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Local Population Differentiation in *Hynobius retardatus* from Hokkaido: An Electrophoretic Analysis (Caudata: Hynobiidae)

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ABSTRACT—Allozyme data are used to examine the genetic relationships among two samples of *Hynobius retardatus* from Obihiro, and a geographically discrete sample from Chitose. The two samples from Obihiro are morphologically distinct from each other, but are found to be electrophoretically closely related, together forming a group distinct from the Chitose sample. The degree of genetic differentiation as measured by the F statistics is also low between the two Obihiro samples. The morphological differentiation seen between these two samples does not seem to be attributable directly to their genetic differentiation, but to the ecological divergence that is yet to be surveyed.

INTRODUCTION

Hynobius retardatus is endemic to Hokkaido of northern Japan and is abundant, widely ranging over much of the island [1]. This species is noted for a significantly smaller number of chromosomes (2n=40) [2] than found in all other congeneric species (2n=56 or 58) [3]. Thus, many studies on this species have hitherto been made mainly from karyological analyses (e.g., [4]), and studies from other fields of biology are relatively few (see [5] for literature review).

Because the geographic range of this species is larger than those of many other congeneric species, the presence of some degree of local population differentiation, both in morphology and genetic structure, is expected, but almost no studies have been made on intraspecific variation within this species.

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Sato and Nakabayashi [6] and Sato [7] found the occurrence of a unique population of *H. retardatus* within a narrow range of Obihiro-shi, Tokachi Plain, eastern Hokkaido. Salamanders from this population are distinguishable from individuals of neighbouring populations of *H. retardatus* in their unusually larger adult body size [population mean ±2SE of total length (TOTL)>175 mm in males and>170 mm in females (Sato, unpublished data)], and probably related to this, in the larger size of egg sac and larger egg diameter. Thus, they are tentatively named the "large" type, in contrast to the *Hynobius retardatus* "common" type (population mean±2SE of TOTL<170 mm in males and<165 mm in females). Although further morphological investigation is required to elucidate the relationships of these two types, it is interesting to study them from a genetic viewpoint.

In studying genetic relatiohships among local populations of caudate amphibians, the technique of electrophoresis has been widely used (e.g., [8–10]), and successfully applied to the Japanese species [11, 12]. Herein, we present analyses of

allozyme data to clarify the relationships of these two types, with another geographically discrete population as a reference.

MATERIALS AND METHODS

For electrophoretic examination, three samples were employed. These included 19 males of the large type and 11 males of the common type from Obihiro-shi (42°44′40″ N, 142°51′15″ E, and 42°42′30″ N, 142°57′15″ E, respectively), and for comparisons, five males of the common type from Shikotsu-ko, Chitose-shi (42°46′20″ N, 141°24′30″ E). The localities of the two Obihiro samples are 8.5 km apart, whereas Shikotsu-ko is 140 km away from Obihiro-shi and is clearly split from the latter locality by the Hidaka mountains and Ishikari plain.

Liver tissues, removed from freshly killed animals and frozen (−84°C), were used throughout the analysis. Voucher specimens were fixed in 10% formalin, later preserved in 70% ethanol and stored in the Biological Laboratory, Kyoto University. Horizontal starch gel electrophoresis was employed using 11.5% potato starch (Connaught) to resolve allozyme products [13]. The allozymes examined, locus designations, and buffer system used are listed in Table 1. Genetic interpretations of allozyme data were based on criteria developed by Selander *et al.* [13]. Enzyme nomenclature and E. C. numbers followed the recommendations of the Nomenclature Committee of the International Union of Biochemistry [14], and the notation of loci, electromorphs and genotypes followed principally Murphy and Crabtree [15]. Electromorphs were designated by letters with "a" representing the most rapidly migrating anodal variant.

The BIOSYS-1 computer program [16] was used to calculate all statistics. Standard estimates of genetic variability, i.e., mean heterozygosity by direct count (H), proportion of polymorphic loci (P), and the mean number of electromorphs per locus (A), were computed for each sample. Variable loci were checked by Chi-square goodness-of-fit tests to determine whether observed genotype frequencies were in Hardy-Weinberg equilibrium in each sample. The expected numbers of each genotype were calculated using Levene's formulae for small samples [17] and pooling. A contingency Chi-square test [18] was performed for inter-sample electromorph frequency heterogeneity. F-statistics were also calculated for all variable loci according to Wright [19].

Overall genetic differentiation among the three samples was estimated using coefficients of Nei's unbiassed genetic distance [20] and modified Rogers' genetic distance [21]. Genetic relationship among samples were estimated from the pairwise matrix of Nei's distance, clustered according to the UPGMA algorithm [22]. This method assumes equal rates of molecular evolution along all branches, but the validity of this assumption is ambiguous. We adopt this method to describe amounts of genetic divergence in this species and to allow comparison to literature accounts of variability in other caudate species. Alternatively, an unrooted Distance Wagner tree [23] was constructed using BIOSYS-1 optimized with the multiple addition criterion of Swofford and Selander [16] with metric measures of modified Rogers' genetic distance coefficients [21].

RESULTS

We studied the variability of 25 presumptive loci. Of these, eight were monomorphic in our sample (Table 1). These included mAat-A, sAcon-A, mMdhp-A, Est-1, Est-2, Pep-C, sSod-1, and sSod-2. Of the remaining 17 polymorphic loci, mAcon-A, Acp-A, Gpi-A, Ldh-B, and Pgm-C were the most variable loci, each with three alleles. In all the polymorphic loci, except for the mMdhp-A locus, the predominant electromorph, occurring at frequencies of 50% or more, was identical in the three samples. In the mMdhp-A locus, the Chitose sample had a predominant electromorph that was absent in the other two samples from Obihiro.

In the three samples, the mean number of electromorphs per locus (A) varied from 1.24–1.68, the percentage of polymorphic loci (P) from 24.0–44.0, and the mean heterozygosity (H) values from 0.029–0.068. The highest polymorphism and heterozygosity were found in the Obihiro-large sample, while the Obihiro-common sample exhibited the lowest variability. Statistically significant ($p<0.05$) deviation from Hardy-Weinberg equilib-

TABLE 1. Electromorph frequencies for 25 loci resolved in three samples of *Hynobius retardatus*. Mitochondrial and supernatant loci are denoted by "m" and "s" prefixes, respectively

Enzymes (commission number)	Locus	Buffer condition*	Obihiro large	Obihiro common	Chitose
Acid phosphatase (3.1.3.2)	Acp-A	B	a 0.08	a 0.23	b 0.90
			b 0.87	b 0.77	c 0.10
			c 0.05		
Acid phosphatase (3.1.3.2)	Acp-B	B	a 0.92	a 1.00	a 1.00
			b 0.08		
Aconitate hydratase (4.2.1.3)	mAcon-A	C	a 0.29	a 0.45	b 0.80
			b 0.58	b 0.55	c 0.20
			c 0.13		
Aconitate hydratase (4.2.1.3)	sAcon-A	C	a 1.00	a 1.00	a 1.00
Aspartate aminotransferase (2.6.1.1)	mAat-A	A	a 1.00	a 1.00	a 1.00
Aspartate aminotransferase (2.6.1.1)	sAat-A	A	a 0.63	a 0.68	a 1.00
			b 0.37	b 0.32	
Creatine kinase (2.7.3.2)	Ck-A	C	a 0.39	a 0.09	b 1.00
			b 0.61	b 0.91	
Dipeptidase (3.4.13.11)	Pep-B	D	b 1.00	a 0.14	a 0.40
				b 0.86	b 0.60
Dipeptidase (3.4.13.11)	Pep-C	D	a 1.00	a 1.00	a 1.00
Esterase (−)	Est-1	A	a 1.00	a 1.00	a 1.00
Esterase (−)	Est-2	A	a 1.00	a 1.00	a 1.00
Glucose-6-phosphate isomerase (5.3.1.9)	Gpi-A	B	a 0.03	b 1.00	b 1.00
			b 0.95		
			c 0.03		
L-iditol dehydrogenase (1.1.1.14)	Iddh-A	A	a 1.00	a 1.00	a 0.70
					b 0.30
Isocitrate dehydrogenase (1.1.1.42)	sIcdh-A	A	a 0.08	b 1.00	b 1.00
			b 0.92		
L-lactate dehydrogenase (1.1.1.27)	Ldh-A	A	a 0.05	b 1.00	b 1.00
			b 0.95		
L-lactate dehydrogenase (1.1.1.27)	Ldh-B	A	a 0.03	b 1.00	b 1.00
			b 0.84		
			c 0.13		
Malte dehydrogenase (1.1.1.37)	mMdh-A	A	b 1.00	b 1.00	a 0.40
					b 0.60
Malate dehydrogenase (1.1.1.37)	sMdh-A	A	b 1.00	b 1.00	a 0.40
					b 0.60
"Malic Enzyme"** (1.1.1.40)	mMdhp-A	A	b 1.00	b 1.00	a 0.80
					b 0.20
"Malic Enzyme"** (1.1.1.40)	sMdhp-A	A	a 1.00	a 1.00	a 1.00
Phosphoglucomutase (5.4.2.2)	Pgm-A	A	a 0.03	b 1.00	b 1.00
			b 0.97		
Phosphoglucomutase (5.4.2.2)	Pgm-C	A	a 0.11	b 0.55	a 0.50
			b 0.84	c 0.45	b 0.50
			c 0.05		
Phosphogluconate dehydrogenase (1.1.1.44)	Pgdh-A	A	a 0.11	b 1.00	a 0.30
			b 0.89		b 0.70
Superoxide dismutase (1.15.1.1)	sSod-1	D	a 1.00	a 1.00	a 1.00
Superoxide dismutase (1.15.1.1)	sSod-2	D	a 1.00	a 1.00	a 1.00

* Buffer system: A=Tris-citrate, pH 7.0. B=Tris-citrate, pH 6.0. C=Tris-citrate, pH 8.0. D=Tris-borate-EDTA, pH 8.0.

** NADP-dependent malate dehydrogenase.

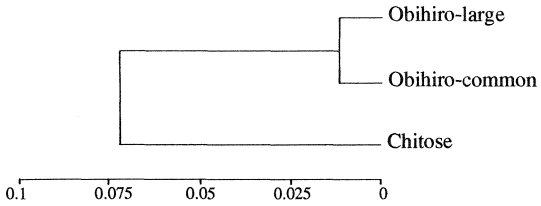

FIG. 1. UPGMA tree constructed from Nei's (1978) unbiased genetic distance for three samples of *Hynobius retardatus*. The percent standard deviation=3.324 and the cophenetic correlation=0.998. The Distance-Wagner method using modified Rogers' D (Wright, 1978) and rooted at the midpoint of the longest path produces the same tree topology.

rium was observed for several loci in all the three samples: mAcon-A, Acp-A, Acp-B, Ck-A, sIcdh-A, Ldh-A, Ldh-B, and Pgdh-A in the Obihiro-large sample; mAcon-A, Acp-A, CK-A, and Pgm-C in the Obihiro-common sample; and mAcon-A, sMdh-A, and mMdh-A in the Chitose sample. All of these deviations were heterozygote deficiencies.

TABLE 2. Summary of F_{ST} values calculated from 17 polymorphic loci for all samples of *Hynobius retardatus* and for Obihiro-large and Obihiro-common types

Locus	All samples	Obihiro-large and common
Acp-A	0.050	0.028
Acp-B	0.053	0.040
sAcon-A	0.105	0.020
sAat-A	0.151	0.003
Ck-A	0.205	0.122
Pep-B	0.118	0.073
Gpi-A	0.027	0.020
Iddh-A	0.222	—
mIcdh-A	0.054	0.041
Ldh-A	0.036	0.027
Ldh-B	0.095	0.073
mMdh-A	0.308	—
sMdh-A	0.308	—
mMdhp-A	0.727	—
Pgm-A	0.018	0.013
Pgm-C	0.207	0.144
Pgdh-A	0.132	0.056
Mean	0.226	0.060

Contingency Chi-square tests for heterogeneity of electromorph frequencies at variable loci between the two samples from Obihiro were not significant ($p>0.01$) except for one case (Pgm-C: $\chi^2=15.24$, $p<0.01$). In order to quantify this spatial homogeneity, F-statistics were calculated among these two samples, and again for all three samples (Table 2). In the two samples from Obihiro, the F_{ST} varied from 0.003 for the sAat-A locus to 0.144 for the Pgm-C locus and averaged 0.060. This value was appreciably lower than the F_{ST} of 0.226 calculated for all three samples.

Figure 1 shows genetic groups clustered by the UPGMA algorithm on the basis of Nei's genetic distance [20]. The two samples from Obihiro formed a cluster ($D\pm SE=0.010\pm0.006$) that is separated from the Chitose sample with an average D of 0.072 ($D\pm SE=0.074\pm0.033$ in the large and 0.070 ± 0.033 in the common type). The topology of the Distance-Wagner tree constructed using modified Rogers' D and rooted at the midpoint of the longest path is identical with the UPGMA tree.

DISCUSSION

The two samples of *H. retardatus* from Obihiro examined here have breeding sites of only 8.5 km apart and seem not to be geographically isolated by any evident barriers. However, the two samples have been reported to be fairly divergent chiefly in adult and egg-sac morphology and, less significantly, in breeding season [6, 7]. Males of the large type have a mean TOTL of 180 mm, while the common type has a TOTL usually less than 159 mm. The common type from Chitose is morphologically indistinguishable from the Obihiro common type. Thus, we first expected the smaples of common-type from Obihiro and Chitose to be genetically more similar to each other than to the Obihiro large type.

The present results, however, do not support this assumption. The values of percentage polymorphic loci (P) and mean heterozygosity (H) found in the three samples of *H. retardatus* (24.0–44.0 and 0.029–0.068) are within the intrapopulational range reported for many urodele species [24], and indicate that this species is almost as variable as other widely ranging Japanese urodeles

such as *Cynops pyrrhogaster* (20.0–53.3 and 0.031–0.147, respectively: [11, 12]) or *Hynobius nigrescens* (9.1–45.5 and 0.015–0.077, respectively: Matsui *et al.*, in preparation). The value of F_{ST} for the three samples (0.226) is even larger than that recorded in the widely spread populations of *Cynops pyrrhogaster* from Tohoku region (0.167: [12]), and indicates the presence of moderately great interpopulational difference in *H. retardatus*.

However, on the basis of the contingency Chi square test, the two samples from Obihiro were judged not to be spatially significant in electromorph frequency differences at most of the variable loci. This conclusion is also supported by the low F_{ST} value calculated for the two populations from Obihiro (0.060). Therefore, notable intersample differentiation is judged to be mainly due to the differentiation between the Chitose sample and the two samples from Obihiro, and morphological and related ecological differentiation found between the latter two samples is hardly attributable to their genetic differentiation as far as the present results have shown.

Actually, genetic groups clustered by both the UPGMA algorithm using Nei's D and by the Distance-Wagner method using modified Rogers' D resulted in a cluster of the two samples from Obihiro that is distinct from the Chitose sample. The distance value of 0.070, found between the two common types, is much larger than that of 0.010 obtained between the two samples from Obihiro. Thus, the two morphologically dissimilar types from Obihiro are judged to be more closely related to each other than either is to the Chitose sample, which is morphologically indistinguishable from the Obihiro common type. The obvious differences in morphology as examplified by the adult body length in the two Obihiro types might, therefore, be largely a result of the probable life history differences of the two types, including food availability, age composition, individual growth speed, time of sexual maturity, and life-span [25, 26].

Turning to overall genetic similarity, the greatest genetic distance value (0.074) obtained among the three populations of *H. retardatus* is much lower than the values generally reported to occur among sister species or even subspecies pairs of Japanese salamanders and newts [11, 27]. Therefore, although the three samples of *H. retardatus* are judged to be genetically somewhat differentiated, they cannot be split at any taxonomic rank genetically.

Using the correlation between D values and time of separation claimed to be unique to urodeles (1D=14 MY [28]), and the greatest difference (Nei's D [20]=0.074) between Obihiro-large sample and Chitose sample, the two were considered to have separated approximately 1×10^6 years B. P. This coincides approximately with the middle of lower Pleistocene. On the other hand, the two samples from Obihiro (D=0.010) are assumed to have separated approximately 1.4×10^5 years B. P., during the Ris-Wurm interglacial period in the middle Pleistocene.

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