	0.123	0.137	0.154
B. j. gar.	China	12	0.297	0.340	0.305	0.273	0.273	0.315	0.351	0.383	0.381	0.321	0.112	—	0.845	0.863	0.296	0.298	0.228	0.126	0.169	0.158	0.162	0.169	0.162
〃	Taiwan	13	0.252	0.299	0.257	0.235	0.240	0.278	0.312	0.356	0.351	0.286	0.366	0.168	—	0.980	0.337	0.341	0.227	0.101	0.138	0.181	0.182	0.200	0.190
B. j. bank.	Taiwan	14	0.219	0.264	0.222	0.202	0.208	0.249	0.277	0.323	0.321	0,254	0.332	0.147	0.020	—	0.352	0.354	0.227	0.118	0.162	0.189	0.194	0.203	0.196
B. b. bufo	Portugal	15	0.980	1.047	0.942	0.958	1.027	0.897	0.941	0.956	0.898	1.001	1.343	1.218	1.087	1.044	—	0.830	0.213	0.171	0.150	0.138	0.133	0.146	0.136
〃	France	16	0.983	1.040	0.958	0.950	1.024	0.895	0.953	0.958	0.898	1.002	1.341	1.209	1.076	1.038	0.187	—	0.205	0.162	0.151	0.145	0.140	0.153	0.130
B. mela.	Taiwan	17	1.098	1.090	1.132	1.251	1.247	1.292	1.345	1.393	1.363	1.249	1.657	1.477	1.484	1.482	1.546	1.586	—	0.314	0.251	0.163	0.154	0.177	0.109
B. vir.	Turkey	18	2.108	2.126	2.087	2.108	2.133	2.081	2.090	2.138	2.161	2.146	2.132	2.069	2.294	2.137	1.769	1.820	1.160	—	0.210	0.220	0.212	0.223	0.148
B. rad.	China	19	1.643	1.501	1.560	1.678	1.814	1.775	1.964	1.976	1.880	1.855	1.896	1.778	1.984	1.819	1.895	1.890	1.383	1.561	—	0.125	0.121	0.132	0.179
B. mar.	New Guinea	20	1.694	1.650	1.682	1.674	1.731	1.693	1.779	1.878	1.776	1.689	2.053	1.846	1.712	1.664	1.984	1.932	1.815	1.512	2.081	—	0.994	0.991	0.116
〃	Ogasawara	21	1.662	1.623	1.672	1.676	1.704	1.701	1.778	1.908	1.806	1.661	2.092	1.819	1.704	1.640	2.020	1.966	1.868	1.552	2.110	0.006	—	0.986	0.121
〃	Philippine	22	1.630	1.591	1.624	1.622	1.674	1.645	1.719	1.823	1.728	1.628	1.988	1.777	1.608	1.597	1.921	1.875	1.730	1.502	2.028	0.010	0.014	—	0.126
B. amer.	America	23	1.874	2.042	1.926	1.813	1.824	1.788	1.767	1.891	1.922	1.690	1.873	1.818	1.661	1.630	1.992	2.043	2.216	1.910	1.719	2.154	2.114	2.070	—

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

and the four eastern populations (2~5) of *B. j. japonicus* were 0.113~0.241. The genetic distance between the Yaku population (9) of *B. j. yakushimensis* and the Odai population (10) of *B. j. torrenticola* was 0.095. Those between the Odai population (10) and the three western populations (6~8) of *B. j. japonicus* were 0.060~0.094, while those between the Odai population (10) and the four eastern populations (2~5) of *B. j. japonicus* were 0.090~0.170.

The Miyako population (11) of *B. j. miyakonis* fairly differed from the populations of *B. j. montanus* (1), *B. j. japonicus* (2~8), *B. j. yakushimensis* (9) and *B. j. torrenticola* (10), as the genetic distances between them were 0.329~0.438. While the genetic distance between the Miyako population (11) of *B. j. miyakonis* and the China population (12) of *B. j. gargarizans* was 0.112, that between the Miyako population (11) and the Taiwan population (13) of *B. j. gargarizans* was 0.366. The Taiwan population (14) of *B. j. bankorensis* was very similar to the Taiwan population (13) of *B. j. gargarizans,* as the genetic distance between them was 0.020. The genetic distance between the Taiwan population (14) of *B. j. bankorensis* and the China population of *B. j. gargarizans* (12) was 0.147. The genetic distances between *B. j. gargarizans* or *B. j. bankorensis* and *B. j. montanus, B. j. japonicus, B. j. yakushimensis* or *B. j. torrenticola* were 0.202~0.383 (Table 7).

c. Specific difference

The smallest genetic distances among seven species were 0.895~1.087 between the two populations (15, 16) of *B. bufo* and the 12 populations (1~10, 13, 14) of the seven subspecies of *B. japonicus* other than the Miyako population (11) of *B. j. miyakonis* and the China population (12) of *B. j. gargarizans,* while the genetic distances between the two populations (15, 16) of *B. bufo* and the Miyako population (11) of *B. j. miyakonis* or the China population (12) of *B. j. gargarizans* were 1.209~1.343. The genetic distances between the Taiwan population (17) of *B. melanostictus* and 12 populations including those of *B. j. montanus* (1), *B. j. japonicus* (2~8), *B. j. yakushimensis* (9), *B. j. torrenticola* (10), *B. viridis* (18) and *B. raddei* (19) were 1.090~1.393. The genetic distances between the Taiwan population (17) of *B. melanostictus* and six populations including the remaining four populations (11~14) of three subspecies of *B. japonicus* and the two populations (15, 16) of *B. bufo* were 1.477~1.657. The genetic distances between the China population (19) of *B. raddei* and 17 populations including the 14 populations (1~14) of the seven subspecies of *B. japonicus,* the two populations (15, 16) of *B. bufo* and the American population (23) of *B. americanus* were 1.501~1.984. The genetic distances between the three populations (20~22) of *B. marinus* and 14 populations including 13 populations (1~10, 12~14) of six subspecies of *B. japonicus* other than the Miyako population (11) of *B. j. miyakonis* and the Taiwan population of *B. melanostictus* (17) were 1.591~1.908. The genetic distances between the three populations (20~22) of *B. marinus* and four populations including the Miyako population (11) of *B. j. miyakonis,* the two populations (15, 16) of *B. bufo* and the China population (19) of *B. raddei* were 1.875~2.110. Those between the American population of *B. americanus* (23) and 17 populations including the 14 populations (1~14) of seven subspecies of *B. japonicus,* two populations (15, 16) of *B. bufo* and the Turkey population (18) of *B. viridis* were 1.630~2.043. While the genetic distances between the Turkey population (18) of *B. viridis* and 14 populations (1~14) of *B. japonicus* were 2.069~2.294, those between the Turkey population (18) of *B. viridis* and the two populations (15, 16) of *B. bufo* were 1.769 and 1.820. Those between the Turkey population (18) of *B. viridis* and four populations including the China population of *B. raddei* (19) and the three populations (20~22) of *B. marinus* were 1.502~1.561 (Table 7).

2. Dendrogram

A dendrogram for the 23 populations belonging to seven species and six subspecies of *Bufo* was drawn by the method of UPGMA (Nei, 1975) on the basis of genetic distances. It was found that *B. americanus* was first produced in North America and *B. marinus* was subsequently produced in South America. The other toads in the eastern hemisphere were divided into two groups, each of which was differentiated into some species in the East and Europe. While the first group produced *B. raddei* and *B. melanostictus* in the East, it produced *B. viridis* in Europe. While the second group produced *B. bufo* in Europe, it produced *B. japonicus* in the Far East. *B. japonicus* was divided into two groups, one of which became two subspecies, *B. j. gargarizans* in China and Taiwan, and *B. j. miyakonis* in Miyako Isl. of Japan, while the other became four subspecies, *B. j. japonicus, B. j. montanus, B. j. torrenticola* and *B. j. yakushimensis* in the mainland and Yaku Isl. of Japan (Fig. 10).

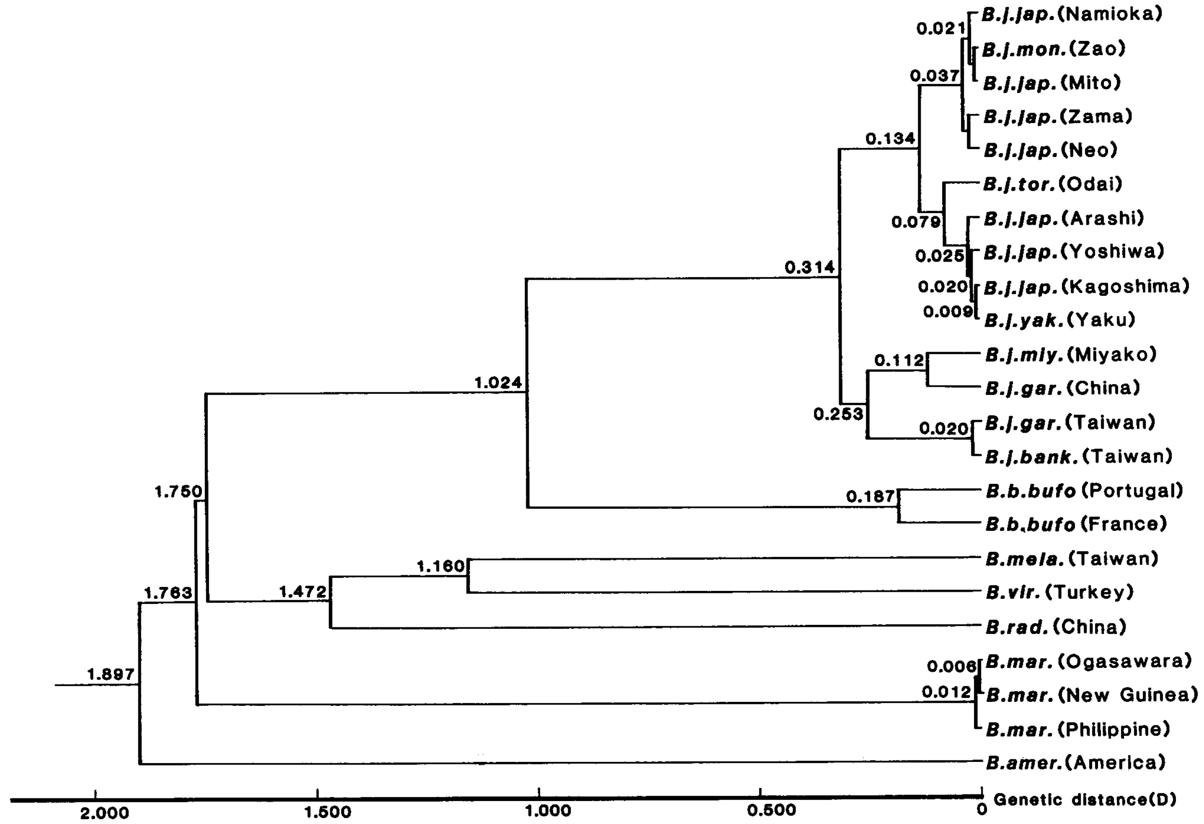
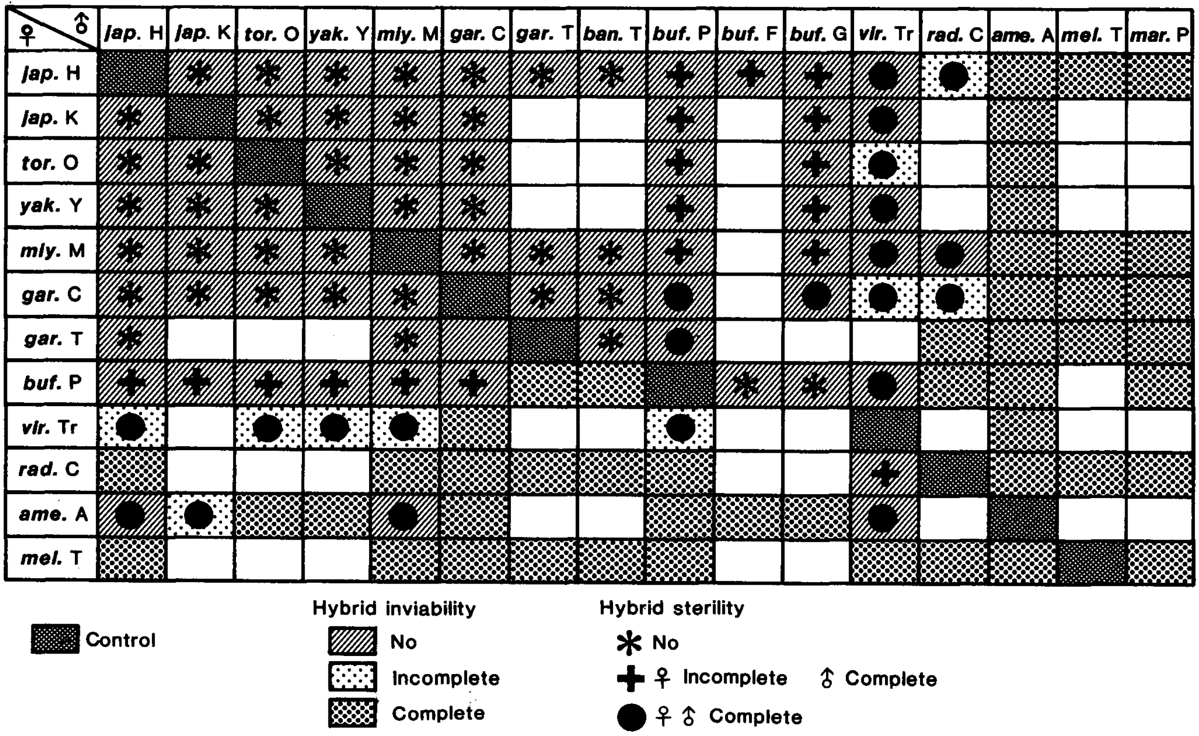


Fig. 10. Dendrogram for 23 populations of *Bufo* species and subspecies based on genetic distances.

Bufo j. bankorensis bears a close resemblance to *B. j. gargarizans,* and had been placed as a synonym of the latter by LIU and HU (1961).

DISCUSSION

KAWAMURA, NISHIOKA and UEDA (1980) have reported on the inter- and intraspecific hybrids among Japanese, European and American toads. Japanese toads included four subspecies of *Bufo japonicus, japonicus* SCHLEGEL, *yakushimensis* OKADA, *miyakonis* OKADA and *torrenticola* MATSUI. European toads included *Bufo bufo* (L.) from Portugal, *Bufo bufo* from France, *Bufo bufo* from Greece and *Bufo viridis* LAURENTI from Turkey. An American toad was *Bufo americanus* HOLBROOK from Michigan. Reciprocal crosses between *Bufo japonicus* and *B. bufo* or *B. viridis* were nearly the same as or somewhat inferior to the control matings in production of metamorphosed toads. Of the reciprocal hybrids between *B. japonicus* and *B. bufo,* males were almost completely sterile, although a small number of male hybrids produced a few offspring by mating with normal females. These offspring all died before completion of metamorphosis. Female hybrids were barely fertile. Their larger eggs became viable triploids by fertilization with normal spermatozoa. All the male hybrids between a female *B. j. japonicus* or *B. j. miyakonis* and a male *B. viridis* were completely sterile, as they have no normal spermatozoa. The male and female hybrids between a female *B. americanus* and a male *B. j. japonicus* were completely sterile and had no germ cells (Fig. 11).



♀ \ ♂	*jap.* H	*jap.* K	*tor.* O	*yak.* Y	*mly.* M	*gar.* C	*gar.* T	*ban.* T	*buf.* P	*buf.* F	*buf.* G	*vir.* Tr	*rad.* C	*ame.* A	*mel.* T	*mar.* P
jap. H	▩	▨ ✱	▨ ✱	▨ ✱	▨ ✱	▨ ✱	▨ ✱	▨ ✱	▨ ✚	▨ ✚	▨ ✚	▨ ●	░ ●	▒	▒	▒
jap. K	▨ ✱	▩	▨ ✱	▨ ✱	▨ ✱	▨ ✱			▨ ✚		▨ ✚	▨ ●		▒		
tor. O	▨ ✱	▨ ✱	▩	▨ ✱	▨ ✱	▨ ✱			▨ ✚		▨ ✚	░ ●		▒		
yak. Y	▨ ✱	▨ ✱	▨ ✱	▩	▨ ✱	▨ ✱			▨ ✚		▨ ✚	▨ ●		▒		
mly. M	▨ ✱	▨ ✱	▨ ✱	▨ ✱	▩	▨ ✱	▨ ✱	▨ ✱	▨ ✚		▨ ✚	▨ ●	▨ ●	▒	▒	▒
gar. C	▨ ✱	▨ ✱	▨ ✱	▨ ✱	▨ ✱	▩	▨ ✱	▨ ✱	▨ ●		▨ ●	░ ●	░ ●	▒	▒	▒
gar. T	▨ ✱				▨ ✱	▨	▩	▨ ✱	▨ ●				▒	▒	▒	▒
buf. P	▨ ✚	▨ ✚	▨ ✚	▨ ✚	▨ ✚	▨ ✚	▒	▒	▩	▨ ✱	▨ ✱	▨ ●	▒	▒		▒
vir. Tr	░ ●		░ ●	░ ●	░ ●	▒			░ ●			▩		▒		
rad. C	▒				▒	▒	▒	▒	▒			▨ ✚	▩	▒	▒	▒
ame. A	▨ ●	░ ●	▒	▒	▨ ●	▒			▒	▒	▒	▨ ●		▩		
mel. T	▒				▒	▒	▒	▒	▒			▒	▒	▒	▩	▒

▩ Control

Hybrid inviability
▨ No
░ Incomplete
▒ Complete

Hybrid sterility
✱ No
✚ ♀ Incomplete ♂ Complete
● ♀ ♂ Complete

Fig. 11. Results of crossing experiments in *Bufo* performed during 1976~1989 by KAWAMURA, NISHIOKA, UEDA and KONDO.

In 1982, KAWAMURA, NISHIOKA, KONDO and WU have reported on the viability of the hybrids among Japanese, Taiwan, European and American toads. The hybrids between females of *B. j. japonicus, B. j. miyakonis, B. j. gargarizans* from China, or *B. bufo* and males of *B. marinus* or *B. melanostictus* became abnormal at the embryonal stage and died before hatching. When the same females were crossed with male *B. raddei,* a small number of hybrids completed metamorphosis. When female *B. raddei* were crossed with male *B. viridis,* most of the hybrids attained to completion of metamorphosis. Thereafter, KAWAMURA, NISHIOKA, UEDA and WU (1982) and KAWAMURA, NISHIOKA, UEDA and KONDO (in preparation) have put together the results of their crossing experiments performed. The toads used in these experiments were six subspecies of *B. japonicus, B. melanostictus, B. bufo, B. viridis, B. americanus, B. raddei* and *B. marinus.* They have confirmed that all reciprocal crosses among six populations including *B. j. japonicus* from Hiroshima and Kagoshima, *B. j. torrenticola, B. j. yakushimensis, B. j. miyakonis* and *B. j. gargarizans* from China did not show any reproductive isolation mechanisms and produced fertile male and female offspring. However, it could not be neglected that the testes of reciprocal hybrids between *B. j. gargarizans* from China and *B. japonicus* including *B. j. japonicus* from Kagoshima and Hiroshima, *B. j. yakushimensis* and *B. j. torrenticola,* contained comparatively few spermatozoa and numerous pycnotic nuclei, while those of reciprocal hybrids between *B. j. gargarizans* and *B. j. miyakonis* were completely normal. When the females of *B. j. japonicus* from Hiroshima, *B. j. miyakonis, and B. j. gargarizans* from China were crossed with males of *B. j. gargarizans* and *B. j. bankorensis* from Taiwan, the hybrids were completely fertile like the foregoing crossing. When the females of *B. j. japonicus* from Hiroshima and Kagoshima, *B. j. torrenticola, B. j. yakushimensis, B. j. miyakonis* and *B. j. gargarizans* from China were crossed with males of *B. bufo* from Portugal and Greece, male hybrids were completely sterile, while female hybrids were somewhat fertile and produced some triploid offspring. When these females were crossed with male *B. viridis,* all the hybrids were completely sterile. These females were isolated from *B. americanus* by hybrid inviability. When females of *B. j. japonicus* from Hiroshima, *B. j. miyakonis* and *B. j. gargarizans* from China were crossed with male *B. raddei,* the hybrids were sterile, although some of them lived for several years. These females were completely isolated from *B. marinus* by hybrid inviability (Fig. 11).

When crosses were made between female *B. raddei* and male *B. japonicus* including *B. j. japonicus* from Hiroshima, *B. j. miyakonis,* and *B. j. gargarizans* from China and Taiwan, *B. americanus, B. melanostictus* and *B. marinus,* all the hybrids were completely inviable. However, the hybrids between female *B. raddei* and male *B. viridis* were viable, although male and female hybrids were completely and incompletely sterile, respectively. When the female *B. melanostictus* were crossed with males of *B. viridis, B. raddei, B. americanus* and *B. marinus,* all the hybrids were completely inviable. However, when the females of *B. bufo* from Portugal were crossed with males of *B. j. japonicus* from Hiroshima and Kagoshima, *B. j. torrenticola, B. j. yakushimensis, B. j. miyakonis* and *B. j. gargarizans* from China, the male hybrids were completely sterile, while the female hybrids produced some triploid offspring. When the female *B. bufo* from Portugal were crossed with males of *B. j. gargarizans* from Taiwan, *B. j. bankorensis, B. raddei, B. americanus* and *B. marinus,* all the hybrids were completely inviable, while all the hybrids between female *B. bufo* and male *B. viridis* were completely sterile. While the hybrids between female *B. viridis* and males of *B. j. japonicus* from Hiroshima, *B. j. torrenticola, B. j. yakushimensis, B. j. miyakonis* and *B. bufo* from Portugal were all completely sterile, the hybrids between female *B. viridis* and male *B. americanus* were inviable. While the hybrids between female *B. americanus* and males of *B. j. japonicus* from Hiroshima, *B. j. japonicus* from Kagoshima, *B. j. miyakonis* and *B. viridis* were all completely sterile, those between female *B. americanus* and males of *B. j. torrenticola, B. j. yakushimensis, B. j. gargarizans* from China and *B. bufo* from Portugal, France and Greece were all completely inviable.

In the present study, the genetic relationships among seven subspecies of *B. japonicus, B. bufo, B. melanostictus, B. viridis, B. raddei, B. marinus* and *B. americanus* were examined by electrophoretic analyses of enzymes and blood proteins extracted from 127 females and 185 males of 23 populations belonging to these species and subspecies. The results of these electrophoretic analyses were compared with those of crossing experiments stated above. It was found that both results remarkably corresponded to each other. The genetic distances among the 14 populations of the seven subspecies of *B. japonicus* were 0.008~0.438. All reciprocal crosses among these 14 populations did not show any kind of reproductive isolation mechanisms. They produced fertile male and female hybrids. Although the genetic distances among seven populations of *B. j. japonicus, B. j. montanus, B. j. yakushimensis* and *B. j. torrenticola* were 0.008~0.241, those among *B. j. miyakonis, B. j. gargarizans* from China and Taiwan and *B. j. bankorensis* were 0.020~0.366. The largest genetic distance among the foregoing 14 populations was 0.438 between *B. j. japonicus* from Namioka and *B. j. miyakonis.* This value seems to be too large for the genetic distance between subspecies. According to HEDGECOCK and AYALA (1974), the genetic distances between populations, between subspecies and between species in three American species of *Taricha* are 0.029±0.010, 0.145±0.027 and 0.466±0.021, respectively. In contrast, KALEZIĆ and HEDGECOCK (1979) have reported that the genetic distances between populations, between subspecies and between species in three European species of *Triturus* are 0.031±0.017, 0.347 and 0.906±0.058, respectively. When the subspecies of *B. japonicus* are compared with those of *Triturus cristatus,* the genetic distances seem not to be too large. The genetic distances between *B. j. gargarizans* from China and Taiwan and seven populations of *B. j. japonicus* are 0.235~0.383, and those between *B. j. gargarizans* from China and Taiwan and *B. j. miyakonis* are 0.112 and 0.366, respectively. These differences in genetic distance seem to show that each of *B. j. japonicus, B. j. miyakonis* and *B. j. gargarizans* belongs to a different subspecies of the same species. In the dendrogram drawn by the method of UPGMA (NEI, 1975) on the basis of genetic distances, *B. japonicus* is evidently divided into two groups of subspecies, one including *miyakonis, gargarizans* and *bankorensis,* and the other including the remaining subspecies, *japonicus, montanus, torrenticola* and *yakushimensis*. These two groups seem to reflect the existence of cytological differences between them, as the hybrids of different subspecies belonging to the same group differ from those of different subspecies belonging to different groups in the amount of normal spermatozoa and pycnotic nuclei in the testes of intersubspecific hybrids, although both kinds of hybrids are completely fertile (KAWAMURA, NISHIOKA and UEDA, 1982; Fig. 11).

The genetic differences among *B. japonicus, B. bufo, B. melanostictus, B. viridis, B. raddei, B. marinus* and *B. americanus* seem to indicate that these taxa are correct, as they are 0.895~2.294. Genetic distances have been calculated in many species of anurans. Those between the Togochi population of *Buergeria buergeri* and the four populations of *Buergeria japonica* are 2.045~2.243, while those among *Rhacophorus arboreus, Rh. schlegelii, Rh. taipeianus* and the three subspecies of *Rh. viridis* are 0.301~0.865 (NISHIOKA, SUMIDA, OHTA and SUZUKI, 1987). Those among *Hyla japonica* from Japan, Korea and Soviet Union, *H. hallowelli* from Amami and *H. chinensis* from Taiwan are 0.596~1.360 (NISHIOKA, SUMIDA and BORKIN, 1990). The genetic distances between six populations of *Rana limnocharis* and two populations of *R. cancrivora* are 1.472~1.715 (NISHIOKA and SUMIDA, 1990). Those between six populations of *Rana t. tagoi* and *R. t. yakushimensis* are 0.182~0.335 (NISHIOKA, OHTA and SUMIDA, 1987). These values seem to show that the nomenclature based on the external morphology is correct on the whole, although there are a few exceptions. However, it is remarkable that there is a species in which the genetic distances between populations are extremely large. In *Rana narina*, there are six populations, including two dwarf-type, three giant-type and one middle-type populations. The genetic distances between the two dwarf-type and three giant-type populations are 0.714~1.079, while those between the two dwarf-type and the middle-type populations are 0.809 and 0.865. The genetic distances between the middle-type and the three giant-type populations are 0.232~0.471. On the basis of genetic distances, each of the dwarf-, middle- and giant-type populations seems to be a real species, as stated previously. In fact, the hybrids between the females of the two dwarf-type populations and the males of the three giant-type populations or the middle-type population, and the reciprocal hybrids between these populations are all lethal at the early developmental stage (NISHIOKA, UEDA and SUMIDA, 1987).

The dendrogram for the seven species shows that *B. japonicus* from the Far East and *B. bufo* from Europe are most closely related among the seven species. The close relationship of these two species seems to correspond to the incomplete sterility between them. While the males of their hybrids are completely sterile, the females are somewhat fertile and produce some large eggs which became triploid offspring by mating with normal diploid males. BOGART (1972) has described that the polyploidy is frequently encountered in hybrid tadpoles of *Bufo*. When the chromosomes can be distinguished, it is found that the female parent contributes the extra set(s) of chromosomes to a polyploid toad. He has listed in the appendix 44 combinations of *Bufo* species which have been found to produce only triploid offspring. Triploid offspring were produced in reciprocal hybrids between the five subspecies of *B. japonicus* and *B. bufo* (KAWAMURA, NISHIOKA and UEDA, 1980; KAWAMURA, NISHOKA, UEDA and KONDO, in preparation). It is remarkable that the hybrids between *Bufo* species which are more remotely related to each other in the dendrogram than those between *B. japonicus* and *B. bufo* are completely sterile or inviable.

ACKNOWLEDGMENTS

The authors are especially indebted to Emeritus Professor Toshijiro KAWAMURA for his encouragement and guidance during the course of this work and for his critical review of the manuscript. The authors are grateful to Professor Dr. Chih-Ye CHANG, Institute of Zoology, Academia Sinica, Professor M. KURAMOTO, Fukuoka University of Education, Dr. M. MATSUI, Kyoto University, Mr. C. S. WANG and Mr. P. S. LIN, National Taiwan University, Professor B. LANZA, Museo Zoologico dell' Università, Italy, and Dr. E. CRESPO, Museu e Laboratório, Zoologico e Antropológica, Faculdade de Ciencias-Universidade, Portugal, who collected and provided valuable specimens to us.

This work was supported by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Science and Culture, Japan.

LITERATURE

BOGART, J. P. 1972. Karyotypes. Evolution in the Genus *Bufo*, edited by W. F. BLAIR. Univ. Texas Press, Austin. pp. 171–195.

BREWER, G. J. 1970. An Introduction to Isozyme Techniques. Academic Press, New York and London. 186 pp.

HARRIS, H. and D. A. HOPKINSON 1976. Handbook of Enzyme Electrophoresis in Human Genetics. North-Holland Publ. Co., Amsterdam.

HEDGECOCK, D. and F. J. AYALA 1974. Evolutionary divergence in the genus *Taricha* (Salamandridae). Copeia, 1974: 738–747.

KALEZIĆ, M. L. and D. HEDGECOCK 1979. Genetic variation and differentiation of three common European newts (*Triturus*) in Yugoslavia. British J. Herpetology, **6**: 49–57.

KAWAMURA, T., M. NISHIOKA, Y. KONDO and Z. WU 1982. Viability of hybrids among Japanese, Chinese, Taiwan, European and American toads. (In Japanese). Jpn. J. Genet., **57**: 677–678.

KAWAMURA, T., M. NISHIOKA, M. SUMIDA and M. RYUZAKI 1990. An electrophoretic study of genetic differentiation in 40 populations of *Bufo japonicus* distributed in Japan. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **10**: 1–51.

KAWAMURA, T., M. NISHIOKA and H. UEDA 1980. Inter- and intraspecific hybrids among Japanese, European and American toads. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **4**: 1–125.

KAWAMURA, T., M. NISHIOKA, H. UEDA and Y. KONDO. Isolating mechanisms among Japanese, Chinese, Taiwan, European and American toads. (In preparation).

KAWAMURA, T., M. NISHIOKA, H. UEDA and Z. WU 1982. Relationship between Japanese and Chinese toads on the basis of hybridization experiments. (In Japanese). Zool. Mag. (Tokyo), **91**: 661.

LEWONTIN, R. C. 1974. The Genetic Basis of Evolutionary Change. Columbia University Press, New York. 346 pp.

LEWONTIN, R. C. and J. L. HUBBY 1966. A molecular approach to the study of genetic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of

Drosophila pseudoobscura. Genetics, **54**: 595–609.

Liu, C.-C. and S.-C. Hu 1961. Chinese Tailless Batrachians. (In Chinese). Ka-sue-shu-ban Sha, Beijing. 364 pp.

Nakamura, K. and S. Ueno 1963. Japanese Reptiles and Amphibians in Colour. (In Japanese). Hoikusha, Osaka, Japan. 214 pp.

Nei, M. 1975. Molecular Population Genetics and Evolution. North-Holland Publ. Co., Amsterdam. 288 pp.

Nishioka, M., S. Ohta and M. Sumida 1987. Intraspecific differentiation of *Rana tagoi* elucidated by electrophoretic analyses of enzymes and blood proteins. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **9**: 97–133.

Nishioka, M., H. Ohtani and M. Sumida 1980. Detection of chromosomes bearing the loci for seven kinds of proteins in Japanese pond frogs. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **4**: 127–184.

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

Nishioka, M., M. Sumida and L. J. Borkin 1990. Biochemical differentiation of the genus *Hyla* distributed in the Far East. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **10**: 93~124.

Nishioka, M., M. Sumida, S. Ohta and H. Suzuki 1987. Speciation of three allied genera, *Buergeria, Rhacophorus* and *Polypedates,* elucidated by the method of electrophoretic analyses. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **9**: 53–96.

Nishioka, M., H. Ueda and M. Sumida 1987. Intraspecific differentiation of *Rana narina* elucidated by crossing experiments and electrophoretic analyses of enzymes and blood proteins. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **9**: 261–303.

Sneath, P. H. A. and R. R. Sokal 1973. Numerical Taxonomy, W. H. Freeman and Co., San Francisco. 573 pp.