日本列島産および琉球列島産アユ間の遺伝的分化

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Substantial Genetic Differentiation in Ayu *Plecoglossus altivelis* of the Japan and Ryukyu Islands

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Genetic differentiation in a sample of Ayu (*Plecoglossus altivelis*) from the Ryukyu Islands and in two samples, an amphidromous population of Kyushu and a landlocked population of Lake Biwa, from the Japan Islands was studied by examining the variation in 28 genetic loci by starch gel electrophoresis. Allele substitutions were observed at virtually 4 loci in the populations of the Japan and Ryukyu Islands. The mean genetic distance between the populations of these two regions was 0.19, which far exceeds the range of distance commonly observed between conspecific populations. Within the Japan Islands, the genetic distance between the amphidromous population of Kyushu and landlocked population of Lake Biwa was much smaller, being 0.02, even though considerable differences in allele frequencies were observed at two loci. These results, along with geological evidence, indicate that Ayu in the Ryukyu Islands has existed as a genetically unique stock isolated from that of the Japan Islands since the middle Pleistocene.

Ayu *Plecoglossus altivelis* is an amphidromous fish which spends its larval and young stages in the sea during the winter and sub-adult and adult stages in river waters in the summer. This species is distributed throughout the Japan Islands and in certain regions near islands such as the Ryukyu Islands, Taiwan Island, the Korean Peninsula, and some parts of the Chinese continent. A previous electrophoretic analysis of proteins showed virtual genetic homogeneity among the populations of Ayu throughout the Japan Islands,[1] though a landlocked population in Lake Biwa was found to be somewhat differentiated from amphidromous populations.[1-3]

The Ryukyu Islands, which are subtropical islands with a long history,[4] are known to have characteristic fauna. There are a few reports suggesting that Ayu in the Ryukyu Islands differ from those of the Japan Islands in behavior and morphological traits.[5-7] In the present study, an attempt was made to assess genetic differences in Ayu of the Japan and Ryukyu Islands. Ayu in the Ryukyu Islands was found to be highly differentiated genetically from that in the Japan Islands.

Materials and Methods

One population of Ayu was sampled at the Sumiyo River in Amami-oshima Island, the Ryukyu Islands, in July 1981. In spite of the considerable effort made to collect the specimens, no fresh specimens in Okinawa Island could be obtained because of the decrease in the number of fish in the waters in this island. Two representative populations of Ayu were collected from the Japan Islands: an amphidromous population caught from the Amori River, Kagoshima Prefecture, Kyushu, in July 1984, and a landlocked population from the Ado River flowing into Lake Biwa in May 1981.

The samples were stored at −20°C and later analyzed by horizontal starch gel electrophoresis. The livers and lateral muscles were removed from specimens stored and homogenized in equal volumes of 0.01 M Tris-HCl buffer (pH 7.1) containing 0.001 M EDTA. The homogenates were absorbed onto filter paper wicks (Whatman No. 3), and subjected to electrophoresis. Starch gels were prepared using both Electrostarch Lot 307 (Otto Hiller, Madison, Wisconsin, U.S.A.) and Connaught starch Lot 383–1 (Connaught Lab., Willowdale, Ontario, Canada) in a 4:1 ratio at a starch concentration of 12%. The staining procedures for specific enzymes were similar to those outlined by SHAW and PRASAD,[8] HARRIS and HOPKINSON,[9] and ALLENDORF *et al.*[10]

The analysed enzymes, their presumed loci, tissue sources, and buffer systems used for electrophoresis are given in Table 1. Some enzymes

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Table 1. Enzymes, their loci, tissue sources, and buffer systems used in electrophoretic survey. Abbreviations and Enzyme Commission numbers are listed after each enzyme

Enzyme	Locus	Tissue*1	Buffer system*2,*3
Aconitate hydratase (AH, 4.2.1.3)	*Ah*	L	TC8.0
Adenylate kinase (AK, 2.7.4.3)	*Ak*	M	TC8.0
Alcohol dehydrogenase (ADH, 1.1.1.1)	*Adh*	L	CAEA (CAPM)
Creatine kinase (CK, 2.7.3.2)	*Ck-2*	M	TC8.0
Fumarate hydratase (FH, 4.2.1.2)	*Fh*	M(L)	CAEA (LiOH)
Glucosephosphate isomerase (GPI, 5.3.1.9)	*Gpi-1*	L	CAEA (CAPM)
	Gpi-2	M	CAEA (CAPM)
Glutamate dehydrogenase (GDH, 1.4.1.3)	*Gdh*	L	TBE8.7
Glutamate oxaloacetate transaminase (GOT, 2.6.1.1)	*Got-1*	L	CAEA (CAPM)
	Got-2	M	CAEA (CAPM)
	Got-3	M	CAEA (CAPM)
Glycerol-3-phosphate dehydrogenase (GPDH, 1.1.1.8)	*Gpdh-1*	L	TBE8.7
	Gpdh-2	M	TBE8.7
Guanine deaminase (GDA, 3.5.4.3)	*Gda*	L	TBE8.7
Isocitrate dehydrogenase (IDH, 1.1.1.42)	*Idh-1*	M	CAEA (TC8.0)
	Idh-2	L	CAEA (TC8.0)
Lactate dehydrogenase (LDH, 1.1.1.27)	*Ldh-1*	M	CAEA
	Ldh-2	M	CAEA
Malate dehydrogenase (MDH, 1.1.1.37)	*Mdh-1*	M	CAEA
	Mdh-2	L	CAEA
	Mdh-3	M	CAEA
Malic enzyme (ME, 1.1.1.40)	*Me-1*	L	CAEA (CAPM)
	Me-2	M	CAEA (CAPM)
Mannosephosphate isomerase (MPI, 5.3.1.8)	*Mpi*	L(M)	TBE8.7
Phosphoglucomutase (PGM, 2.7.5.1)	*Pgm*	M	TC8.0
Phosphogluconate dehydrogenase (PGDH, 1.1.1.44)	*Pgdh*	L	CAEA
Sorbitol dehydrogenase (SDH, 1.1.1.14)	*Sdh*	L	TC8.0
Superoxide dismutase (SOD, 1.15.1.1)	*Sod*	L	TBE 8.7.

*1 L: Liver, M: Muscle

*2 TC8.0: Tris-citric acid, pH8.0[11]
CAEA: Citric acid-aminopropyl diethanolamine, pH 7.0[11]
CAPM: Citric acid-aminopropyl morpholine, pH 6.0[11]
LiOH: Tris-citric acid, pH 8.5 (gel), Lithium hydroxide-boric acid, pH 8.1 (electrode)[12]
TBE 8.7: Tris-boric acid-EDTA, pH 8.7[13]

*3 Second buffer system in parentheses

were electrophorased in the second buffer system for reference, in addition to the standard buffer system. No general protein staining was carried out in this study, since the main consistent band of general muscle proteins formerly scored[1] was found to be creatine kinase-2, as indicated by a comparison of their electrophoretic patterns with those of Ayu and other fish. The esterase locus was omitted from analysis in this study, since it was apparent that an allozyme, assumed to be predominant in the former report,[1] contained many variants so similar in mobility that genotypes could not be accurately scored.

Alleles were lettered alphabetically in order of decreasing anodal mobility of their protein products. The mobility relative to that of the most common alleles in Lake Biwa when using the standard buffer system is also given in Table 2. Each locus was named by an italicized abbrivation of the enzyme name, with a numerical suffix, when multiple loci were included, in the same order mode as above.

The distribution of observed genotypes in each population was compared to that expected from the HARDY-WEINBERG equilibrium, using the Chi-square test. This test was also used to examine the independence of absolute allele frequency between populations. Genetic differences among populations were quantified by NEI's[14] genetic distance.

Results and Discussion

Genetic Interpretation of Zymograms

Eleven of the 28 enzyme systems examined in this study were not analysed in our previous study[1]

and are described briefly as follows.

AH, AK, GDH, and SDH—Each of these systems was represented by a single invariant band, indicating the locus encoded for each to be monomorphic in the present populations.

CK—A single fast-migrating band was detected in liver extracts, and one slow-migrating band was observed in muscle extracts. These were assumed to be coded by two loci, *Ck-1* and *2*. The products of *Ck-1* stained only weakly, and were not consistently clear enough to permit reliable scoring.

FH—This enzyme was detected in both liver and muscle extracts. Since the extracts showed only one band in the LiOH buffer system, FH was assumed to be determined by a single monomorphic locus, though an additional band was usually observed in the CAEA buffer system.

GDA—The activity of this enzyme was found in liver extracts. Heterozygotes had three bands, indicating the dimeric structure of this enzyme.

GPDH—This enzyme was represented by two systems, one appearing in liver extracts and the other in muscle extracts and was controlled by two loci, *Gpdh-1* and *2*, respectively. The *Gpdh-2* locus was scored in our previous study.[1] The products of *Gpdh-1* appeared as a weak band along with a few sub-bands. Since *Gpdh-1* could not be scored in the Sumiyo River sample, it was not included in the analysis of genetic distance, but was so in the heterozygosity estimates for the other two samples for which scoring was possible.

GPI—The products of the liver-specific locus *Gpi-1* were found to migrate rapidly toward the anode, and those of the muscle-predominant locus *Gpi-2* to move more slowly. Heterozygotes were three-banded for each locus. The *Gpi-1* locus was highly polymorphic in the samples from the Japan Islands, as reported by TANIGUCHI *et al.*[3]

MPI—The activity of this enzyme was detected in both liver and muscle extracts. The heterozygotes exhibited a two band pattern, consistent with the known monomeric structure of this enzyme.

Genetic Variation within Populations

Of 28 loci examined, 12 (43%) were polymorphic at least in one population. None deviated significantly from the HARDY-WEINBERG equilibrium in any population. Allele frequencies are given in Table 2.

Increased electrophoretic migration distance and double-checking with different buffer systems in the present study made it possible to detect new alleles not found in our previous study[1] at the loci *Got-1* and *2*, *Gpdh-2*, *Idh-2*, and *Pgdh*. However, the frequencies of these new alleles were low. Some of the *aa* homozygotes at the *Me-1* locus had a wide band or one of somewhat irregular mobility. Thus, there is the possibility that alleles other than those described in the present study may be present at this locus and whose product mobility is closely similar to that of the common allele.

Table 2. Allele frequencies in three populations of *P. altivelis*

Locus*	Allele	Relative mobility	L. Biwa (N=30)	Amori R. (N=51)	Sumiyo R. (N=45)
Gpi-1	*a*	120	.440	.696	1.000
	b	100	.560	.294	
	d	80		.010	
Gpi-2	*a*	350			.013
	b	240		.010	.987
	c	100	1.000	.990	
Got-1	*a*	100	1.000	.912	1.000
	b	90		.088	
Got-2	*a*	125		.029	
	b	100	1.000	.971	1.000
Gda	*a*	100	.980	.980	1.000
	b	94	.020	.010	
	c	87		.010	
Idh-1	*a*	100	1.000	1.000	
	b	90			1.000
Idh-2	*b*	110		.010	
	c	100	1.000	.990	1.000
Ldh-2	*a*	190			1.000
	b	100	1.000	1.000	
Me-1	*a*	100	1.000	.961	1.000
	b	50		.039	
Mpi	*a*	110	.059	.010	
	b	105	.147	.912	1.000
	c	100	.794	.078	
Pgm	*b*	120	.017		
	c	100	.967	1.000	1.000
	d	80	.017		
Pgdh	*b*	100	.974	.971	1.000
	c	90	.026	.029	
Sod	*a*	270	.017		
	b	200			1.000
	c	100	.983	.980	
	d	0		.020	

* The following loci were fixed for the same allele in all three populations: *Ah*, *Ak*, *Adh*, *Ck-2*, *Fh*, *Gdh*, *Got-3*, *Ldh-1*, *Mdh-1*, *2*, and *3*, *Me-2*, and *Sdh*. In addition, *Gpdh-1* was scored in the two samples from Lake Biwa and Amori River, and fixed identically.

Table 3. Estimates of genetic variability in three populations of *P. altivelis*

Population	Proportion of loci polymorphic*1	Average heterozygosity*2
L. Biwa	0.214	0.037
Amori R.	0.393	0.040
Sumiyo R.	0.037	0.001

*1 A locus was treated as polymorphic in the case of any variation

*2 Estimated from allele frequencies

The alleles *b* and *c* at the *Pgm* locus were the same as the previously reported alleles *130* and *100*, respectively, and the alleles *a*, *b*, and *c* at the locus *Sod* corresponded to the alleles *350*, *225*, and *100*, respectively.

Estimates of genetic variability are summarized in Table 3. The average heterozygosity of the Amori River sample (4.0%) was somewhat higher than that of the Lake Biwa sample (3.7%), but the sample size of the former was also larger. These heterozygosity values were higher than those reported for samples from the Japan Islands by NISHIDA and TAKAHASHI[1]. This is principally due to two polymorphic loci (*Gpi-1* and *Mpi*) not included in their study. Values similar to those obtained in this study were reported by TANIGUCHI *et al.*[3] for the Lake Biwa population and the amphidromous populations in Shikoku, the Japan Islands, respectively; their study included the *Gpi-1* locus but not the *Mpi* locus.

The present heterozygosity values for populations in the Japan Islands are typical of the level of average heterozygosity observed in marine and freshwater fishes.[15] In contrast, estimates of genetic variability in the Sumiyo River population was very low (Table 3).

Comparison of Landlocked and Amphidromous Populations within the Japan Islands

Considerable differences in allele frequencies were observed at two loci, *Mpi* ($P<0.005$) and *Gpi-1* ($P<0.005$), between the Lake Biwa and Amori River populations. The allele *b* at the locus *Mpi* was predominant in the Amori River population but had only a low frequency in the Lake Biwa population. Thus, the *Mpi* locus may possibly be a means for evaluating the effects of the transportation of Ayu from Lake Biwa to other waters.

The genetic distance between the populations of Lake Biwa and Amori River was 0.025 (Table 4). This value is larger than the values formerly estimated by NISHIDA[2] and TANIGUCHI *et al.*[3] who used a fewer number of loci. Given that proteins may evolve at relatively constant rates, the genetic distance can be used to estimate the time of the evolutionary divergence of two lineages. Using NEI's[16] formula, the genetic distance value between the two populations was found to correspond to a divergence time of about 1×10^5 years. This estimation suggests that the origin of the landlocked population of Lake Biwa occurred at least before the last glacial period. This supports the hypothesis presented by KAWANABE[17,18] that the invasion of Ayu into Lake Biwa took place in later inter-glacial ages and the Lake Biwa population survived the glacial ages, at least up through the Würm period.

Table 4. NEI's genetic identity (above diagonal) and genetic distance (below diagonal) for pairs of populations of *P. altivelis*

	L. Biwa	Amori R.	Sumiyo R.
L. Biwa		0.976	0.813
Amori R.	0.025		0.847
Sumiyo R.	0.208	0.166	

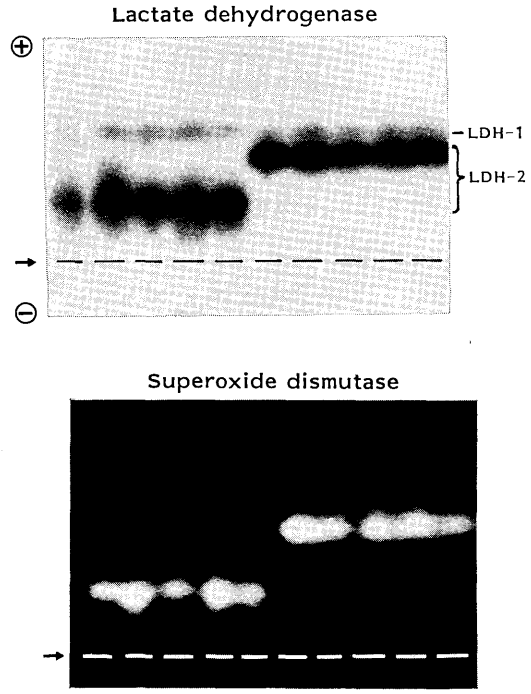

Fig. 1. Lactate dehydrogenase and Superoxide dismutase phenotypes of 5 specimens of *P. altivelis* from Lake Biwa in the Japan Islands (positions 1–5 from the left) and 5 specimens of *P. altivelis* from Sumiyo River in the Ryukyu Islands (6–10). Arrow denotes origin.

Genetic Differentiation in the Ayu Populations of the Japan and Ryukyu Islands

Large genetic differences were found in the Ayu populations of the Japan and Ryukyu Islands. The Sumiyo River sample from the Ryukyus was fixed for unique alleles at the *Idh-1*, *Ldh-2*, and *Sod* loci, and nearly so for alleles rare in the other two samples from the Japan Islands at the *Gpi-2* locus (Table 2). Figure 1 shows zymograms indicating allele substitution at *Ldh-2* and *Sod*. Genetic distances between the Ryukyus population and each of the other two populations of the Japan Islands were large (Table 4), averaging 0.19.

The results of allelic substitution at several loci and the high value of the mean genetic distance indicate the essential absence of gene exchange between the populations of these two regions. Off the Ryukyu Islands, the Kuroshio current flows northward to southern parts of the Japan Islands. Larval transport is generally considered to be by the Kuroshio for many marine fish from southern areas such as the Ryukyu Islands to southern Japan.[19] There has been no attempt to examine genetic homogeneity (or heterogeneity) in fish populations between the two regions except for a study on anemonefish (*Amphiprion clarki*) by BELL *et al.*[20] They observed small genetic differences among populations of this species along southern Japan to the Ryukyus, and attributed this to the genetic exchange among them. But our results indicate that populations of Ayu in the Japan and Ryukyu Islands are completely isolated, as suspected by KAWANABE.[21] In Ayu, larval dispersal by currents must be much less than that in the case of other marine fish.

The mean genetic distance of 0.19 between populations of the Japan and Ryukyu Islands, using the same calibrations as above, suggests the divergence between them to have occurred about 1 million years ago. Taking the paleogeographic evidence of the Ryukyu Islands also into consideration, this estimation provides insight into the possible origin of Ayu in the Ryukyus. KIZAKI and OSHIRO[4] have summarized geological data on the Ryukyu Islands, and made speculations regarding the paleogeography of these islands. They consider that the Ryukyu Islands at one time underwent an upheaval in the early Pleistocene period, causing them to be almost completely connected to the Japan Islands, the only break being the Tokara Channel. Since that time, the Tokara Channel has continued to exist there, separating Amami-oshima Island from southern Japan. During this period of upheaval, there was ample opportunity for genetic exchange between populations of Ayu of the Ryukyus and Japan Islands, because the Tokara Channel was then narrower. With the subsequent sinking of the islands, widening of the Tokara Channel may have isolated the populations of these regions, allowing genetic divergence. Ayu of the Ryukyus may thus have evolved as a distinct genetic unit for a considerable period of the middle and late Quaternary.

A fair number of genetic distance values between populations of various taxa has now been estimated for fish. These values are useful for interpreting the taxonomic relationship to other fish groups. SHAKLEE *et al.*[22] reviewed and compiled genetic distance values for conspecific populations, species and generic levels in many marine and freshwater fishes. They found the genetic distance between conspecific populations to average 0.05 (range 0.002–0.065) and between species, 0.30 (0.025–0.609). The present value for the genetic distance between Ayu of the Japan and Ryukyu Islands far exceeds the range of values commonly observed between conspecific populations.

Some papers suggest that Ayu in the Ryukyus differ from those in the Japan Islands. KAWANABE[5] found differences in territorial behavior between populations of the two regions. INOHA and SESOKO[6] and SHOKITA *et al.*[7] suggest that a few meristic traits in Ayu of the Ryukyus differ from those reported for Ayu in the Japan Islands. A more extensive and synthetic study of geographic populations of Ayu has confirmed this suggestion.[23] Apparently, Ayu in the Ryukyus is a genetically unique stock with specific behavior and morphology, and should be granted a taxonomic status as a subspecies. In view of its genetic uniqueness and taxonomic irreplaceability, attention should be directed to the fact that this unique stock is now being threatened on both Okinawa and Amami-oshima Islands as a result of the artificial change in its habitats.

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