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Biochemical Differentiation of the Genus *Hyla* Distributed in the Far East

By

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ABSTRACT

Electrophoretic patterns of 14 enzymes and four blood proteins were examined in 261 frogs of 13 populations belonging to three *Hyla* species, *H. japonica, H. hallowelli* and *H. chinensis*. It was found that these enzymes and blood proteins were controlled by genes at 26 loci. The numbers of alleles and phenotypes at the 26 loci were one to nine, 4.0 on the average, and one to 17, 6.1 on the average, respectively. The mean proportions of heterozygous and polymorphic loci and mean number of alleles per locus in the 13 populations were 11.7%, 37.3% and 1.47 on the average, respectively.

The genetic distances among the 13 populations of the three *Hyla* species were estimated on the basis of gene frequencies at the 26 loci by the method of NEI (1975). Those among the 11 populations of Sakhalin, Kunashiri, Sapporo, Setana, Hirosaki, Ichinoseki, Odawara, Maibara, Hiroshima, Tsushima and Korea of *H. japonica* were 0.012~0.201. Those between the Tsushima and the other 10 populations of *H. japonica* were 0.050~0.132, while those between the Korea and nine other populations excluding the Tsushima were 0.137~0.201. The genetic distances between *H. hallowelli* from Amami and the 11 populations of *H. japonica* were 0.974~1.131, while those between *H. chinensis* from Taiwan and the 11 populations of *H. japonica* were 1.177 ~1.360. The genetic distance between *H. hallowelli* and *H. chinensis* was 0.596.

The phylogenetic relationship of the 13 populations of the three *Hyla* species was examined by drawing a dendrogram by the UPGMA method. It was found that *H. japonica* was first divided from the others which later became *H. hallowelli* and *H. chinensis*. From *H. japonica,* the Korea population first diverged, and then the remaining populations were roughly divided into three groups.

INTRODUCTION

STEJNEGER (1907) reported on *Hyla arborea japonica* GUENTHER distributed all over Japan. According to him, there is no record of the species in any of the Ryukyu Islands or Taiwan. He also reported on *Hyla stepheni* BOULENGER collected from middle Ussuri and from Port Hamilton Island, Korea, and *Hyla chinensis* GUENTHER obtained in Taiwan. OKADA (1931) described two species and two subspecies; *Hyla arborea japonica* GUENTHER distributed widely in Japan and

Korea, *Hyla arborea stepheni* BOULENGER collected from Port Hamilton Island and Seoul, Korea, *Hyla chinensis* GUENTHER distributed commonly in Taiwan and southern and eastern China, and *Hyla hallowelli* THOMPSON distributed on three small islands, Kikaigashima, Amamioshima and Tokunoshima of the Ryukyu Islands. SHANNON (1956), YANG (1962) and WEBB, JONES and BYERS (1962) placed *Hyla stepheni* as a synonym of *Hyla arborea japonica.*

The crosses between Japanese and European *Hyla* were first performed by DAITO (1968). He obtained many hybrids between *H. arborea japonica* and *H. arborea sarda.* As the male and female were almost completely sterile, he considered that *H. arborea japonica* should be a valid species. Thereafter, the position of *H. arborea japonica* as a valid species was confirmed by KAWAMURA, NISHIOKA and UEDA (1972, 1990) and KURAMOTO (1984). Although the frogs belonging to *Hyla japonica* distributed in Japan and adjacent territory are very similar to each other in color and pattern, it is well known that there is a distinct difference in body size between two populations. For example, the frogs of the Higashihiroshima population are generally smaller than those of the Hiroshima population, while the frogs of the Tsushima and Sapporo populations are evidently larger than the latter.

In the present study, the genetic relationships among 11 populations of *Hyla japonica* distributed in Japan, Sakhalin, Kunashiri and Korea, the Amami population of *H. hallowelli* and the Taiwan population of *H. chinensis* were biochemically examined by electrophoresis in order to clarify the intraspecific differentiation of *H. japonica* as well as the interspecific relationships of the three *Hyla* species.

A preliminary report has been published by NISHIOKA and SUMIDA (1984).

MATERIALS AND METHODS

In the years from 1982 to 1988, 261 mature males and females of three species of the genus *Hyla* were collected from Japan and surrounding countries (Fig. 1). Of these species, *Hyla japonica* included 11 populations, the Kunashiri of the Kuril Islands and the Sakhalin, two populations of Hokkaido, the Sapporo and Setana, six populations of Honshu and an adjacent island, the Hirosaki, Ichinoseki, Odawara, Maibara, Hiroshima and Tsushima, and the Suwon population of Korea. The populations of the other two species were the Amami of *Hyla hallowelli* and the Taiwan of *Hyla chinensis.* The number of frogs and sex ratio in each of these populations are shown in Table 1.

Thirteen enzymes extracted from skeletal muscles, one enzyme extracted from livers and four kinds of blood proteins were analyzed by the method of horizontal starch-gel electrophoresis. The names of enzymes and blood proteins analyzed and their abbreviations, E. C. Nos. and the kinds of samples and buffer systems used in the electrophoresis are shown in Table 2. The method of electrophoresis has been described in detail by NISHIOKA, OHTANI and SUMIDA (1980). The detection of each enzyme was performed by the agar overlay method of BREWER (1970) and HARRIS and HOPKINSON (1976) with a slight modification. The

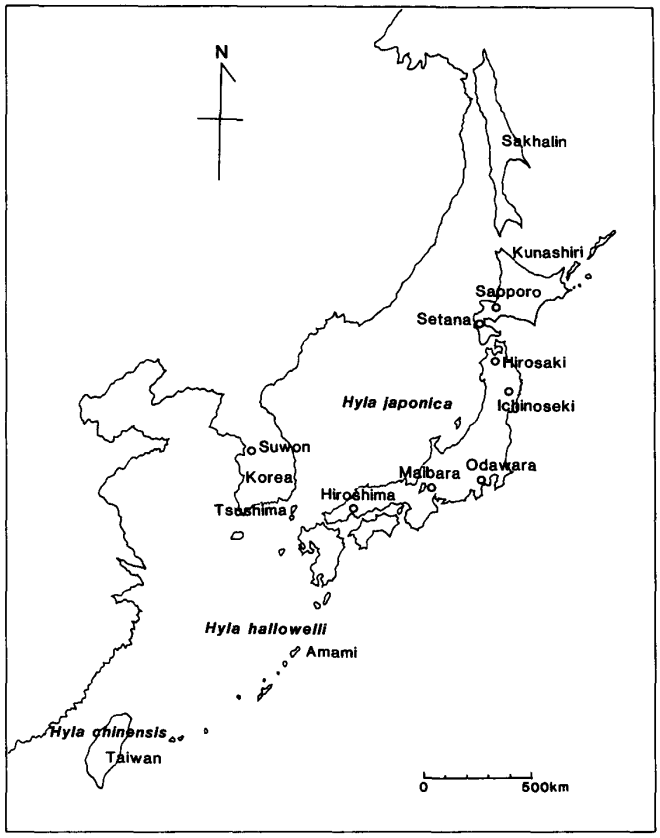

Fig. 1. Map showing localities of 13 populations used in this study.

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

Species	Locality	Collector	No. of frogs		
			Total	Male	Female
Hyla japonica	Sakhalin	Borkin	10	5	5
〃	Kunashiri	〃	2	2	0
〃	Sapporo	Katagiri	90	83	7
〃	Setana	Goto	30	24	6
〃	Hirosaki	Saitoh	13	5	8
〃	Ichinoseki	Sumida	3	2	1
〃	Odawara	〃	5	3	2
〃	Maibara	〃	25	18	7
〃	Hiroshima	〃	58	50	8
〃	Tsushima	〃	6	2	4
〃	Korea	Kuramoto	3	3	0
Hyla hallowelli	Amami	Suzuki	3	3	0
Hyla chinensis	Taiwan	Kuramoto	13	4	9
Total			261	204	57

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	〃	〃
Mannose phosphate isomerase	MPI	5.3.1.8	〃	T-C pH 7.0
Peptidase	Pep	3.4.3.1	Liver	T-B-E pH 8.0
6-Phosphogluconate dehydrogenase	6-PGD	1.1.1.44	Skeletal muscle	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	〃
Serum protein-C	Prot-C	—	〃	〃
Serum protein-E	Prot-E	—	〃	〃
Hemoglobin	Hb	—	Erythrocyte	T-B-E pH 8.6

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

detection of blood proteins was made with amido-black staining.

A locus of multiple alleles was considered to be polymorphic, when each of the alleles existed in a frequency of more than 1% at this locus. The genetic variations of local populations were shown by the average heterozygosity and the proportion of polymorphic loci (LEWONTIN, 1974). The genetic distances among local populations were estimated by NEI's method (1975). A dendrogram for the 13 populations of the three *Hyla* species was drawn by the unweighted pair-group arithmetic average (UPGMA) clustering method (SNEATH and SOKAL, 1973; NEI, 1975) on the basis of the genetic distance (D).

OBSERVATION

I. Electrophoretic patterns and multiple alleles

Electrophoretic patterns of 14 enzymes and four blood proteins were examined in 261 frogs of the 13 populations belonging to the three *Hyla* species. It was found that these enzymes and blood proteins were controlled by genes at 26 loci. At the Prot-E locus, a phenotype was produced by a single allele, *a*. At the four loci of AAT-A, AK, CK and MDH-B, there were two or three phenotypes produced by two alleles, *a* and *b*. At the five loci of GPI, LDH-A, PGM, SOD-A and Pep-C, three to six phenotypes produced by three alleles, *a*~*c*, were

TABLE 3
Number of phenotypes and alleles at 26 loci in 13 populations of *Hyla japonica*, *H. hallowelli* and *H. chinensis*

Locus	No. of phenotypes	No. of alleles
AAT-A	2	2
AAT-B	6	4
ADA	15	6
AK	3	2
CK	3	2
α-GDH	8	6
GPI	3	3
IDH-A	7	5
IDH-B	4	4
LDH-A	4	3
LDH-B	6	5
MDH-A	4	4
MDH-B	3	2
MPI	15	7
Pep-A	5	4
Pep-B	7	4
Pep-C	3	3
Pep-D	10	7
6-PGD	7	4
PGM	6	3
SOD-A	5	3
SOD-B	5	4
Ab	17	9
Prot-C	5	4
Prot-E	1	1
Hb	4	4
Average	6.1	4.0

observed. At the nine loci of AAT-B, IDH-B, MDH-A, Pep-A, Pep-B, 6-PGD, SOD-B, Prot-C and Hb, four to seven phenotypes produced by four alleles, *a*~*d*, were observed. At the two loci of LDH-B and IDH-A, there were six or seven phenotypes produced by five alleles, *a*~*e*, while at the two loci of ADA and α-GDH, there were eight or 15 phenotypes produced by six alleles, *a*~*f*. At the two loci of Pep-D and MPI, there were 10 or 15 phenotypes produced by seven alleles, *a*~*g*, while at the Ab locus, there were 17 phenotypes produced by nine alleles, *a*~*i* (Table 3; Fig. 2).

The alleles at these 26 loci were one to nine, 4.0 on the average, in number. The phenotypes were one to 17, 6.1 on the average, in number (Table 3).

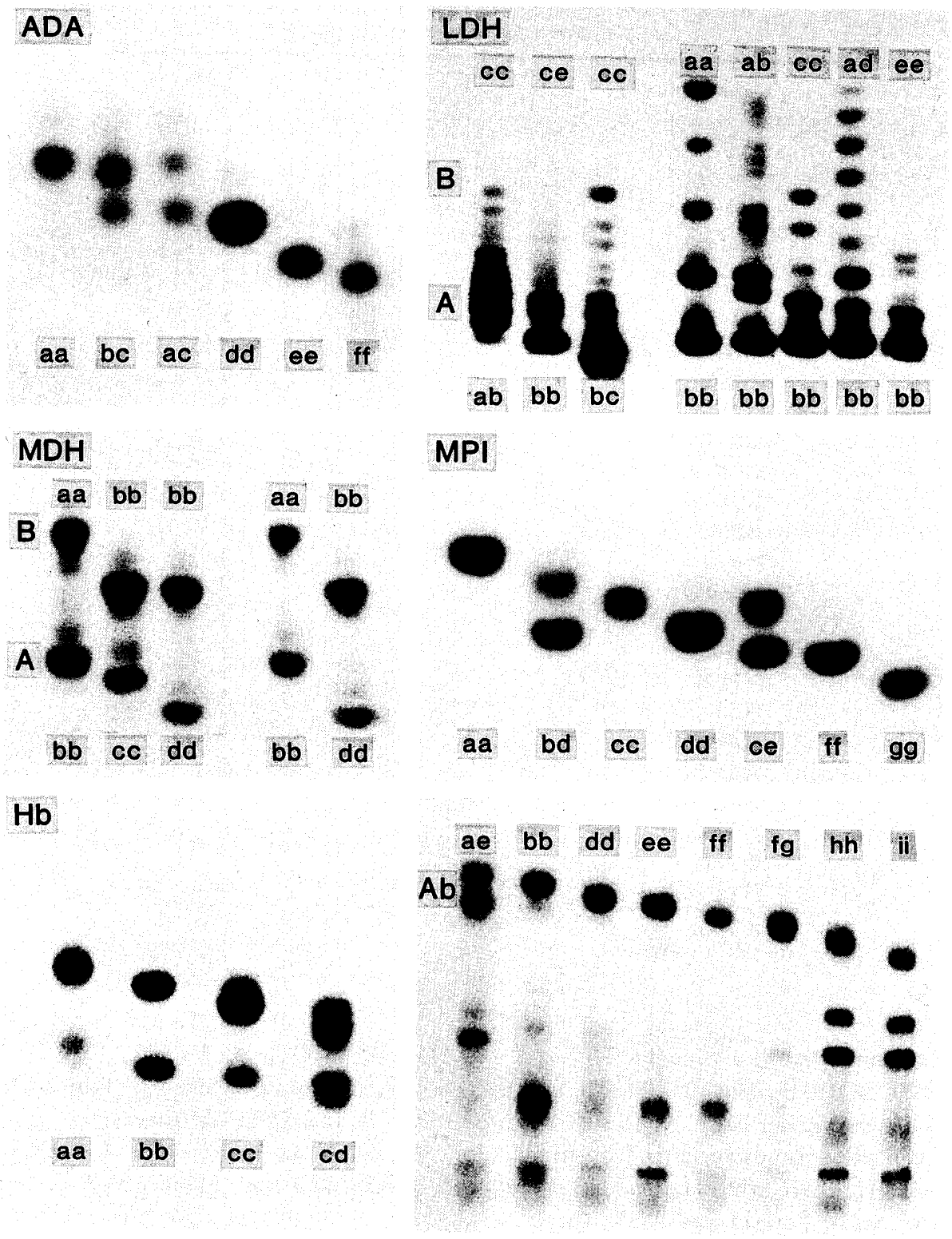

Fig. 2. Electrophoretic patterns of four enzymes, ADA, LDH, MDH and MPI, and two blood proteins, Hb and Ab, in 13 populations of *Hyla japonica*, *H. hallowelli* and *H. chinensis*.

TABLE 4

Gene frequencies at 25 loci in 13 populations of *Hyla japonica*, *H. hallowelli* and *H. chinensis*

Species		*H. japonica*											*H. hallowelli*	*H. chinensis*
Locality		Sakhalin	Kunashiri	Sapporo	Setana	Hirosaki	Ichinoseki	Odawara	Maibara	Hiroshima	Tsushima	Korea	Amami	Taiwan
Sample size		10	2	90	30	13	3	5	25	58	6	3	3	13
Locus	Allele													
1) AAT-A	*a*				0.083	0.115				0.017				
	b	1.000	1.000	1.000	0.917	0.885	1.000	1.000	1.000	0.983	1.000	1.000	1.000	1.000
2) AAT-B	*a*													0.038
	b								0.040		0.083		0.167	0.962
	c												0.833	
	d	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.960	1.000	0.917	1.000		
3) ADA	*a*									0.086	0.333	1.000		
	b										0.167			
	c							0.100	0.020	0.784	0.417		0.167	
	d			0.011	0.250	0.192		0.100		0.121	0.083			0.885
	e	0.350	0.500	0.583	0.333	0.538	0.833		0.520	0.009			0.833	
	f	0.650	0.500	0.406	0.417	0.269	0.167	0.800	0.460					0.115
4) AK	*a*	1.000	1.000	1.000	1.000	0.923	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
	b					0.077								1.000
5) CK	*a*			0.011	0.150	0.500	0.167	0.200	0.280	0.129	0.083	0.500	1.000	1.000
	b	1.000	1.000	0.989	0.850	0.500	0.833	0.800	0.720	0.871	0.917	0.500		
6) α-GDH	*a*	0.050		0.017	0.100	0.192	0.333	0.500	0.160	0.560	1.000	0.833		
	b												1.000	
	c	0.950	1.000	0.983	0.900	0.808	0.667	0.500	0.620	0.440				
	d											0.167		
	e													1.000
	f								0.220					
7) GPI	*a*				0.083									0.231
	b	1.000	1.000	1.000	0.917	1.000	0.833	1.000	1.000	1.000	1.000	1.000	1.000	0.769
	c						0.167							
8) IDH-A	*a*	1.000	1.000	0.861	1.000	1.000	1.000	1.000	1.000	0.991	1.000	1.000		
	b													0.577
	c			0.139						0.009				
	d												1.000	
	e													0.423
9) IDH-B	*a*									0.043				
	b													0.038
	c	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.957	1.000	1.000	1.000	0.885
	d													0.077

Continued-2

Species		*H. japonica*											*H. hallowelli*	*H. chinensis*
Locality		Sakhalin	Kunashiri	Sapporo	Setana	Hirosaki	Ichinoseki	Odawara	Maibara	Hiroshima	Tsushima	Korea	Amami	Taiwan
Sample size		10	2	90	30	13	3	5	25	58	6	3	3	13
Locus	Allele													
10) LDH-A	*a*								0.020	0.017				
	b	1.000	1.000	0.967	0.750	0.846	1.000	1.000	0.980	0.983	1.000	1.000	1.000	1.000
	c			0.033	0.250	0.154								
11) LDH-B	*a*												0.833	0.846
	b													0.154
	c	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.983	0.333	0.167		
	d												0.167	
	e									0.017	0.667	0.833		
12) MDH-A	*a*				0.017									
	b	1.000	1.000	1.000	0.983	1.000	1.000	1.000	1.000	1.000	1.000	1.000		
	c													1.000
	d												1.000	
13) MDH-B	*a*	1.000	1.000	1.000	1.000	0.923	1.000	0.900	1.000	1.000	1.000	1.000		
	b					0.077		0.100					1.000	1.000
14) MPI	*a*													0.269
	b										0.167			
	c												1.000	0.615
	d				0.017	0.077	0.167	0.400	0.160	0.543	0.167			
	e				0.033	0.462	0.167							0.115
	f	1.000	1.000	1.000	0.933	0.308	0.667	0.600	0.640	0.388	0.667	1.000		
	g				0.017	0.154			0.200	0.069				
15) Pep-A	*a*												1.000	0.846
	b													0.154
	c	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.940	0.917	0.833		
	d									0.060	0.083	0.167		
16) Pep-B	*a*													0.154
	b					0.154				0.086				
	c	1.000	1.000	1.000	1.000	0.846	1.000	1.000	0.840	0.914	1.000	1.000	0.667	0.615
	d								0.160				0.333	0.231
17) Pep-C	*a*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		
	b													1.000
	c												1.000	

Continued-3

Species		*H. japonica*											*H. hallowelli*	*H. chinensis*
Locality		Sakha-lin	Kuna-shiri	Sappo-ro	Setana	Hiro-saki	Ichinoseki	Odawara	Maibara	Hiro-shima	Tsushima	Korea	Amami	Taiwan
Sample size		10	2	90	30	13	3	5	25	58	6	3	3	13
Locus	Allele													
18) Pep-D	*a*									0.060				
	b	1.000	1.000	0.694	1.000	1.000	1.000	0.600	0.620	0.647	0.583	0.167		
	c			0.306				0.300	0.380	0.026	0.417	0.667		
	d												1.000	0.731
	e											0.167		
	f							0.100		0.267				
	g													0.269
19) 6-PGD	*a*				0.050		0.167	0.100	0.240	0.069	0.083	0.167		
	b	1.000	1.000	1.000	0.950	1.000	0.833	0.900	0.740	0.871	0.917	0.833		
	c								0.020	0.060				
	d												1.000	1.000
20) PGM	*a*	1.000	0.250	0.983	0.867	0.731	1.000	0.800	0.760	1.000	1.000	0.833		
	b		0.750	0.011	0.033	0.269		0.200	0.240			0.167	1.000	0.962
	c			0.006	0.100									0.038
21) SOD-A	*a*									0.060				
	b													0.385
	c	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.940	1.000	1.000	1.000	0.615
22) SOD-B	*a*	0.050	0.250	0.394	0.233	0.346	0.167	0.600	0.840	0.129	0.083			
	b	0.950	0.750	0.606	0.767	0.654	0.833	0.400	0.160	0.871	0.917	1.000		
	c													1.000
	d												1.000	
23) Ab	*a*									0.018				
	b	0.625	1.000	0.625	0.759	0.833	0.833	0.167	0.280	0.281	0.500			0.071
	c	0.375		0.375		0.167	0.167	0.833	0.560	0.219	0.333			
	d													0.929
	e				0.224					0.149				
	f				0.017				0.160	0.333	0.167		0.167	
	g												0.833	
	h											0.333		
	i											0.667		
24) Prot-C	*a*								0.280	0.132				
	b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.720	0.868	1.000	1.000		
	c												1.000	
	d													1.000
25) Hb	*a*												1.000	
	b													1.000
	c	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.982	0.917	1.000		
	d									0.018	0.083			

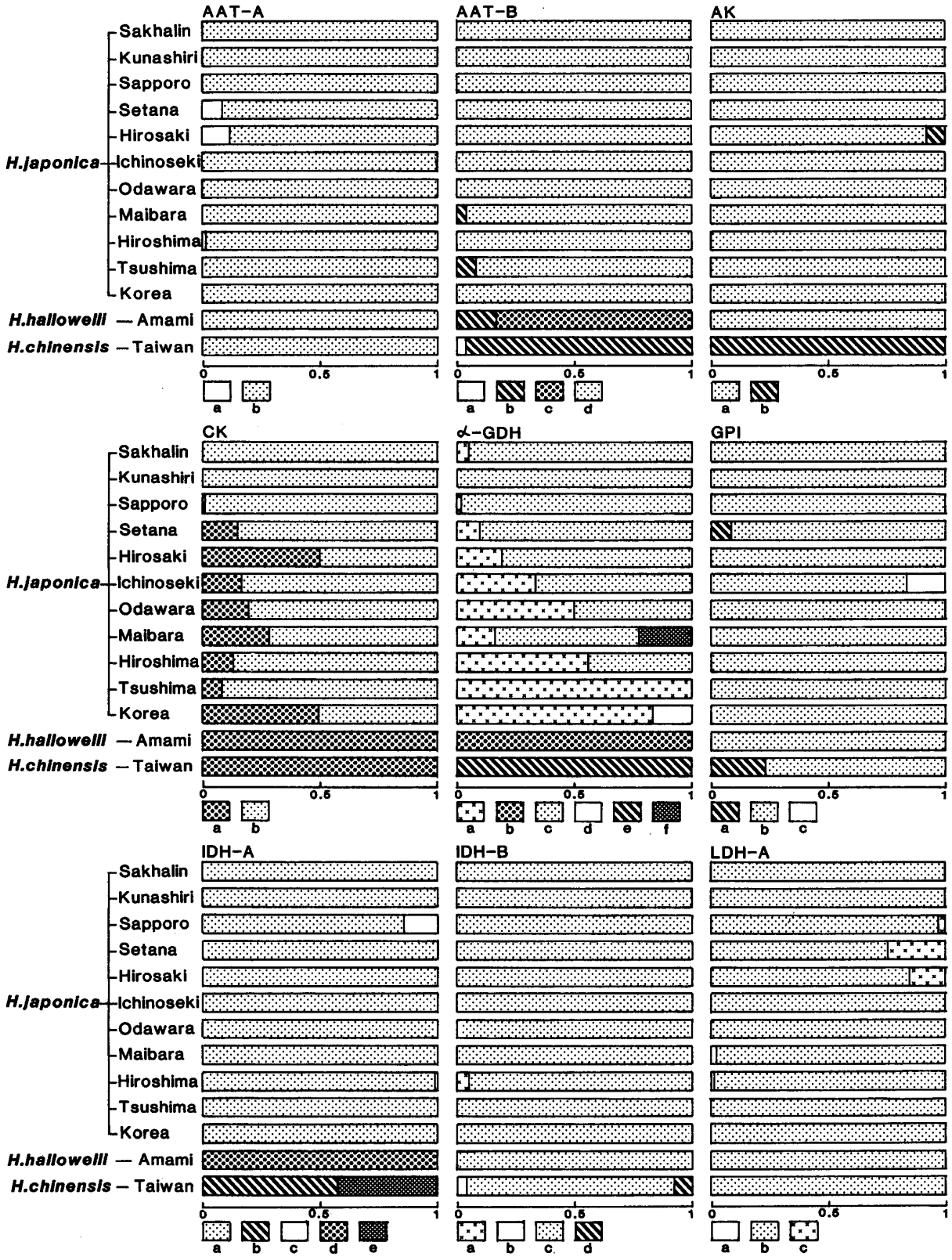

Fig. 3. Gene frequencies at nine loci, AAT-A, AAT-B, AK, CK, α-GDH, GPI, IDH-A, IDH-B and LDH-A, in 13 populations of *Hyla japonica, H. hallowelli* and *H. chinensis*.

II. Gene frequency

1. AAT-A locus

At the AAT-A locus in the 13 populations of the three *Hyla* species distributed in the Far East, two phenotypes, BB and AB, produced by two alleles, *a* and *b*, were observed. All the 160 frogs belonging to the eight populations of *H. japonica* other than the populations of Setana, Hirosaki and Hiroshima, and the two populations of *H. hallowelli* and *H. chinensis* showed a homozygous BB band produced by allele *b*. Of the 30 frogs of the Setana, 13 frogs of the Hirosaki and 58 frogs of the Hiroshima population of *H. japonica*, 25, 10 and 56, respectively, also showed a homozygous BB band produced by allele *b*, while the remaining five, three and two frogs, respectively, showed a heterozygous AB band. In these three populations, allele *b* was 0.917, 0.885 and 0.983 in frequency, respectively, while allele *a* was only 0.083, 0.115 and 0.017, respectively (Table 4; Fig. 3).

2. AAT-B locus

At the AAT-B locus in the 13 populations of the three *Hyla* species, six phenotypes, BB, CC, DD, AB, BC and BD, produced by four alleles, *a*~*d*, were observed. All the 214 frogs belonging to the nine populations of *H. japonica* other than the Maibara and Tsushima populations showed a homozygous DD band produced by allele *d*. Of the 25 frogs of the Maibara population and the six frogs of the Tsushima population, 23 and five, respectively, also showed a homozygous DD band, while the remaining two and one, respectively, showed a heterozygous BD band. In these two populations, allele *d* was 0.960 and 0.917 in frequency, respectively, while allele *b* was 0.040 and 0.083, respectively. In the Amami population of *H. hallowelli*, two of the three frogs showed a homozygous CC band, while the other showed a heterozygous BC band. Alleles *c* and *b* were 0.833 and 0.167 in frequency, respectively. In the Taiwan population of *H. chinensis*, 12 of the 13 frogs showed a homozygous BB band, while the remainder showed a heterozygous AB band. Alleles *b* and *a* were 0.962 and 0.038 in frequency, respectively.

In the 11 populations of *H. japonica*, allele *d* was overwhelmingly abundant, and allele *b* was slightly found only in the Maibara and Tsushima populations. In the two populations of *H. hallowelli* and *H. chinensis*, alleles *c* and *b*, respectively, were overwhelmingly abundant, while alleles *b* and *a*, respectively, were slightly found (Table 4; Fig. 3).

3. ADA locus

At the ADA locus in the 13 populations of the three *Hyla* species, 15 phenotypes, AA, CC, DD, EE, FF, AC, AD, BC, BD, CD, CE, CF, DE, DF and EF, produced by six alleles, *a*~*f*, were found.

In *H. japonica*, one, four and five of the 10 frogs of the Sakhalin population showed EE, FF and EF bands, respectively. One and one of the two frogs of the

Kunashiri population showed EE and FF bands, respectively. In the Sapporo population, 32, 17, 2 and 39 of the 90 frogs showed EE, FF, DE and EF bands, respectively. In the Setana population, three, two and six of the 30 frogs showed homozygous DD, EE and FF bands, respectively, while the other six, three and 10 showed heterozygous DE, DF and EF bands, respectively. In the Hirosaki population, three and one of the 13 frogs showed homozygous EE and FF bands, respectively, while the other four, one and four showed heterozygous DE, DF and EF bands, respectively. Of the three frogs of the Ichinoseki population, two and one showed EE and EF bands, respectively. Of the five frogs of the Odawara population, three, one and one showed FF, CF and DF bands, respectively. Of the 25 frogs of the Maibara population, seven, five, one and 12 showed EE, FF, CF and EF bands, respectively. In the Hiroshima population, one and 35 of the 58 frogs showed homozygous AA and CC bands, respectively, while the other seven, one, 13 and one showed heterozygous AC, AD, CD and CE bands, respectively. In the Tsushima population, one and one of the six frogs showed homozygous AA and CC bands, respectively, while the other two, one and one showed heterozygous AC, BC and BD bands, respectively. All the three frogs of the Korean population showed an AA band.

Of the three frogs of the Amami population of *H. hallowelli,* two and one showed EE and CE bands, respectively. In *H. chinensis,* 10 and three of the 13 frogs of the Taiwan population showed DD and DF bands, respectively.

In the seven northern populations of *H. japonica* of Sakhalin, Kunashiri, Sapporo, Setana, Hirosaki, Ichinoseki and Maibara, their gene accumulation was mostly occupied by alleles *e* and *f*. Allele *e* was 0.350~0.833 in frequency, while allele *f* was 0.167~0.650. In addition to these two alleles, allele *d* was 0.011 ~0.250 in frequency in the three populations of Sapporo, Setana and Hirosaki, and allele *c* was 0.020 in the Maibara population. In the Odawara population, allele *f* was high in frequency, being 0.800, and in addition there were alleles *c* and *d,* each of which was 0.100 in frequency. In the Hiroshima population, allele *c* was high in frequency, being 0.784, and in addition there were alleles *a, d* and *e* in the frequencies of 0.009~0.121. In the Tsushima population, alleles *a* and *c* were 0.333 and 0.417 in frequency, respectively, and in addition alleles *b* and *d* were 0.167 and 0.083, respectively. In the Korea population, there was only allele *a*. In the Amami population of *H. hallowelli,* allele *e* was high in frequency, being 0.833, and in addition there was allele *c* in frequency of 0.167. In the Taiwan population of *H. chinensis,* allele *d* was high in frequency, being 0.885, and in addition allele *f* was found in frequency of 0.115 (Table 4; Fig. 5).

4. AK locus

At the AK locus in the 13 populations of the three *Hyla* species, three phenotypes, AA, BB and AB, produced by two alleles, *a* and *b,* were observed. All the 235 frogs belonging to the 11 populations including the 10 populations of *H. japonica* other than the Hirosaki and the Amami population of *H. hallowelli* showed only a homozygous AA band produced by allele *a*. In the Hirosaki

population, 11 and two of the 13 frogs showed AA and AB bands, respectively. In this population, alleles *a* and *b* were 0.923 and 0.077 in frequency, respectively. In the Taiwan population of *H. chinensis*, all the 13 frogs showed a homozygous BB band produced by allele *b* (Table 4; Fig. 3).

5. CK locus

At the CK locus in the 13 populations of the three *Hyla* species, three phenotypes, AA, BB and AB, produced by two alleles, *a* and *b*, were observed. In the Sakhalin and Kunashiri populations of *H. japonica*, all the 12 frogs showed only a BB band produced by allele *b*. In the four populations of Sapporo, Ichinoseki, Odawara and Tsushima, 88 of the 90 frogs, two of the three frogs, three of the five frogs and five of the six frogs, respectively, showed a homozygous BB band, and the other two, one, two and one showed a heterozygous AB band. In the five populations of Setana, Hirosaki, Maibara, Hiroshima and Korea, one of the 30 frogs, four of the 13 frogs, two of the 25 frogs, one of the 58 frogs and one of the three frogs, respectively, showed a homozygous AA band, 22, four, 13, 44 and one, respectively, showed a homozygous BB band, and the remaining seven, five, 10, 13 and one, respectively, showed a heterozygous AB band.

In the nine populations of *H. japonica* other than the populations of Hirosaki and Korea, allele *b* was overwhelmingly high in frequency, being 0.720~1.000. In the two populations of Hirosaki and Korea, each of alleles *a* and *b* was 0.500 in frequency. All the 16 frogs belonging to the two populations of *H. hallowelli* and *H. chinensis* showed a homozygous AA band produced by allele *a* (Table 4; Fig. 3).

6. α-GDH locus

At the α-GDH locus in the 13 populations of the three *Hyla* species, eight phenotypes, AA, BB, CC, EE, AC, AD, AF and CF, produced by six alleles, *a*~*f*, were observed. In the three populations of Sakhalin, Sapporo and Ichinoseki of *H. japonica*, nine of the 10 frogs, 87 of the 90 frogs and one of the three frogs, respectively, showed a homozygous CC band, while the remaining one, three and two showed a heterozygous AC band. In the four populations of Setana, Hirosaki, Odawara and Hiroshima, one of the 30 frogs, one of the 13 frogs, one of the five frogs and 17 of the 58 frogs showed a homozygous AA band, 25, nine, one and 10 showed a homozygous CC band, and the remaining four, three, three and 31 showed a heterozygous AC band.

In the five populations of Sakhalin, Sapporo, Setana, Hirosaki and Ichinoseki, allele *c* was high in frequency, being 0.667~0.983, and allele *a* was 0.017~0.333. The two frogs of the Kunashiri population had only allele *c*. In the Odawara population, each of alleles *a* and *c* was 0.500 in frequency, while in the Hiroshima population, alleles *a* and *c* were 0.560 and 0.440, respectively. In the Maibara population, seven, seven, one and 10 of the 25 frogs showed CC, AC, AF and CF bands, respectively. In this population, alleles *c*, *f* and *a* were 0.620, 0.220 and 0.160 in frequency, respectively. All the six frogs in the Tsushima population showed a homozygous AA band produced by allele *a*. Of the three frogs of

Korea, two and one showed AA and AD bands, respectively. Alleles *a* and *d* were 0.833 and 0.167 in frequency, respectively. All the three frogs of *H. hallowelli* showed a homozygous BB band produced by allele *b*, while the 13 frogs of *H. chinensis* showed a homozygous EE band produced by allele *e* (Table 4; Fig. 3).

7. GPI locus

At the GPI locus in the 13 populations of the three *Hyla* species, three phenotypes, BB, AB and BC, produced by three alleles, *a*, *b* and *c*, were observed. In the nine populations of *H. japonica* other than the Setana and Ichinoseki populations and the Amami population of *H. hallowelli*, all the 215 frogs showed a homozygous BB band produced by allele *b*. In the Setana population of *H. japonica* and the Taiwan population of *H. chinensis*, 25 of the 30 frogs and seven of the 13 frogs, respectively, showed a homozygous BB band, and the other five and six, respectively, showed a heterozygous AB band. In these two populations, allele *b* was very high in frequency, being 0.917 and 0.769, respectively, while allele *a* was 0.083 and 0.231, respectively. In the Ichinoseki population of *H. japonica*, two and one of the three frogs showed BB and BC bands, respectively. In this population, alleles *b* and *c* were 0.833 and 0.167 in frequency, respectively (Table 4; Fig. 3).

8. IDH-A locus

At the IDH-A locus in the 13 populations of the three *Hyla* species, seven phenotypes, AA, BB, CC, DD, EE, AC and BE, produced by five alleles, *a*~*e*, were observed. In the nine populations of *H. japonica* other than the populations of Sapporo and Hiroshima, all the 97 frogs showed a homozygous AA band produced by allele *a*. In the Sapporo population, 68 and three of the 90 frogs showed homozygous AA and CC bands, respectively, and the other 19 showed a heterozygous AC band. Alleles *a* and *c* were 0.861 and 0.139 in frequency, respectively. In the Hiroshima population, 57 of the 58 frogs showed a homozygous AA band, while the remainder showed a heterozygous AC band. Alleles *a* and *c* were 0.991 and 0.009 in frequency, respectively. All the three frogs of the Amami population of *H. hallowelli* showed a homozygous DD band produced by allele *d*. Of the 13 frogs of the Taiwan population of *H. chinensis*, six, four and three showed BB, EE and BE bands, respectively. Alleles *b* and *e* were 0.577 and 0.423 in frequency, respectively (Table 4; Fig. 3).

9. IDH-B locus

At the IDH-B locus in the 13 populations of the three *Hyla* species, four phenotypes, CC, AC, BC and CD, produced by four alleles, *a*~*d*, were observed. All the 190 frogs belonging to the 10 populations of *H. japonica* other than the Hiroshima and the Amami population of *H. hallowelli* showed a homozygous CC band produced by allele *c*, while in the Hiroshima population, 53 and five of the 58 frogs showed CC and AC bands, respectively. In this population, alleles *c* and *a* were 0.957 and 0.043 in frequency, respectively. In the Taiwan population of *H.*

chinensis, 10, one and two of the 13 frogs showed CC, BC and CD bands, respectively. In this population, alleles *c, b* and *d* were 0.885, 0.038 and 0.077 in frequency, respectively (Table 4; Fig. 3).

10. LDH-A locus

At the LDH-A locus in the 13 populations of the three *Hyla* species, four phenotypes, BB, CC, AB and BC, produced by three alleles, *a, b* and *c,* were observed. In the eight populations including the Sakhalin, Kunashiri, Ichinoseki, Odawara, Tsushima and Korea populations of *H. japonica,* the Amami population of *H. hallowelli* and the Taiwan population of *H. chinensis,* all the 45 frogs showed a homozygous BB band produced by allele *b*. In the Sapporo population of *H. japonica,* 84 and six of the 90 frogs showed BB and BC bands, respectively. In the Setana and Hirosaki populations of the same species, 19 of the 30 frogs and 10 of the 13 frogs, respectively, showed a homozygous BB band, four and one, respectively, showed a homozygous CC band, and the remaining seven and two, respectively, showed a heterozygous BC band. In these three populations, allele *b* was overwhelmingly high in frequency, being 0.750~0.967, while allele *c* was 0.033~0.250. In the Maibara and Hiroshima populations, 24 of the 25 frogs and 56 of the 58 frogs, respectively, showed a homozygous BB band and the remaining one and two, respectively, showed a heterozygous AB band. In these populations, allele *b* was overwhelmingly high in frequency, being 0.980 and 0.983, respectively, while allele *a* was 0.020 and 0.017, respectively (Table 4; Fig. 3).

11. LDH-B locus

At the LDH-B locus in the 13 populations of the three *Hyla* species, six phenotypes, AA, CC, EE, AB, AD and CE, produced by five alleles, *a~e,* were observed. In the eight populations of *H. japonica* other than the Hiroshima, Tsushima and Korea populations, all the 178 frogs showed a homozygous CC band produced by allele *c*. In the Hiroshima population, 56 and two of the 58 frogs showed CC and CE bands, respectively. In this population, alleles *c* and *e* were 0.983 and 0.017 in frequency, respectively. In the Tsushima population, one, three and two of the six frogs showed CC, EE and CE bands, respectively. In the Korea population, two and one of the three frogs showed EE and CE bands, respectively. In these two populations, allele *e* was 0.667 and 0.833 in frequency, while allele *c* was 0.333 and 0.167. In the Amami population of *H. hallowelli,* two and one of the three frogs showed AA and AD bands, respectively. Alleles *a* and *d* were 0.833 and 0.167 in frequency, respectively. In the Taiwan population of *H. chinensis,* nine and four of the 13 frogs showed AA and AB bands, respectively. Alleles *a* and *b* were 0.846 and 0.154 in frequency, respectively (Table 4; Fig. 4).

12. MDH-A locus

At the MDH-A locus in the 13 populations of the three *Hyla* species, four phenotypes, BB, CC, DD and AB, produced by four alleles, *a~d,* were observed. In the 10 populations of *H. japonica* other than the Setana, all the 215 frogs showed

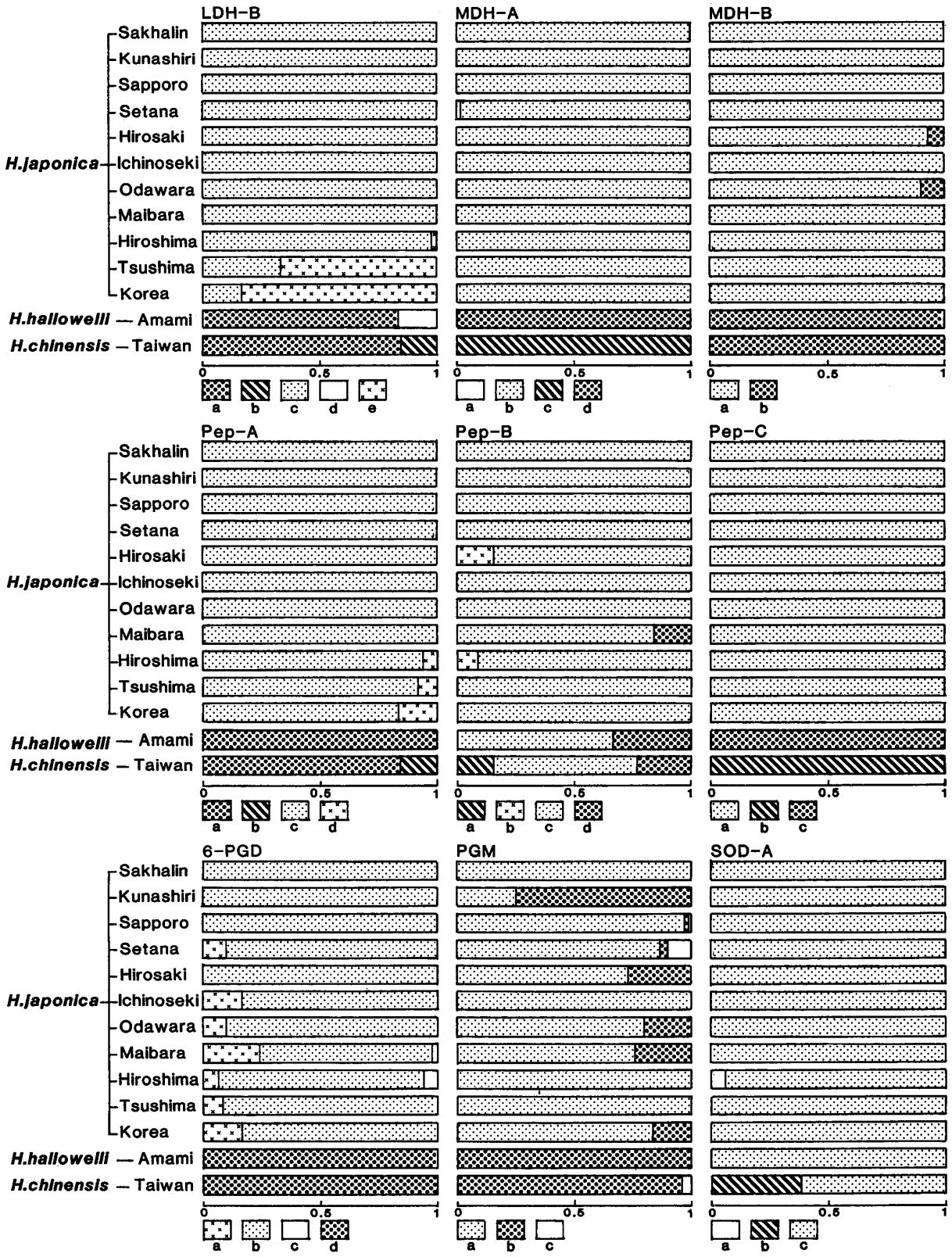

Fig. 4. Gene frequencies at nine loci, LDH-B, MDH-A, MDH-B, Pep-A, Pep-B, Pep-C, 6-PGD, PGM and SOD-A, in 13 populations of *Hyla japonica, H. hallowelli* and *H. chinensis*.

a homozygous BB band produced by allele *b*. In the Setana population, 29 and one of the 30 frogs showed BB and AB bands, respectively. Alleles *b* and *a* were 0.983 and 0.017 in frequency, respectively. In the Amami population of *H. hallowelli*, all the three frogs showed a homozygous DD band produced by allele *d*, while all the 13 frogs in the Taiwan population of *H. chinensis* showed a homozygous CC band produced by allele *c* (Table 4; Fig. 4).

13. MDH-B locus

At the MDH-B locus in the 13 populations of the three *Hyla* species, three phenotypes, AA, BB and AB, produced by two alleles, *a* and *b*, were observed. All the 227 frogs of the nine populations of *H. japonica* other than the Hirosaki and Odawara showed a homozygous AA band produced by allele *a*. In the Hirosaki and Odawara populations, 11 of the 13 frogs and four of the five frogs, respectively, showed a homozygous AA band, while the other two and one showed a heterozygous AB band. In these populations, allele *a* was 0.923 and 0.900 in frequency, while allele *b* was 0.077 and 0.100. All the 16 frogs of the Amami population of *H. hallowelli* and the Taiwan population of *H. chinensis* showed a homozygous BB band produced by allele *b* (Table 4; Fig. 4).

14. MPI locus

At the MPI locus in the 13 populations of the three *Hyla* species, 15 phenotypes, AA, CC, DD, EE, FF, GG, AC, BD, CE, DE, DF, DG, EF, EG and FG, produced by seven alleles, *a*~*g*, were observed. In the *H. japonica*, all the 105 frogs of the four populations of Sakhalin, Kunashiri, Sapporo and Korea showed a homozygous FF band produced by allele *f*. In the Setana population, 26 of the 30 frogs showed a homozygous FF band produced by allele *f*. One, two and one of the other four frogs showed heterozygous DF, EF and FG bands, respectively. While allele *f* was overwhelmingly high in frequency, being 0.933, alleles *d*, *e* and *g* were 0.017, 0.033 and 0.017, respectively. In the Hirosaki population, three of the 13 frogs showed a homozygous EE band and one, one, four, one and three of the other 10 frogs showed heterozygous DE, DF, EF, EG and FG bands, respectively. In this population, alleles *e*, *f*, *g* and *d* were 0.462, 0.308, 0.154 and 0.077 in frequency, respectively. In the Ichinoseki population, one, one and one of the three frogs showed FF, DF and EF bands, respectively. Alleles *d*, *e* and *f* were 0.167, 0.167 and 0.667 in frequency, respectively. In the Odawara population, one, two and two of the five frogs showed DD, FF and DF bands, respectively. Alleles *d* and *f* were 0.400 and 0.600 in frequency, respectively. In the Maibara population, two, 12 and two of the 25 frogs showed homozygous DD, FF and GG bands, respectively, while three, one and five of the other nine frogs showed heterozygous DF, DG and FG bands, respectively. In this population, alleles *f*, *g* and *d* were 0.640, 0.200 and 0.160 in frequency, respectively. In the Hiroshima population, 19, nine and one of the 58 frogs showed homozygous DD, FF and GG bands, respectively, while 23, two and four of the other 29 frogs showed heterozygous DF, DG and FG bands, respectively. In this population, alleles *d*, *f* and *g*

were 0.543, 0.388 and 0.069 in frequency, respectively. In the Tsushima population, four and two of the six frogs showed FF and BD bands, respectively. Alleles *f*, *b* and *d* were 0.667, 0.167 and 0.167 in frequency, respectively. All the three frogs of the Amami population of *H. hallowelli* showed a homozygous CC band produced by allele *c*. In the Taiwan population of *H. chinensis*, two, five, three and three of the 13 frogs showed AA, CC, AC and CE bands, respectively. In this population, alleles *c*, *a* and *e* were 0.615, 0.269 and 0.115 in frequency, respectively (Table 4; Fig. 5).

In the four populations of *H. japonica* of Sakhalin, Kunashiri, Sapporo and Korea, there was only allele *f*. In the five populations of Setana, Ichinoseki, Odawara, Maibara and Tsushima, allele *f* was abundant in frequency, and in the Hirosaki population, allele *e* was abundant and allele *f* was fairly abundant. In the Hiroshima population, allele *d* was abundant and allele *f* was fairly abundant. In *H. hallowelli* and *H. chinensis*, allele *c* was abundant, while alleles *a* and *e* were additionally found in *H. chinensis*.

15. Pep-A locus

At the Pep-A locus in the 13 populations of the three *Hyla* species, five phenotypes, AA, BB, CC, AB and CD, produced by four alleles, *a*~*d*, were observed. All the 178 frogs of the eight populations of *H. japonica* showed a homozygous CC band produced by allele *c*. In the three populations of Hiroshima, Tsushima and Korea, 51 of the 58 frogs, five of the six frogs and two of the three frogs, respectively, showed a homozygous CC band, while seven, one and one of the other nine frogs showed a heterozygous CD band. In these three populations, allele *c* was overwhelmingly high in frequency, being 0.833~0.940, while allele *d* was 0.060~0.167. All the three frogs of the Amami population of *H. hallowelli* showed a homozygous AA band produced by allele *a*. In the Taiwan population of *H. chinensis*, 10 and one of the 13 frogs showed AA and BB bands, respectively, while the remaining two showed a heterozygous AB band. In this population, alleles *a* and *b* were 0.846 and 0.154 in frequency, respectively (Table 4; Fig. 4).

16. Pep-B locus

At the Pep-B locus in the 13 populations of the three *Hyla* species, seven phenotypes, BB, CC, DD, AC, AD, BC and CD, produced by four alleles, *a*~*d*, were observed. All the 149 frogs of the eight populations of *H. japonica* other than the Hirosaki, Maibara and Hiroshima populations showed a homozygous CC band produced by allele *c*. In the Hirosaki and Hiroshima populations, one and 10 of the 13 frogs and one and 49 of the 58 frogs, respectively, showed homozygous BB and CC bands, respectively. The remaining two and eight frogs, respectively, showed a heterozygous BC band. In the Maibara population, 17 and eight of the 25 frogs showed CC and CD bands, respectively. In these three populations, allele *c* was overwhelmingly high in frequency, being 0.840~0.914, allele *b* was found in the frequencies of 0.154 and 0.086 in the Hirosaki and Hiroshima populations, respectively, and allele *d* was found in the frequency of 0.160 in the

Maibara population.

In the Amami population of *H. hallowelli,* one and two of the three frogs showed CC and CD bands, respectively. Alleles *c* and *d* were 0.667 and 0.333 in frequency, respectively. In the Taiwan population of *H. chinensis,* five and one of the 13 frogs showed homozygous CC and DD bands, respectively, while three, one and three of the other seven frogs showed heterozygous AC, AD and CD bands, respectively. Alleles *c, d* and *a* were 0.615, 0.231 and 0.154 in frequency, respectively (Table 4; Fig. 4).

17. Pep–C locus

At the Pep–C locus in the 13 populations of the three *Hyla* species, three phenotypes, AA, BB and CC, produced by three alleles, *a, b* and *c,* were observed. All the 245 frogs of the 11 populations of *H. japonica* showed a homozygous AA band produced by allele *a.* All the three frogs of the Amami population of *H. hallowelli* showed a homozygous CC band produced by allele *c,* while all the 13 frogs of the Taiwan population of *H. chinensis* showed a homozygous BB band produced by allele *b* (Table 4; Fig. 4).

18. Pep–D locus

At the Pep–D locus in the 13 populations of the three *Hyla* species, 10 phenotypes, BB, CC, DD, FF, AB, AF, BC, BF, CE and DG, produced by seven alleles, *a*~*g,* were observed. In *H. japonica,* all the 58 frogs of the five populations of Sakhalin, Kunashiri, Setana, Hirosaki and Ichinoseki showed a homozygous BB band produced by allele *b.* In three other populations of Sapporo, Maibara and Tsushima, 45 of the 90 frogs, 10 of the 25 frogs and two of the six frogs, respectively, showed a homozygous BB band, 10, four and one frogs, respectively, showed a homozygous CC band, and the remaining 35, 11 and three frogs, respectively, showed a heterozygous BC band. In the Odawara population, two, one, one and one of the five frogs showed BB, CC, BC and BF bands, respectively. In the Hiroshima population, 24, five, five, two, three and 19 of the 58 frogs showed BB, FF, AB, AF, BC and BF bands, respectively. In the latter five populations, allele *b* was high in frequency, being 0.583~0.694, and allele *c* was 0.026~0.417. In addition, there was allele *f* in the frequency of 0.100 in the Odawara population, and alleles *f* and *a* were found in the frequency of 0.267 and 0.060, respectively, in the Hiroshima population. In the Korea population of the same species, each of the three frogs showed CC, BC or CE band. In this population, alleles *c, b* and *e* were 0.667, 0.167 and 0.167 in frequency, respectively.

All the three frogs of the Amami population of *H. hallowelli* showed a homozygous DD band produced by allele *d.* In the Taiwan population of *H. chinensis,* six of the 13 frogs showed a homozygous DD band and the other seven showed a heterozygous DG band. Alleles *d* and *g* were 0.731 and 0.269 in frequency, respectively (Table 4; Fig. 5).

19. 6-PGD locus

At the 6-PGD locus in the 13 populations of the three *Hyla* species, seven phenotypes, AA, BB, CC, DD, AB, AC and BC, produced by four alleles, *a*~*d*, were observed. In the four populations of Sakhalin, Kunashiri, Sapporo and Hirosaki of *H. japonica*, all the 115 frogs showed a homozygous BB band produced by allele *b*. In the Setana of the other seven populations, one and 28 of the 30 frogs showed homozygous AA and BB bands, respectively, while the remainder showed a heterozygous AB band. In the Ichinoseki, Odawara, Tsushima and Korea populations, two of the three frogs, four of the five frogs, five of the six frogs and two of the three frogs, respectively, showed a homozygous BB band, while the remaining one frog in each population showed a heterozygous AB band. In the Maibara population, two and 14 of the 25 frogs showed homozygous AA and BB bands, respectively, and the other eight and one showed heterozygous AB and BC bands, respectively. In the Hiroshima population, 45 and one of the 58 frogs showed homozygous BB and CC bands, respectively, while seven, one and four of the remaining 12 frogs showed heterozygous AB, AC and BC bands, respectively. In these seven populations, allele *b* was very high in frequency, being 0.740~0.950, and there was allele *a* in the frequencies of 0.050~0.240. In the Maibara and Hiroshima populations, there was allele *c* in the frequencies of 0.020 and 0.060, respectively, in addition to alleles *a* and *b*.

In the two populations of *H. hallowelli* and *H. chinensis*, all the 16 frogs showed a homozygous DD band produced by allele *d* (Table 4; Fig. 4).

20. PGM locus

At the PGM locus in the 13 populations of the three *Hyla* species, six phenotypes, AA, BB, CC, AB, AC and BC, produced by three alleles, *a*, *b* and *c*, were observed. In the four populations of Sakhalin, Ichinoseki, Hiroshima and Tsushima, of the 11 populations of *H. japonica*, all the 77 frogs showed a homozygous AA band produced by allele *a*. In the Hirosaki and Maibara populations, eight of the 13 frogs and 14 of the 25 frogs, respectively, showed a homozygous AA band, and two and one, respectively, showed a homozygous BB band. The remaining three and 10 frogs, respectively, in the two populations showed a heterozygous AB band. In the Odawara and Korea populations, three of the five frogs and two of the three frogs, respectively, showed a homozygous AA band, while the other two and one, respectively, showed a heterozygous AB band. In the Sapporo population, 87 of the 90 frogs showed a homozygous AA band, and two and one of the other frogs showed heterozygous AB and AC bands, respectively. In the Setana population, 23 and one of the 30 frogs showed homozygous AA and CC bands, respectively, while two and four of the remaining six frogs showed heterozygous AB and AC bands, respectively. In the latter six populations, allele *a* was overwhelmingly high in frequency, being 0.731~0.983, and allele *b* was 0.011~0.269. There was allele *c* in the frequencies of 0.006 and 0.100 in the Sapporo and Setana populations, respectively, in addition to alleles *a* and *b*. In

the Kunashiri population, one of the two frogs showed a homozygous BB band, while the other showed a heterozygous AB band. Alleles *b* and *a* were 0.750 and 0.250 in frequency, respectively.

All the three frogs in the Amami population of *H. hallowelli* showed a homozygous BB band produced by allele *b*. In the Taiwan population of *H. chinensis*, 12 of the 13 frogs showed a homozygous BB band, while the remainder showed a heterozygous BC band. Alleles *b* and *c* were 0.962 and 0.038 in frequency, respectively (Table 4; Fig. 4).

21. SOD-A locus

At the SOD-A locus in the 13 populations of the three *Hyla* species, five phenotypes, AA, BB, CC, AC and BC, produced by three alleles, *a*, *b* and *c*, were observed. In the 10 populations of *H. japonica* other than the Hiroshima population and in the Amami population of *H. hallowelli*, all the 190 frogs showed a homozygous CC band produced by allele *c*. In the Hiroshima population, one and 52 of the 58 frogs showed homozygous AA and CC bands, respectively, and the remaining five showed a heterozygous AC band. Alleles *c* and *a* were 0.940 and 0.060 in frequency, respectively. In the Taiwan population of *H. chinensis*, two, five and six of the 13 frogs showed BB, CC and BC bands, respectively. Alleles *c* and *b* were 0.615 and 0.385 in frequency, respectively (Table 4; Fig. 4).

22. SOD-B locus

At the SOD-B locus in the 13 populations of the three *Hyla* species, five phenotypes, AA, BB, CC, DD and AB, produced by four alleles, *a*~*d*, were observed. In the five populations of Sakhalin, Kunashiri, Ichinoseki, Hiroshima and Tsushima of *H. japonica*, nine of the 10 frogs, one of the two frogs, two of the three frogs, 43 of the 58 frogs and five of the six frogs, respectively, showed a homozygous BB band, while the other one, one, one, 15 and one, respectively, showed a heterozygous AB band. In four other populations of Sapporo, Setana, Hirosaki and Odawara, 13 of the 90 frogs, one of the 30 frogs, one of the 13 frogs and two of the five frogs showed a homozygous AA band, 32, 17, five and one, respectively, showed a homozygous BB band, and the remaining 45, 12, seven and two, respectively, showed a heterozygous AB band. In the Maibara population, 17 and eight of the 25 frogs showed AA and AB bands, respectively. In the eight populations of *H. japonica* other than the Odawara and Maibara populations, allele *b* was very high in frequency, being 0.606~0.950, while there was allele *a* in the frequencies of 0.050~0.394. In the Odawara and Maibara populations, allele *a* was high in frequency, being 0.600 and 0.840, respectively, while allele *b* was 0.400 and 0.160, respectively. All the three frogs of the remaining Korea population showed a homozygous BB band produced by allele *b*. All the three frogs of the Amami population of *H. hallowelli* showed a homozygous DD band produced by allele *d*. All the 13 frogs of the Taiwan population of *H. chinensis* showed a homozygous CC band produced by allele *c* (Table 4; Fig. 5).

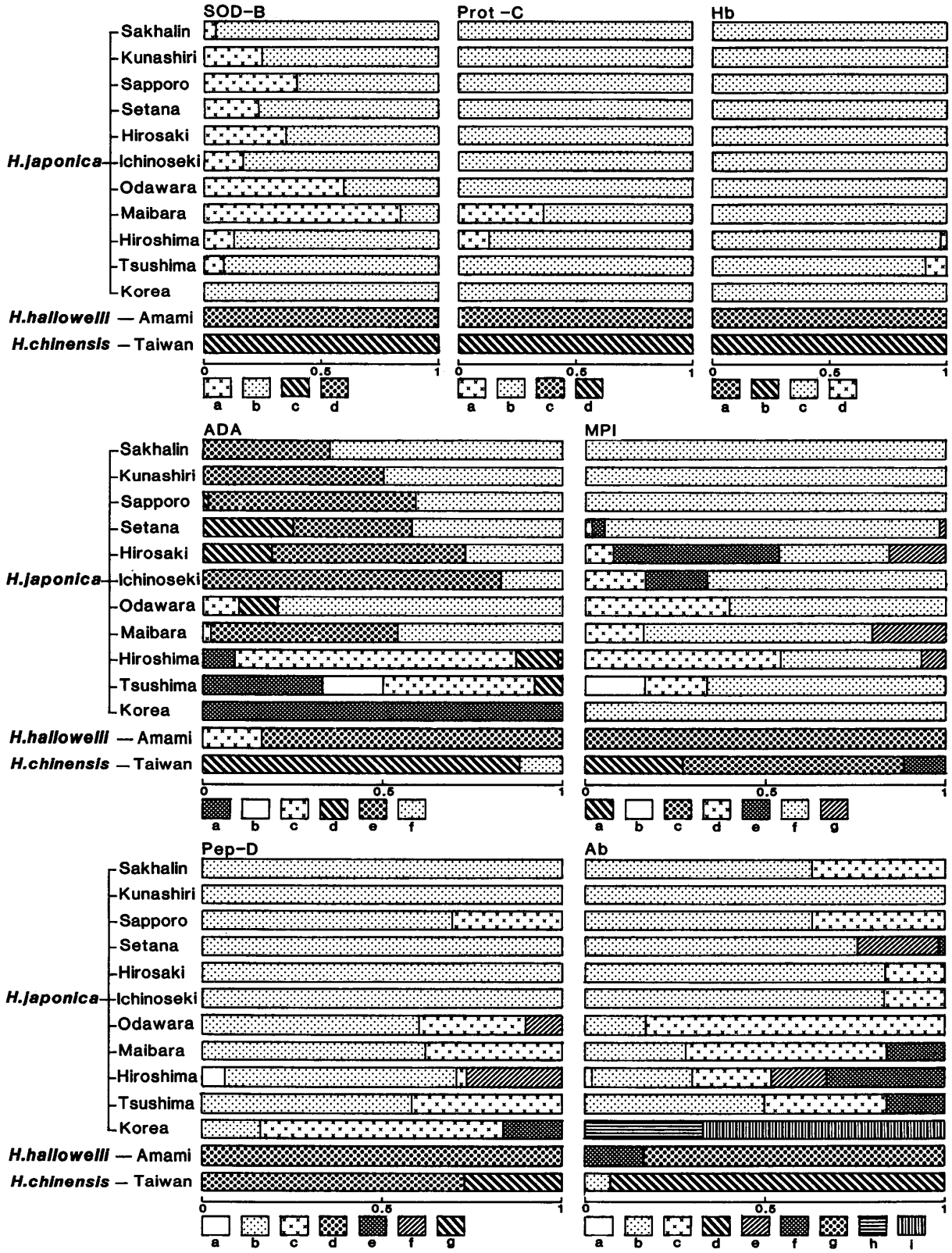

Fig. 5. Gene frequencies at seven loci, SOD-B, Prot-C, Hb, ADA, MPI, Pep-D and Ab, in 13 populations of *Hyla japonica*, *H. hallowelli* and *H. chinensis*.

The 10 populations of *H. japonica* other than the Korea population contained alleles *a* and *b*. While allele *a* was abundant in the Odawara and Maibara populations, allele *b* was abundant in the other eight populations. The Korea population contained only allele *b*. The Amami population of *H. hallowelli* contained only allele *d*, while the Taiwan population of *H. chinensis* contained only allele *c*.

23. Ab locus

At the Ab locus in the 13 populations of the three *Hyla* species, 17 phenotypes, BB, CC, DD, EE, FF, GG, HH, II, AE, BC, BD, BE, BF, CE, CF, EF and FG, produced by nine alleles, *a*~*i*, were observed. In the 11 populations of *H. japonica*, three of the eight frogs of the Sakhalin population and 35 of the 88 frogs of the Sapporo population showed a homozygous BB band, one and 13 others, respectively, showed a homozygous CC band, and the remaining four and 40, respectively, showed a heterozygous BC band. In the Setana population, 18, two, eight and one of the 29 frogs showed BB, EE, BE and EF bands, respectively. In the Hirosaki and Ichinoseki populations, six of the nine frogs and two of the three frogs showed a homozygous BB band, while the other three and one showed a heterozygous BC band. In the Maibara population, two and 10 of the 25 frogs showed homozygous BB and CC bands, respectively, while five, five and three of the remaining 13 frogs showed heterozygous BC, BF and CF bands, respectively. In the Hiroshima population, six, four, one, and eight of the 57 frogs showed homozygous BB, CC, EE and FF bands, respectively, while two, four, five, 11, five, eight and three of the remaining 38 frogs showed heterozygous AE, BC, BE, BF, CE, CF and EF bands, respectively. In the Tsushima population, one and one of the six frogs showed homozygous BB and CC bands, respectively, while two and two of the remaining four frogs showed heterozygous BC and BF bands, respectively. In the Korea population, one and two of the three frogs showed homozygous HH and II bands, respectively. The two frogs of the Kunashiri population showed a homozygous BB band.

In the seven populations of *H. japonica* other than the four populations of Odawara, Maibara, Hiroshima and Korea, allele *b* was high in frequency, being 0.500~1.000. In the five populations other than the Kunashiri, Setana and the foregoing four populations, allele *c* was secondarily high in frequency, being 0.167~0.375. The Kunashiri population had only allele *b*. The Setana population had alleles *e* and *f* in the frequencies of 0.224 and 0.017 in addition to allele *b*. The Tsushima population had allele *f* in the frequency of 0.167 in addition to alleles *b* and *c*. In the Odawara and Maibara populations, allele *c* was high in frequency, being 0.833 and 0.560, respectively, while allele *b* was 0.167 and 0.280, respectively. Allele *f* was also found in the Maibara population in the frequency of 0.160. In the Hiroshima population, there were alleles *f*, *b*, *c*, *e* and *a* in the frequencies of 0.333, 0.281, 0.219, 0.149 and 0.018, respectively. In the Korea population, alleles *i* and *h* were 0.667 and 0.333, respectively.

In the Amami population of *H. hallowelli*, two and one of the three frogs showed

GG and FG bands, respectively. Alleles *g* and *f* were 0.833 and 0.167, respectively. In the Taiwan population of *H. chinensis,* six and one of the seven frogs showed DD and BD bands, respectively. Alleles *d* and *b* were 0.929 and 0.071, respectively (Table 4; Fig. 5).

24. Prot-C locus

At the Prot-C locus in the 13 populations of the three *Hyla* species, five phenotypes, AA, BB, CC, DD and AB, produced by four alleles, *a*~*d,* were observed. In the nine populations of *H. japonica* other than the Maibara and Hiroshima populations, all the 151 frogs showed a homozygous BB band produced by allele *b*. In the Maibara and Hiroshima populations, three of the 25 frogs and three of the 57 frogs, respectively, showed a homozygous AA band, 14 and 45, respectively, showed a homozygous BB band, and the remaining eight and nine, respectively, showed a heterozygous AB band. In these two populations, allele *b* was 0.720 and 0.868, respectively, while allele *a* was 0.280 and 0.132, respectively.

In the Amami population of *H. hallowelli,* all the three frogs showed a homozygous CC band produced by allele *c,* while in the Taiwan population of *H. chinensis,* all the seven frogs showed a homozygous DD band produced by allele *d* (Table 4; Fig. 5).

25. Prot-E locus

At the Prot-E locus in the 13 populations of the three *Hyla* species, all the 243 frogs showed a homozygous AA band produced by allele *a*.

26. Hb locus

At the Hb locus in the 13 populations of the three *Hyla* species, four phenotypes, AA, BB, CC and CD, produced by four alleles, *a*~*d,* were observed. In the nine populations of *H. japonica* other than the Hiroshima and Tsushima populations, all the 170 frogs showed a homozygous CC band produced by allele *c*. In the Hiroshima and Tsushima populations, 55 of the 57 frogs and five of the six frogs, respectively, showed a homozygous CC band produced by allele *c,* while the remaining two and one, respectively, showed a heterozygous CD band. In these two populations, allele *c* was 0.982 and 0.917, respectively, while allele *d* was 0.018 and 0.083, respectively.

All the three frogs of *H. hallowelli* showed a homozygous AA band produced by allele *a*, while all the seven frogs of *H. chinensis* showed a homozygous BB band produced by allele *b* (Table 4; Fig. 5).

III. Genetic differentiation

1. Fixation index (Fst)

At the 26 loci analyzed in 261 frogs belonging to the 13 populations of the three *Hyla* species, the fixation indexes (Fst) were calculated according to WRIGHT (1978). When the gene frequencies at a definite locus are the same in all the 13

populations, the fixation index is zero, while this is 1.000 when there is a population-specific allele in one or more populations.

a. Fixation indexes in the 13 populations of the three *Hyla* species

At the Pep–C locus, *H. japonica, H. chinensis* and *H. hallowelli* had only alleles *a, b* and *c,* respectively. Thus, this locus was 1.000 in fixation index, and showed that it was completely differentiated in each species. When the values in fixation index were arranged from higher to lower, four loci of MDH–A, Hb, AK and MDH–B in the 13 populations were 0.991~0.911, and showed that the genetic differentiation was nearly complete in these loci. Five loci of Prot–C, AAT–B, IDH–A, Pep–A and LDH–B were 0.850~0.772, and somewhat lower in genetic differentiation. Six loci of 6–PGD, α–GDH, PGM, SOD–B, Pep–D and CK

TABLE 5
Fixation index at 26 loci in 13 populations of *Hyla japonica, H. hallowelli* and *H. chinensis*

Locus	Fixation index (Fst)	
	13 populations (*jap. hal. chi.*)	11 populations (*jap.*)
AAT–A	0.080	0.077
AAT–B	0.844	0.058
ADA	0.453	0.401
AK	0.928	0.070
CK	0.522	0.195
α–GDH	0.646	0.470
GPI	0.159	0.124
IDH–A	0.805	0.119
IDH–B	0.069	0.039
LDH–A	0.157	0.152
LDH–B	0.772	0.711
MDH–A	0.991	0.015
MDH–B	0.911	0.075
MPI	0.474	0.275
Pep–A	0.796	0.098
Pep–B	0.200	0.117
Pep–C	1.000	0
Pep–D	0.532	0.320
6–PGD	0.669	0.084
PGM	0.618	0.335
SOD–A	0.321	0.055
SOD–B	0.567	0.289
Ab	0.471	0.344
Prot–C	0.850	0.203
Prot–E	0	0
Hb	0.949	0.063

were 0.669~0.522, four loci of MPI, Ab, ADA and SOD-A were 0.474~0.321, and three loci of Pep-B, GPI and LDH-A were 0.200~0.157. Two loci of AAT-A and IDH-B were 0.080 and 0.069, respectively, and showed that the genetical differentiation was very slight. The Prot-E locus was zero in fixation index and indicated that this locus was not genetically differentiated (Table 5).

b. Fixation indexes in the 11 populations of *H. japonica*

When fixation indexes were calculated in the 11 populations of *H. japonica,* it was found that these populations were most differentiated at the LDH-B locus, being 0.711. The second largest value in fixation index was 0.470 at the α-GDH locus. Six loci of ADA, Ab, PGM, Pep-D, SOD-B and MPI were 0.401~0.275, six loci of Prot-C, CK, LDH-A, GPI, IDH-A and Pep-B were 0.203~0.117, eight loci of Pep-A, 6-PGD, AAT-A, MDH-B, AK, Hb, AAT-B and SOD-B were 0.098~0.055, and two loci of IDH-B and MDH-A were 0.039 and 0.015, respectively, which were extremely slight in genetic differentiation. Two loci of Pep-C and Prot-E were zero in fixation index and indicated that these loci had not genetically differentiated (Table 5).

2. Proportion of heterozygous loci

In each of the 13 populations of the three *Hyla* species, the mean proportion of heterozygous loci per individual was estimated at 26 analyzed loci. It was found that the lowest were 3.8% and 4.4% in the Kunashiri and Sakhalin populations of

TABLE 6

Genetic variabilities at 26 loci in 13 populations of *Hyla japonica, H. hallowelli* and *H. chinensis*

Species	Locality	Sample size	Mean proportion of heterozygous loci per individual (%)	Mean proportion of polymorphic loci per population (%)	Mean number of alleles per locus
Hyla japonica	Sakhalin	10	4.4(4.3)	15.4	1.15
〃	Kunashiri	2	3.8(4.8)	11.5	1.12
〃	Sapporo	90	8.3(8.7)	34.6	1.42
〃	Setana	30	10.3(11.5)	46.2	1.65
〃	Hirosaki	13	15.8(16.2)	46.2	1.58
〃	Ichinoseki	3	12.8(10.0)	30.8	1.35
〃	Odawara	5	14.8(13.9)	38.5	1.46
〃	Maibara	25	18.5(18.7)	50.0	1.69
〃	Hiroshima	58	15.4(16.1)	69.2	2.04
〃	Tsushima	6	13.5(14.0)	42.3	1.58
〃	Korea	3	10.3(10.9)	30.8	1.31
H. hallowelli	Amami	3	7.7(6.0)	19.2	1.19
H. chinensis	Taiwan	13	15.9(15.4)	50.0	1.62
Average (Total)		20.1 (261)	11.7(11.6)	37.3	1.47

Parentheses show expected values.

H. japonica, respectively. The mean proportions of heterozygous loci were 7.7 ~10.3% in four populations, including the Amami population of *H. hallowelli* and the Sapporo, Setana and Korea populations of *H. japonica.* The Ichinoseki and Tsushima populations of the latter species were 12.8% and 13.5%, respectively. Four populations including the Odawara, Hiroshima and Hirosaki populations of *H. japonica* and the Taiwan population of *H. chinensis* were 14.8~15.9%. The highest was 18.5% in the Maibara population of *H. japonica.* The mean proportions of heterozygous loci in the 13 populations were 11.7% on the average. When the mean proportion in each population was compared with the expected value, there was no large difference between the actual and expected values in most of the 13 populations, although there were differences of 2.8% and 1.7% between the two values in the Ichinoseki population of *H. japonica* and the Amami population of *H. hallowelli,* respectively (Table 6).

3. Proportion of polymorphic loci

The proportion of polymorphic loci containing multiple alleles at the rate of more than 1% was estimated in each of the 26 loci. The results showed that the Kunashiri population of *H. japonica* was the lowest in this proportion, being 11.5%. The Sakhalin population of the same species was 15.4% and the Amami population of *H. hallowelli* was 19.2%. Five populations of Korea, Ichinoseki, Sapporo, Odawara and Tsushima of *H. japonica* were 30.8%, 30.8%, 34.6%, 38.5% and 42.3%, respectively, and four populations including the Setana and Hirosaki populations of *H. japonica,* the Taiwan population of *H. chinensis* and the Maibara population of *H. japonica* were 46.2%, 46.2%, 50.0% and 50.0%, respectively. The highest was 69.2% in the Hiroshima population. The mean proportions of polymorphic loci in the 13 populations were 37.3% on the average (Table 6).

4. Mean number of alleles per locus

Mean number of alleles at the 26 loci was counted in the 13 populations of the three *Hyla* species. It was found that the smallest number was 1.12 in the Kunashiri population of *H. japonica.* In the Sakhalin population of the same species and the Amami population of *H. hallowelli,* the mean numbers of alleles were 1.15 and 1.19, respectively. They were 1.31, 1.35, 1.42 and 1.46 in the Korea, Ichinoseki, Sapporo and Odawara populations of *H. japonica,* respectively. In the Tsushima and Hirosaki populations of *H. japonica,* the Taiwan population of *H. chinensis* and the Setana and Maibara populations of *H. japonica,* they were 1.58, 1.58, 1.62, 1.65 and 1.69, respectively. The highest number was 2.04 in the Hiroshima population of *H. japonica.* The mean numbers of alleles in the 13 populations were 1.47 on the average (Table 6).

IV. Genetic distance and dendrogram

1. Genetic distance

The genetic distances among the 13 populations of the three *Hyla* species

TABLE 7

Genetic distance among 13 populations of *Hyla japonica, H. hallowelli* and *H. chinensis*

Species	Locality	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
H. japonica	Sakhalin (1)	—											
〃	Kunashiri (2)	0.031	—										
〃	Sapporo (3)	0.012	0.034	—									
〃	Setana (4)	0.013	0.028	0.017	—								
〃	Hirosaki (5)	0.042	0.043	0.041	0.027	—							
〃	Ichinoseki (6)	0.022	0.041	0.023	0.020	0.024	—						
〃	Odawara (7)	0.050	0.083	0.045	0.062	0.066	0.066	—					
〃	Maibara (8)	0.058	0.071	0.035	0.058	0.054	0.055	0.028	—				
〃	Hiroshima (9)	0.064	0.102	0.070	0.063	0.072	0.056	0.057	0.078	—			
〃	Tsushima (10)	0.091	0.132	0.095	0.095	0.111	0.080	0.081	0.112	0.050	—		
〃	Korea (11)	0.167	0.201	0.163	0.167	0.184	0.160	0.151	0.173	0.137	0.059	—	
H. hallowelli	Amami (12)	1.131	1.024	1.085	1.123	0.997	1.051	1.050	0.974	1.086	1.090	1.049	—
H. chinensis	Taiwan (13)	1.360	1.243	1.342	1.318	1.177	1.326	1.217	1.211	1.299	1.282	1.238	0.596

were estimated on the basis of gene frequencies at the 26 loci controlling enzymes and blood proteins by the method of NEI (1975). It was found that those among the 11 populations of *H. japonica* were 0.012~0.201. While the genetic distances among the six northeastern populations of Sakhalin, Kunashiri, Sapporo, Setana, Hirosaki and Ichinoseki were 0.012~0.043, those among the four southwestern populations of Odawara, Maibara, Hiroshima and Tsushima were 0.028~0.112. The genetic distances between the six northeastern and the four southwestern populations were 0.035~0.132. The genetic distances between the Tsushima and the other 10 populations of *H. japonica* were 0.050~0.132, while those between the Korea and nine other populations excluding the Tsushima population were 0.137~0.201. The genetic distance between the Tsushima and Korea populations was 0.059.

The genetic distances between the Amami population of *H. hallowelli* and the 11 populations of *H. japonica* were 0.974~1.131, while those between the Taiwan population of *H. chinensis* and the 11 populations of *H. japonica* were 1.177~1.360. The genetic distance between the Amami and Taiwan populations was 0.596 (Table 7).

2. Dendrogram

For the 13 populations of the three *Hyla* species, seven dendrograms were drawn on the basis of genetic distances by seven different methods. As there were no remarkable differences among these dendrograms, the phylogenetic relationship of the 13 populations of the three *Hyla* species was evaluated by drawing a dendrogram by the UPGMA method (SNEATH and SOKAL, 1973; NEI, 1975) which is most commonly utilized.

As shown in Fig. 6, it is found that *H. japonica* was first divided from the others which later became two species, *H. hallowelli* and *H. chinensis*. On the other hand, the Korea population first diverged from *H. japonica*, and then the remaining

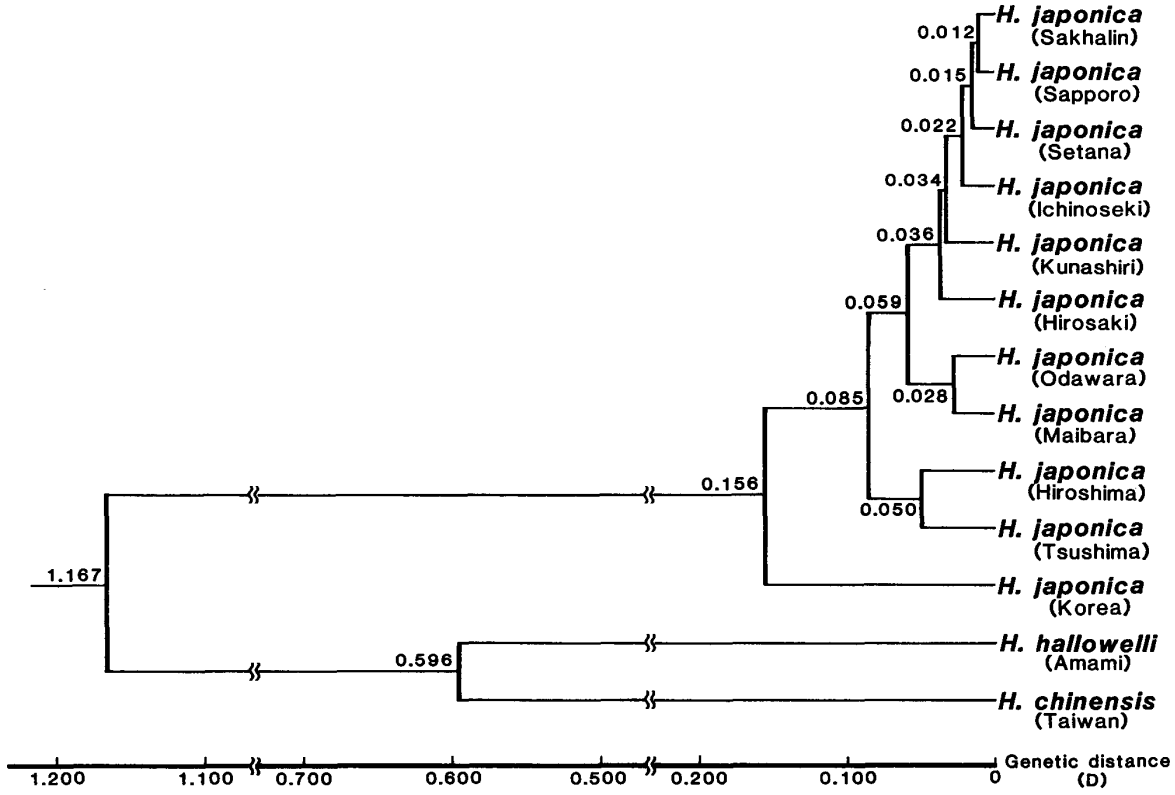

Fig. 6. Dendrogram for 13 populations of *Hyla japonica*, *H. hallowelli* and *H. chinensis* based on genetic distances.

populations were roughly divided into three groups. The first group contained the Hiroshima and Tsushima populations, the second group contained the Odawara and Maibara populations and the third group contained the Hirosaki, Kunashiri, Ichinoseki, Setana, Sapporo and Sakhalin populations.

DISCUSSION

The claim that *Hyla arborea japonica* distributed widely in Japan and the adjacent territories is a valid species was first made by DAITO (1968) in our laboratory who confirmed that the male and female hybrids between a male *H. arborea sarda* from Corsica, France and two female *H. arborea japonica* from Hiroshima, Japan were almost completely sterile. Soon thereafter, KAWAMURA, NISHIOKA and UEDA (1972, 1990) reported similar crosses, using a male *H. arborea arborea* from Luxembourg and female *H. arborea japonica* from Hiroshima, Tsushima and Korea, and confirmed that all the hybrids were completely sterile (KAWAMURA and NISHIOKA, 1977). KURAMOTO (1984) also found that the ovaries of female hybrids between female *H. arborea japonica* and male *H. arborea sarda* from Corsica and *H. arborea arborea* from Austria and Italy were abnormal, suggesting that fertility of adult hybrids would be very low. All the crossing experiments performed by DAITO, KAWAMURA *et al.* and KURAMOTO showed that *H. arborea japonica* is a valid species and should be called *Hyla japonica*.

The existence of reproductively isolating mechanisms among the Hiroshima, Tsushima and Korea populations of *H. japonica* was examined by KAWAMURA and NISHIOKA (1977) and KAWAMURA, NISHIOKA and UEDA (1972, 1990). According to them, reciprocal hybrids between the Hiroshima and Tsushima populations, between the Hiroshima and Korea populations and between the Tsushima and Korea populations in *Hyla japonica* were all nearly normal in fertilization, while they were fairly inferior to the parental populations in rate of metamorphosis. While almost all the mature females of each kind of hybrids had abundant normal eggs, the mature males had testes which were somewhat abnormal in inner structure and contained some degenerating germ cells in addition to normal spermatozoa. When the male and female hybrids were mated with females and males of the parental populations, the backcrosses were generally inferior to the controls in developmental capacity, in addition to a slight morphological difference among the three populations. On the other hand, KURAMOTO (1984) reported that the hybrids between female *H. japonica* and male *H. hallowelli* or *H. chinensis* were abnormal in ovarian structure at metamorphosis.

The present study clarified the genetic relationships among 11 populations of *Hyla japonica,* a population of *H. hallowelli* and a population of *H. chinensis* by electrophoretic analyses of enzymes and blood proteins. The genetic distances among the 11 populations of *H. japonica* collected from Sakhalin, Kunashiri of the Kuril Islands, Sapporo and Setana of Hokkaido, Hirosaki, Ichinoseki, Odawara, Maibara and Hiroshima of Honshu, Tsushima Isl. and Korea were 0.012~0.201. Those between the Tsushima and the other 10 populations of *H. japonica* were 0.050~0.132, while those between the Korea and nine other populations excluding the Tsushima were 0.137~0.201. On the other hand, the genetic distances between *H. hallowelli* from Amami and the 11 populations of *H. japonica* were 0.974~1.131, and those between *H. chinensis* from Taiwan and the 11 populations of *H. japonica* were 1.177~1.360.

A comparison between genetic distance values and taxonomic ranks was made by HEDGECOCK and AYALA (1974) and KALEZIĆ and HEDGECOCK (1979) in American and European urodeles, respectively. HEDGECOCK and AYALA calculated genetic distances among three populations of *Taricha rivularis,* two populations of *T. granulosa,* three populations of *T. torosa torosa* and a population of *T. t. sierrae.* According to them, the genetic distances between populations, between subspecies and between species were 0.029 ± 0.010, 0.145 ± 0.027 and 0.466 ± 0.021, respectively. HEDGECOCK (1976) additionally reported that the genetic distance between two subspecies of *Taricha torosa, T. t. torosa* and *T. t. sierrae,* was 0.104~0.309. KALEZIĆ and HEDGECOCK examined the three populations of *Triturus v. vulgaris,* two populations of *T. a. alpestris,* two populations of *T. cristatus dobrogicus* and a population of *T. c. karelinii.* The genetic distances between populations, between subspecies and between species were 0.031 ± 0.017, 0.347 and 0.906 ± 0.058, respectively. If the genetic distances like 0.145, 0.104 ~0.309 and 0.347 are the standard for subspecies, the Korea population of *H. japonica* may possess the qualification necessary for the position of subspecies. In

contrast to the Korea population, the Tsushima population seems to be insufficient in genetic distance to be given a position of subspecies. While the genetic distances of this population were 0.132, 0.111 and 0.112 from the Kunashiri, Hirosaki and Maibara populations, respectively, those were only 0.080, 0.081 and 0.050 from the Ichinoseki, Odawara and Hiroshima populations, respectively. It was interesting that the populations distributed in Sakhalin, Kunashiri of the Kuril Islands, Sapporo and Setana of Hokkaido, and Hirosaki and Ichinoseki of northeastern Japan are genetically very close with one another. The genetic distances among them were 0.012~0.043. It was confirmed that *H. japonica* is remotely related to *H. hallowelli* from Amami and *H. chinensis* from Taiwan. The genetic distances between *H. hallowelli* and the 11 populations of *H. japonica* were 0.974~1.131, while those between *H. chinensis* and the 11 populations of *H. japonica* were 1.177~1.360.

A more conspicuous genetic differentiation than that of *H. japonica* was reported by CASE, HANELINE and SMITH (1975) in *H. regilla* distributed along the Pacific coast of North America. The 17 populations examined by them were divided into three groups. The genetic distances among the three groups were 0.01~0.49, while in each of the three groups, they were 0.03~0.15, 0.03~0.07 or 0.01~0.17 among populations. A genetic differentiation somewhat similar to that of *H. japonica* was reported by NEVO and YANG (1979) in *H. arborea savignyi* distributed in Israel. By analyses of enzymes controlled at 27 loci, they found that the genetic distances among eight populations were 0.002~0.141. RALIN and SELANDER (1979) calculated genetic distances among nine populations of diploid *H. chrysoscelis* and three populations of sibling tetraploid *H. versicolor* distributed in southeastern North America. The genetic distances among the nine populations of *H. chrysoscelis* were 0.001~0.138, while those between *H. chrysoscelis* and *H. versicolor* were 0.036~0.108. It seems interesting that the populations of *H. chrysoscelis* showed a similar range of genetic distances to the populations of *H. japonica* except for the Korea population as well as the populations of *H. arborea savignyi*.

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