Japanese Journal of Ichthyology
Vol. 39, No. 4 1993

魚類学雑誌
39巻4号 1993年

An Electrophoretic Study of Genetic Differentiation of a Japanese Freshwater Goby, *Rhinogobius flumineus*

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Abstract Genetic differentiation among 34 populations of the fluvial land-locked goby, *Rhinogobius flumineus*, endemic to southwestern Japan, was investigated by electrophoretic methods. Twenty-three loci, which were presumed to correspond to 15 enzymes and one non-enzymatic protein, were scored. Genetic differentiation within the species was high compared with other amphidromous or peripheral fishes, probably due to more restricted gene exchanges between adjacent populations.

From the allelic constitution, 5 population groups could be recognized. The largest group, distributed in the western part of Japan, included 21 populations with low genetic differentiation (mean genetic distance; 0.04). Six populations distributed in the eastern part of Japan, bounded by the Suzuka Mountains, constituted the second largest group (mean genetic distance; 0.02). The most divergent group, distributed at the eastern edge of the species' range, had a unique allelic constitution, not only when compared with other groups but also within populations of the group itself. The geographical patterns of the genetic groups were discussed in relation to the geological history of the Japanese Archipelago since the Pleistocene.

Rhinogobius flumineus (Mizuno) is a common freshwater goby endemic to southwestern Japan, living mainly in mountain streams (Mizuno, 1960). It spawns smaller numbers of, but larger eggs than all other species of *Rhinogobius* in Japan (Mizuno, 1961a, 1987) and is clearly distinguishable from them by some meristic characteristics (Mizuno, 1961b; Nishijima, 1968). Unlike other *Rhinogobius* species and almost all Japanese freshwater gobies, the species has no planktonic larval stage, adopting a benthic life immediately after hatching (Mizuno, 1960). It spends its whole life in streams and probably cannot inhabit lakes or ponds (Mizuno, 1963b). Mizuno (1963a) suggested the possibility of a polypatric origin from a common ancestor, perhaps a diadromous species of *Rhinogobius*.

In general, dispersal of fluvial land-locked fishes between river systems is restricted because they are isolated by seas or mountains. Their genetic differentiation is usually greater than in diadromous or peripheral fishes (e.g., Menezes et al., 1990).

R. flumineus has not been transferred artificially between rivers, unlike commercially useful fishes. Therefore, it can be expected that the species' present distribution substantially reflects the geological history of the Japanese Archipelago.

In this study, we used an electrophoretic method to analyze the extent of genetic differentiation among populations of *R. flumineus*. The relationship between ancient geographic features and the dispersion of the species was also considered.

Materials and Methods

Samples were collected from 34 rivers in 33 river systems between 1988 and 1990 (Fig. 1). Details of sampling localities, numbers of specimens, dates of collection and abbreviations for local populations are given in Table 1. All the samples were transported live to the laboratory and stored at −20°C or −80°C until used. Skeletal muscle and livers were dissected for electrophoretic analyses. Horizontal starch-gel electrophoresis was carried out, procedures basically following Taniguchi and Numachi (1978) and Taniguchi et al. (1978). The buffer systems used were citric acid-aminopropyl morpholine

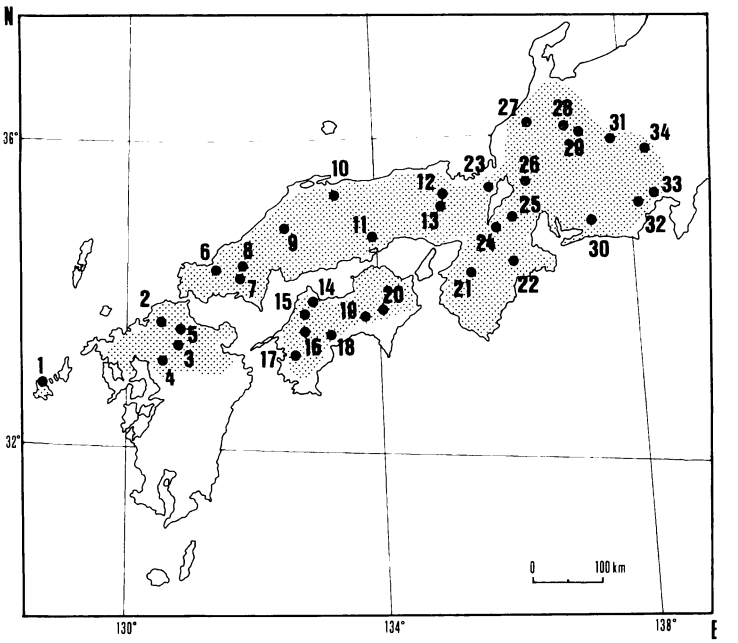

Fig. 1. Sampling locations and the distribution (dotted areas) of *Rhinogobius flumineus*.

(C-APM) at pH 6.0 and 7.0 (Clayton and Tretiak, 1972, modified) and citric acid-Tris (C-T) at pH 8.0 (Numachi et al., 1979). Staining procedures followed Harris and Hopkinson (1976) and Shaw and Prasad (1970), with slight modifications.

The analyzed enzymes, their presumed loci, tissue sources and the buffer systems used are shown in Table 2. Identification of loci and alleles, and genetic nomenclature and inscriptions followed Taniguchi et al. (1983) and Shaklee et al. (1989), respectively. Allele frequencies were calculated directly from observed genotypes. The distribution of observed genotypes was compared with that expected from the Hardy-Weinberg equilibrium by use of a Chi-square test. Genetic distances were computed among all pairwise comparisons of 34 populations of *R. flumineus* by use of Nei's formula (Nei, 1972). F_{ST} and G_{ST} values (Nei, 1987) were computed to indicate the extent of genetic differentiation among populations.

Results

Genetic control of enzymes. Twenty-three loci presumed to correspond to 15 enzymes and one non-enzymatic protein were scored in this study. Of these loci, 16 included genetic variations between populations. The allele frequencies for polymorphic loci are given in Table 3. Deviations of observed numbers of genotypes from the values expected from the Hardy-Weinberg equilibrium were not significant for almost all loci of all sample populations except in the case of the appearance of a very rare allele ($p<0.05$) or of more than three alleles at a locus. Of the 16 loci that varied, 15 were polymorphic ($p<0.95$) or fixed for different alleles among populations. A brief description of the enzymes analyzed is as follows:

Aspartate aminotransferase (AAT; EC: 2.6.1.1): Three loci coded for AAT, which is a dimeric enzyme. The liver-specific *AAT-1** and the muscle-dominant *AAT-2** in the anodal zone, and *AAT-3** in the cathodal zone were identified. The heterodimer between *AAT-1** and *AAT-2** was observed (Fig 2).

Acid phosphatase (ACP; EC: 3.1.3.2): The liver-specific *ACP** was identified, and polymorphism of its allele was seen in only one population.

α-Glycerophosphate dehydrogenase (GPD; EC: 1.1.1.8): Our interpretation was the same as that of Masuda et al. (1989). Five alleles were seen, *GPD*d* being dominant in almost all populations (Fig. 2).

Glucosephosphate isomerase (GPI; EC: 5.3.1.9): *GPI-1**, *GPI-2** and their heterodimer are the same as in Masuda et al. (1989). Three alleles for *GPI-1** and five alleles for *GPI-2** were identified (Fig. 2).

Isocitrate dehydrogenase (IDHP; EC: 1.1.1.42): The muscle-specific *IDHP-1** and the liver-specific

*IDHP-2** are the same as *IDH-2** and *IDH-1**, respectively, in Masuda et al. (1989). A total of three alleles in each locus were identified (Fig. 2).

Lactate dehydrogenase (LDH; EC: 1.1.1.27): Two loci were seen in muscle and liver extracts, but only the dominant *LDH-1**, which was monomorphic in every population, was scored.

Malate dehydrogenase (MDH; EC: 1.1.1.37): The muscle-specific, but recessive *MDH-1 **, the most dominant *MDH-2** and the least anodal *MDH-3** were identified. *MDH-1** and *MDH-2** were usually very close to each other, and the heterodimer between them was seen in muscle extract (Fig. 2). *MDH-2** is the same as *MDH** in Masuda et al. (1989).

Malic enzyme (ME; EC: 1.1.1.40): Two loci coded for ME. The monomorphic *ME-1**, the same as *ME** in Masuda et al. (1989), and the less anodal *ME-2** were identified in muscle extract. The rare allele, *ME-2*b*, was seen in only one population, with no heterozygotes being seen (Fig. 2).

Mannose-6-phosphate isomerase (MPI; EC: 5.3.1.8): Monomeric MPI was observed in muscle extract. A total of four alleles appeared to be present at the locus *MPI** (Fig. 2).

6-Phosphogluconate dehydrogenase (PGDH; EC: 1.1.1.44): The liver-specific *PGDH** was identified, and a rare polymorphism seen in one population.

Phosphoglucomutase (PGM; EC: 2.7.5.1): PGM is a monomeric enzyme, as is MPI. A total of six

Table 1. Localities of populations, their abbreviations, numbers of specimens and dates of collection of *Rhinogobius flumineus* examined electrophoretically in this study. Numbers 1–34 are as in Fig. 1

Locality			No. of specimens	Date of collection	Abbreviation
No.	Prefecture	River (-River system)			
1	Nagasaki	R. Wani	24	Oct. 1988	WAN
2	Fukuoka	R. Tatara	20	Sep. 1990	TAT
3		R. Chikugo	16	Sep. 1988	CHI
4	Kumamoto	R. Nabeharu (-R. Yabe)	20	Aug. 1989	NAB
5	Oita	R. Ima	22	Aug. 1989	IMA
6	Yamaguchi	R. Abu	20	Aug. 1989	ABU
7		R. Fukatani (-R. Nishiki)	22	June 1988	NIS
8	Shimane	R. Kanoashikawachi (-R. Takatsu)	24	June 1988	TAK
9		R. Izuha (-R. Gou)	8	Sep. 1989	IZU
10		R. Hino	11	Aug. 1989	HIN
11	Okayama	R. Asahi	20	Sep. 1989	ASA
12	Hyougo	R. Takeda (-R. Yura)	20	Sep. 1989	KAK
13		R. Miyata (-R. Kako)	20	Sep. 1989	YUR
14	Ehime	R. Shigenobu	19	Apr. 1989	SGE
15		R. Tobe (-R. Shigenobu)	24	June 1988	TOB
16		R. Kawabe (-R. Hiji)	20	May 1988	KAW
17		R. Meguro (-R. Shimanto)	20	Aug. 1990	MEG
18	Kouchi	R. Sakaore (-R. Niyodo)	20	Oct. 1990	SAK
19		R. Monobe	18	May 1989	MON
20	Tokushima	R. Naka	22	May 1989	NAK
21	Nara	R. Yoshino (-R. Kino)	16	Sep. 1989	YOS
22	Mie	R. Shoujidani (-R. Kushida)	20	Sep. 1989	KUS
23	Shiga	R. Ado	20	May 1989	ADO
24		R. Shigaraki (-R. Yodo)	23	May 1989	SGA
25		R. Yasu	21	May 1989	YAS
26	Gifu	R. Fujiko (-R. Ibi)	21	Oct. 1990	FUJ
27	Ishikawa	R. Sai	20	May 1989	SAI
28	Gifu	R. Miya (-R. Jintsuu)	21	July 1988	MIY
29		R. Hida (-R. Kiso)	20	July 1988	HID
30	Aichi	R. Ooi (-R. Toyo)	24	Sep. 1989	TOY
31	Nagano	R. Mibu (-R. Tenryuu)	23	Oct. 1990	MIB
32	Shizuoka	R. Warashina (-R. Abe)	22	Oct. 1990	WAR
33		R. Okitsu	25	Oct. 1990	OKI
34	Yamanashi	R. Kamanashi (-R. Fuji)	25	July 1988	KAM

alleles appeared to be present, but heterozygotes between alleles *a, *c, *e and *b, *d, *f were not seen (Fig. 2).

Sarcoplasmic protein (PROT): Some stained zones were observed in the analysis of muscle extract, and the most dominant, *PROT**, the same as *PROT-1** in Masuda et al. (1989), was scored.

D-3-Hydroxybutyrate dehydrogenase (HBDH; EC: 1.1.1.30), sorbitol dehydrogenase (SDH; EC: 1.1.1.14), superoxide dismutase (SOD; EC: 1.15.1.1) and xanthine dehydrogenase (XDH; EC: 1.2.1.37) were all monomorphic and showed no variations.

Genetic variability. The genetic variability of all populations is shown in Table 4. Proportions of polymorphic loci ($p<0.99$) in each population ranged from 0 to 0.30 (average 0.14). Observed heterozygosities ranged from 0 to 0.08 (average 0.04) and those expected ranged from 0 to 0.07 (average 0.04). The average ratio of observed/expected heterozygosity (H_o/H_e) was 0.92.

Genetic distances. The values of the genetic distance (D) between pairs of all 34 populations ranged from 2.8×10^{-4} to 0.35 (Table 5). The minimum D value was obtained in the pairwise comparison between the Takeda River population (Locality 12) and the Miyata River population (Locality 13) as in Fig. 3. These two rivers are adjacent but flow down opposite sea slopes (Figs. 1 and 4). The specimens from the two rivers were obtained from the river-heads, which include an area where the rivers are parallel, only a few hundred meters distant from each other. In this area, some irrigation channels connect the two rivers, enabling some fishes, such as loach, to move easily between the rivers. No obvious watershed is evident. The minimum D value might reflect genetic exchange between the two river populations owing to a landform-like spill over or as a consequence of stream-capture in more recent times, as Kimizuka and Kobayashi (1983) have pointed out regarding *Cobitis biwae* distribution in the area. The pairwise comparisons of the Kamanashi River population (Locality 34), at the eastern limit of the species' distribution, with other populations gave higher D values than any other comparisons.

A dendrogram (Fig. 3) based on the cluster analysis was made by the group-average method. Based

Table 2. Enzymes and proteins examined, loci detected, buffers used and tissues assayed

Enzyme and protein (E.C.)	Locus	Buffer[1]	Tissue[2]
Aspartate aminotransferase (2.6.1.1)	*AAT-1**	II	L
	*AAT-2**	II	M
	*AAT-3**	II	M, L
Acid phosphatase (3.1.3.2)	*ACP**	I	L
α-Glycerophosphate dehydrogenase (1.1.1.8)	*GPD**	II, III	M
Glucosephosphate isomerase (5.3.1.9)	*GPI-1**	II	M
	*GPI-2**	II	M
D-3-Hydroxybutyrate dehydrogenase (1.1.1.30)	*HBDH**	II	M
Isocitrate dehydrogenase (1.1.1.42)	*IDHP-1**	II	M
	*IDHP-2**	I	L
Lactate dehydrogenase (1.1.1.27)	*LDH-1**	II	M, L
Malate dehydrogenase (1.1.1.37)	*MDH-1**	I	M
	*MDH-2**	I	M, L
	*MDH-3**	I	M, L
Malic enzyme (1.1.1.40)	*ME-1**	III	M
	*ME-2**	III	M
Mannose-6-phosphate isomerase (5.3.1.8)	*MPI**	II, III	M
6-Phosphogluconate dehydrogenase (1.1.1.44)	*PGDH**	II	L
Phosphoglucomutase (2.7.5.1)	*PGM**	II	M
Sorbitol dehydrogenase (1.1.1.14)	*SDH**	I	M
Superoxide dismutase (1.15.1.1)	*SOD**	II, III	M
Xanthine dehydrogenase (1.2.1.37)	*XDH**	III	M
Sarcoplasmic protein	*PROT**	III	M

[1] I: citric acid-aminopropyl morpholine, pH 6.0; II: citric acid-aminopropyl morpholine, pH 7.0; III: citric acid-Tris, pH 8.0.

[2] M: muscle; L: liver.

Table 3. Allele frequence at 16 loci that are polymorphic or fixed for different alleles for populations of *Rhinogobius flumineus*. Abbreviations are as in Table 1

Locus	Allele	Population																
		1 WAN	2 TAT	3 CHI	4 NAB	5 IMA	6 ABU	7 NIS	8 TAK	9 IZU	10 HIN	11 ASA	12 YUR	13 KAK	14 SGE	15 TOB	16 KAW	17 MEG
*AAT-1**	*a																	
	*b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*c																	
	*d																	
*AAT-2**	*a					0.068							0.050		0.026			
	*b	1.000	1.000	1.000	1.000	0.932	1.000	1.000	1.000	1.000	1.000	1.000	0.950	1.000	0.974	1.000	1.000	1.000
*AAT-3**	*a					0.341												
	*b	0.917	1.000	1.000	1.000	0.659	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*c	0.083																
*ACP**	*a	0.860	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*b	0.140																
*GPD**	*a								0.521									
	*b		0.450	0.219	0.075	0.114				0.125	1.000							
	*c								0.479									
	*d	0.563	0.550	0.781	0.925	0.886	0.975	1.000		0.875		1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*e	0.437					0.025											
*GPI-1**	*a																	
	*b	0.979	0.050	1.000	1.000	0.705	0.350	1.000	1.000	1.000	1.000	0.975	1.000	0.975	1.000	1.000	1.000	1.000
	*c	0.021	0.950			0.295	0.650					0.025		0.025				
*GPI-2**	*a																	
	*b							0.182		0.813		0.850	0.325	0.325	0.237	0.272	0.500	0.900
	*c	1.000					0.050						0.075	0.025	0.053	0.042		
	*d		1.000	1.000	1.000	1.000	0.950	0.818	1.000	0.187	1.000	0.150	0.600	0.650	0.710	0.686	0.500	0.100
	*e																	
*IDHP-1**	*a								0.062									
	*b	1.000	1.000	1.000	1.000	0.977	1.000	1.000	0.938	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*c					0.023												
*IDHP-2**	*a					0.205	0.300											
	*b	1.000	1.000	1.000	1.000	0.795	0.700	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*c																	
*MDH-1**	*a																	
	*b	1.000	0.150	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*c		0.850															
*MDH-2**	*a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*b																	
*MDH-3**	*a															0.021		
	*b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.979	1.000	1.000
	*c																	
*ME-2**	*a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*b																	
*MPI**	*a																	
	*b		1.000	1.000	1.000	1.000	1.000	1.000	0.938	0.437		0.800	0.975	0.950	1.000	0.958	1.000	1.000
	*c	1.000							0.062	0.563	1.000	0.200	0.025	0.050		0.042		
	*d																	
*PGDH**	*a																	
	*b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
*PGM**	*a																	
	*b							0.864				0.375			0.579	0.354		
	*c																	
	*d	1.000	1.000	1.000	1.000	1.000	1.000	0.136	0.979	1.000	1.000	0.625	1.000	1.000	0.421	0.646	1.000	1.000
	*e																	
	*f								0.021									

Table 3. Continued

Locus	Allele	Population																
		18 SAK	19 MON	20 NAK	21 YOS	22 KUS	23 ADO	24 SGA	25 YAS	26 FUJ	27 SAI	28 MIY	29 HID	30 TOY	31 MIB	32 WAR	33 OKI	34 KAM
*AAT-1**	*a																	1.000
	*b	0.950	1.000	0.886	1.000	0.175	1.000	1.000	1.000	0.119	0.025		0.050	0.271	1.000	1.000	1.000	
	*c	0.050		0.114		0.675				0.762	0.975	0.929	0.925	0.688				
	*d					0.150				0.119		0.071	0.025	0.041				
*AAT-2**	*a	0.375																
	*b	0.625	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
*AAT-3**	*a																1.000	1.000
	*b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		
	*c																	
*ACP**	*a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*b																	
*GPD**	*a																	
	*b																	
	*c																	
	*d	1.000	1.000	0.864	1.000	0.450	1.000	1.000	0.952	0.786	0.950	0.667	0.400	0.417	1.000	1.000	0.480	0.780
	*e			0.136		0.550			0.048	0.214	0.050	0.333	0.600	0.583			0.520	0.220
*GPI-1**	*a														0.022			
	*b	1.000	1.000	1.000	0.938	0.975	1.000	1.000	0.952	1.000	1.000	1.000	0.975	1.000	0.978	1.000		0.700
	*c				0.062	0.025			0.048				0.025				1.000	0.300
*GPI-2**	*a						0.600	0.043										
	*b	0.475	0.972	0.295				0.957	0.952			0.024		0.125				
	*c				0.375													
	*d	0.525	0.028	0.705		0.975	0.400		0.048	0.976	1.000	0.976	1.000	0.875	1.000	1.000	0.960	0.440
	*e				0.625	0.025				0.024							0.040	0.560
*IDHP-1**	*a						0.075											
	*b	1.000	1.000	1.000		1.000	0.925	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.978	1.000	1.000	1.000
	*c				1.000										0.022			
*IDHP-2**	*a																	
	*b	1.000	1.000	1.000	0.062	1.000	1.000	1.000	1.000	0.810	0.450	0.024	0.400	1.000	1.000	1.000	1.000	1.000
	*c				0.938					0.190	0.550	0.976	0.600					
*MDH-1**	*a			0.068														
	*b	1.000	1.000	0.932	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000	1.000	1.000
	*c														1.000			
*MDH-2**	*a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000		
	*b														1.000		1.000	1.000
*MDH-3**	*a													0.042				
	*b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.895	1.000	1.000	1.000	1.000
	*c													0.063				
*ME-2**	*a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000	1.000
	*b															1.000		
*MPI**	*a			0.091				0.130	0.048									
	*b	1.000	1.000	0.909	0.969			0.630	0.571	0.024								
	*c				0.031	1.000	1.000	0.240	0.381	0.976	1.000	1.000	1.000	1.000	0.978	0.568	1.000	1.000
	*d														0.022	0.432		
*PGDH**	*a									0.024								
	*b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.976	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
*PGM**	*a																	0.020
	*b	0.025	0.472	0.045		0.175							0.025			0.819		
	*c														1.000		0.440	0.680
	*d	0.975	0.528	0.955	0.938	0.825	1.000	1.000	1.000	0.929	1.000	1.000	0.975	0.917		0.136		
	*e																0.560	0.300
	*f				0.062					0.071				0.083		0.045		

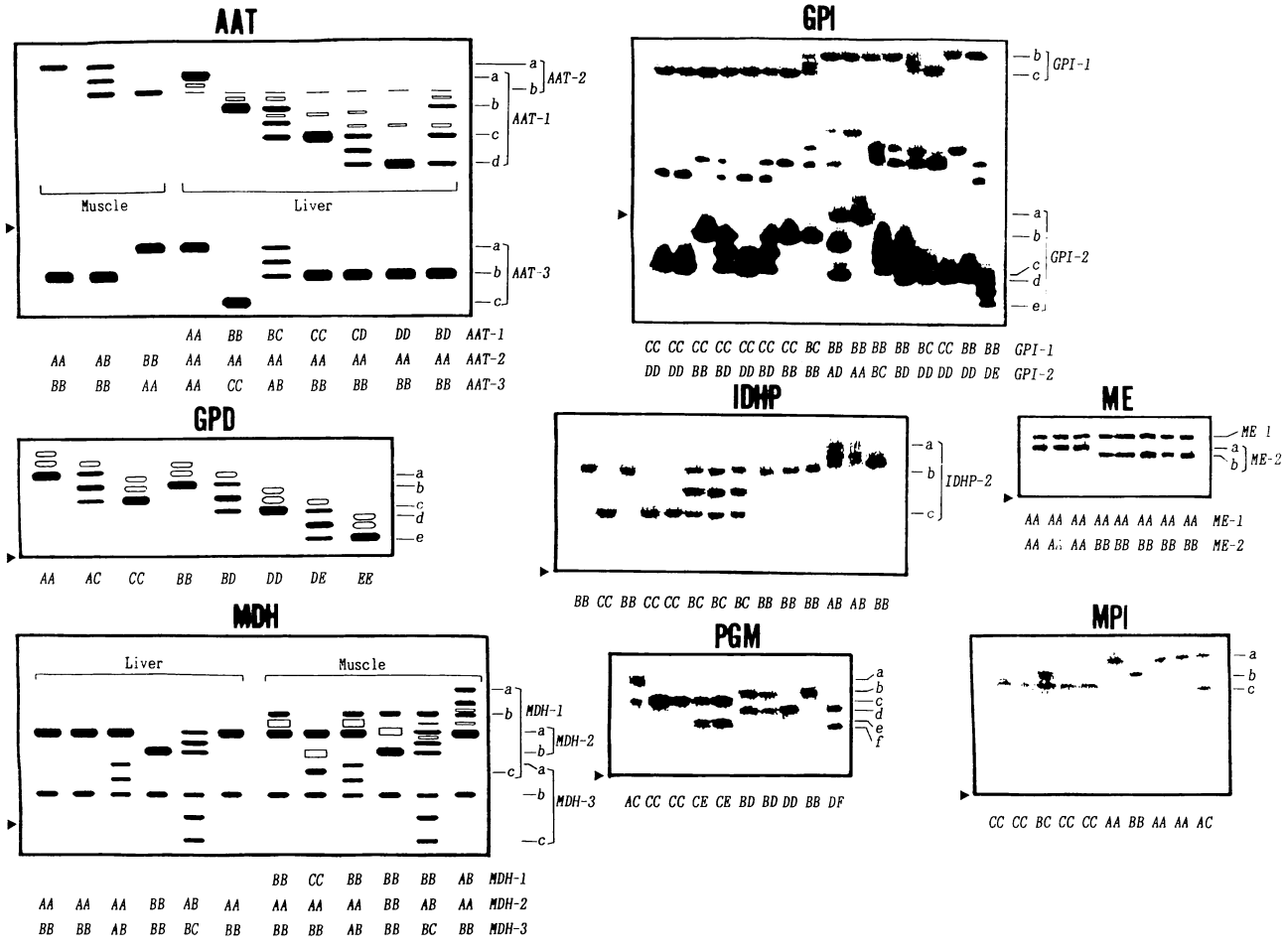

Fig. 2. Examples of electropherograms of isozymes. Closed triangles indicate the sample origin.

on the dendrogram pattern and the geographic distribution pattern of populations, five major groups, including one (4) with a single population, can be conveniently recognized (Fig. 3). Group 4, the Warashina River population (Locality 32), was rather similar to groups 2 and 3 in the allelic constitution of many loci (Table 3), but it differed from them by having the locus *ME-2** fixed for the allele *ME-2*b*, which was not seen in any other populations.

The sampled locations of each group are shown in Fig. 4. Group 1 comprises three populations that are near the Japan Sea (Sea of Japan) slope, but are very distant from one another. Some differences in allelic constitution were seen among these three populations (Table 3). Group 2 is made up of six populations that are comparatively close to each other in the eastern part of Japan (Fig. 4). Groups 1 and 2 are distinguishable from one another by the frequency of the major allele at the locus *AAT-1**. Group 3 includes 21 populations, which are distributed in the western part of Japan, from the Kyusyu region to near Lake Biwa in the Honsyu region. It is distinguishable from groups 1 and 2 by the allelic constitution of the locus *MPI** (Table 3). Three eastern

Table 4. Genetic variabilities at 23 loci for populations of *Rhinogobius flumineus*. P: criterion for polymorphism is lower than 0.99 in major allele frequency. P*: polymorphism is lower than 0.95 in major allele frequency. Abbreviations are as in Table 1

Population	Proportion of P	Proportion of P*	Allele/Locus	Average heterozygosity Observed (H_o)	Average heterozygosity Expected (H_e)	H_o/H_e
WAN	0.174	0.130	1.174	0.038	0.041	0.927
TAT	0.130	0.130	1.130	0.043	0.037	1.162
CHI	0.043	0.043	1.043	0.019	0.015	1.267
NAB	0.043	0.043	1.043	0.007	0.006	1.167
IMA	0.261	0.217	1.261	0.063	0.068	0.926
ABU	0.174	0.130	1.174	0.046	0.044	1.045
NIS	0.087	0.087	1.087	0.024	0.023	1.043
TAK	0.174	0.130	1.174	0.022	0.034	0.647
IZU	0.130	0.130	1.130	0.033	0.044	0.750
HIN	0	0	1.000	0	0	0
ASA	0.174	0.130	1.174	0.043	0.048	0.896
YUR	0.130	0.087	1.174	0.026	0.029	0.897
KAK	0.130	0.087	1.174	0.030	0.027	1.111
SGE	0.130	0.087	1.174	0.041	0.042	0.976
TOB	0.174	0.087	1.217	0.034	0.045	0.756
KAW	0.043	0.043	1.043	0.017	0.022	0.772
MEG	0.043	0.043	1.043	0.009	0.008	1.125
SAK	0.174	0.130	1.174	0.054	0.048	1.125
MON	0.087	0.043	1.087	0.029	0.024	1.208
NAK	0.261	0.217	1.304	0.075	0.071	1.056
YOS	0.217	0.174	1.217	0.035	0.038	0.921
KUS	0.217	0.130	1.261	0.061	0.060	1.017
ADO	0.087	0.087	1.087	0.028	0.027	1.037
SGA	0.087	0.043	1.130	0.015	0.027	0.556
YAS	0.174	0.043	1.217	0.033	0.035	0.943
FUJ	0.304	0.130	1.348	0.058	0.057	1.018
SAI	0.130	0.087	1.130	0.024	0.028	0.857
MIY	0.174	0.087	1.174	0.023	0.029	0.793
HID	0.217	0.130	1.217	0.054	0.053	1.019
TOY	0.217	0.217	1.304	0.054	0.065	0.831
MIB	0.130	0	1.130	0.006	0.006	1.000
WAR	0.087	0.087	1.130	0.026	0.035	0.743
OKI	0.130	0.087	1.130	0.045	0.046	0.978
KAM	0.174	0.174	1.217	0.061	0.074	0.824
Mean	0.144	0.102	1.161	0.035	0.037	0.923

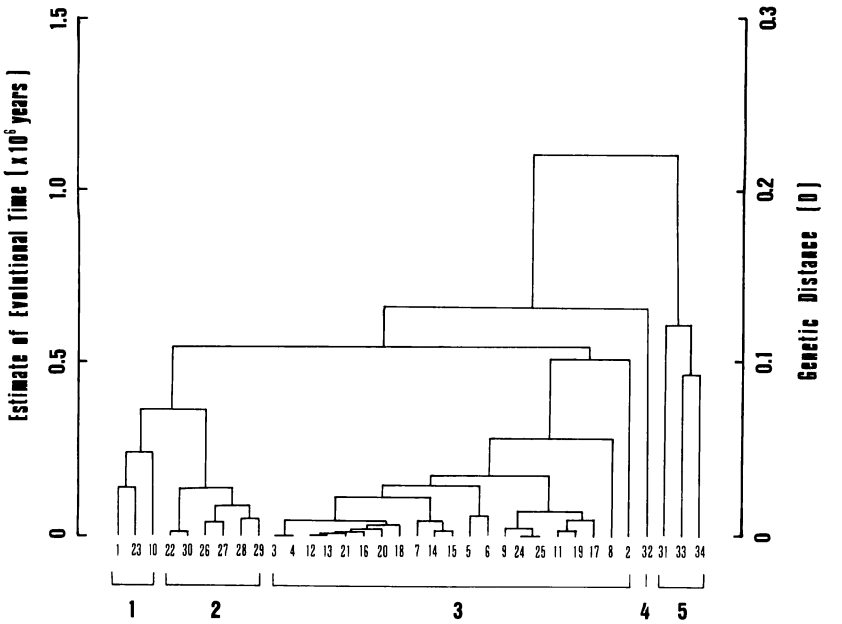

Fig. 3. Dendrogram based on genetic distances among populations of *Rhinogobius flumineus*. Estimate of evolutional time is calculated by Nei's (1975) formula. Numbers 1–34 are as in Fig. 1.

populations in group 5 (Fig. 4) are distinguishable not only from other groups by complete allelic replacement at the loci *MDH-2** and *PGM**, but also from one another, at the loci *AAT-1** and *AAT-3** (Table 3).

D values among the five groups ranged from 0.07 ±0.01 to 0.23±0.18 (Table 5), being generally equivalent to the intrageneric or intraspecific values (Nei, 1975; Wallis and Beardmore, 1984). The average D value among populations in group 5 was 0.11 ±0.04, higher than those within other groups (Table 5).

Genetic differentiation as indicated by F_{ST} and G_{ST}. The values of F_{ST} and G_{ST} for the total population and for each group are shown in Table 6. The F_{ST} values for some groups were much lower than those for the total population at the loci *AAT-1*, 2*, 3*, GPI-1*, MDH-1*, 2*, ME-2*, MPI** and *PGM**. The G_{ST} value for the overall population of *R. flumineus* was 0.70, a value larger than usual for diadromous fishes but nearly equivalent to those of freshwater fishes (Menezes et al., 1990). The G_{ST} value for each group in *R. flumineus* was also lower than that of the total population. The value for group 2 was about half of the other values, indicating higher genetic uniformity of this group. In contrast, genetic uniformity of group 5 was the lowest of all the groups, the G_{ST} value being nearly equal to that of

Table 5. Average D values with 95% confidence intervals for inter- and intragroup comparisons in the total population of *Rhinogobius flumineus*. Ranges are in parentheses. Groups 1–5 are as in Fig. 3

	Group 1	Group 2	Group 3	Group 4	Group 5
Group 1	0.041±0.045 (0.027–0.062)	0.071±0.014 (0.027–0.125)	0.083±0.007 (0.034–0.155)	0.115±0.049 (0.100–0.137)	0.185±0.042 (0.104–0.256)
Group 2		0.021±0.008 (0.003–0.052)	0.121±0.006 (0.070–0.236)	0.149±0.029 (0.122–0.192)	0.207±0.024 (0.129–0.296)
Group 3			0.035±0.004 (0.000–0.144)	0.129±0.012 (0.084–0.215)	0.224±0.016 (0.131–0.348)
Group 5				0.227±0.179 (0.144–0.277)	0.112±0.041 (0.093–0.122)

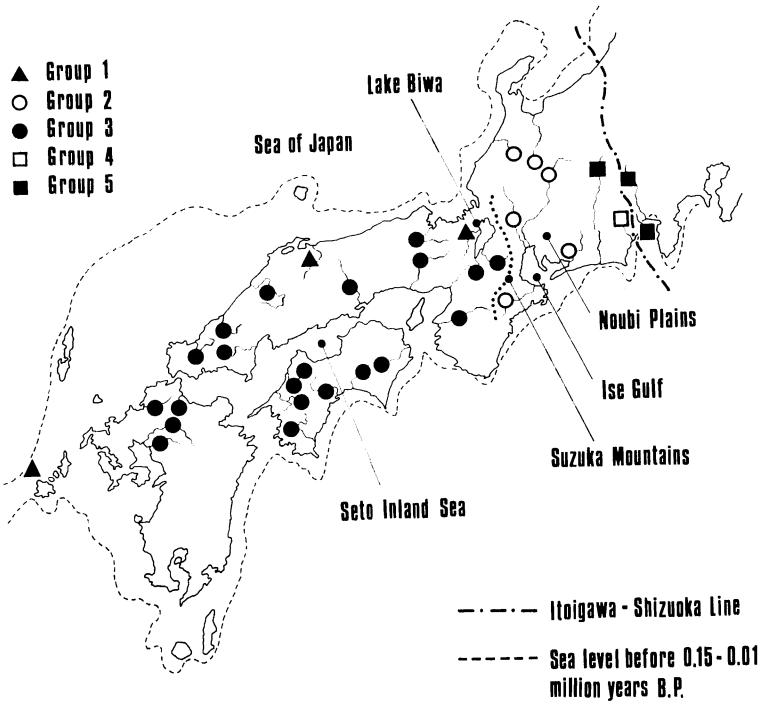

Fig. 4. Distribution of regional groups of *Rhinogobius flumineus*, and topographical and geological features mentioned in this paper. The ancient sea level is based on data from Minato et al. (1965). Groups 1–5 are as in Fig. 3.

Table 6. F_{ST} and G_{ST} values for comparisons between total and group populations of *Rhinogobius flumineus*. Numbers 1–5 are Groups 1–5 in Fig. 3

Locus	F_{ST}					
	Total	1	2	1+2	3	5
*AAT-1**	0.822	—	0.087	0.654	0.087	1.000
*AAT-2**	0.275	—	—	—	0.268	—
*AAT-3**	0.867	0.057	—	0.074	0.330	1.000
*ACP**	0.136	0.098	—	0.126	—	—
*GPD**	0.485	0.725	0.180	0.434	0.444	0.244
*GPI-1**	0.698	0.014	0.017	0.016	0.657	0.691
*GPI-2**	0.565	0.500	0.063	0.433	0.508	0.407
*IDHP-1**	0.053	0.051	—	0.067	0.048	0.015
*IDHP-2**	0.591	—	0.532	0.613	0.219	—
*MDH-1**	0.787	—	—	—	0.783	—
*MDH-2**	1.000	—	—	—	—	—
*MDH-3**	0.067	—	0.067	0.072	0.020	—
*ME-2**	1.000	—	—	—	—	—
*MPI**	0.826	—	0.020	0.021	0.292	0.015
*PGDH**	0.023	—	0.020	0.021	—	—
*PGM**	0.640	—	0.081	0.095	0.499	0.251
G_{ST}	0.698	0.541	0.254	0.498	0.478	0.617

the overall population. In group 3, the G_{ST} value would have been lower (0.40) had not two populations (TAT and TAK) exhibited a high frequency of unique alleles that were not seen in the other populations (Table 3).

Groups 1 and 2 could have been considered as a single group (Fig. 3), since the G_{ST} value of group 1+2 is equivalent to that of group 1. However, they should be considered as separate groups, on the basis of the genetic and geographic uniformity among populations in group 2 (Table 6, Fig. 4) and the difference in allelic constitution between the two groups (Table 3). In group 1, unique allelic constitution at the locus *GPD** or at the locus *GPI-2** was seen in each of three populations (Table 3). For these reasons, and considering the considerable geographic distances between group 1 populations, group 1 appears not to be uniform as other groups, either genetically or geographically. The apparent overlap between groups 1 and 2 may be a consequence of the coincidence of their allelic constitutions at some loci.

Discussion

The extent of genetic differences within the total population. In general, higher gene polymorphism is maintained in larger populations in which the members can interbreed (Nei, 1983). The size of exchangeable populations in truly freshwater fishes is considered to be smaller than in diadromous or peripheral fishes. Average values of the proportion of loci that were polymorphic ($p<0.95$) and the heterozygosity in the populations of *R. flumineus* were estimated to be 0.10 and 0.04, respectively (Table 4), these values being within the range for fishes previously reported (Nevo, 1978). The values are nearly equivalent to those for fluvial dace, *Tribolodon hakonensis* (0.22 and 0.04; Hanzawa et al., 1988), but are higher than those for land-locked stickleback, *Gasterosteus aculeatus* (0.04 and 0.01; Taniguchi et al., 1990) and fluvial, land-locked sculpin, *Cottus nozawae* (0.03 and 0.01; Goto and Andoh, 1990) in Japan. The heterozygosity expected for *R. flumineus* is also between those for anadromous smelt, *Hypomesus transpacificus nipponensis* and for the pond smelt, *H. olidus* (Okubo and Kudo, 1986). The results may be interpreted as indicating that genetic variability in the populations of *R. flumineus* is relatively high compared to other land-locked fish species and that the mean size of exchangeable populations of *R. flumineus* is relatively large.

The G_{ST} value of the total population is large compared to other fluvial fishes (Buth, 1980; Ferris et al., 1982), being equivalent, for example, to the interspecific value for five fluvial darter species, the *Etheostoma variatum* complex (McKeown et al., 1984). The mean D value of the total population of *R. flumineus* is 0.12 (0.88 in the mean genetic identity), within the range of conspecific values for fishes (Thorpe, 1983). It is also within the range of values for local populations of fluvial fish species, but is larger than those of other diadromous or marine fishes (Menezes et. al., 1990).

Genetic differences within and between groups. The populations of *R. flumineus* can be divided into five groups, although group 4 has but a single member, and groups 1 and 5 are not very uniform (Fig. 3). Group 4 and the three populations in group 5, which occur near the eastern edges of the distribution range of the species (Fig. 4), show complete allelic replacement from one another and from other groups (Table 3). Each of these four populations might have been influenced by a bottleneck effect.

Group 5, which has highly divergent populations, is genetically very different from the other groups. In contrast, the degree of genetic uniformity in the populations comprising groups 2 and 3 is comparatively high, and complete allelic replacement was hardly seen (Table 3). Two populations in group 3 (TAT and TAK) might have been subject to a bottleneck effect, because they had a high frequency of the allele *MDH-1*c*, and exhibited allelic replacement at the locus *GPD**, respectively. These features were not seen in other populations (Table 3). Except for the two former populations (labelled 2 and 8 in Fig. 3), groups 2 and 3 can each be considered to reflect truly genetically close populations.

The D values for the five groups were close to the interspecific values for other fishes (Wallis and Beardmore, 1984) and include the values equivalent among some other species of *Rhinogobius* (Masuda et al., 1989).

Taxonomic level among three regional groups. In groups 2 and 3, complete allelic replacement among populations was seen only in two populations (TAT and TAK) in group 3. Populations in each group would be able to exchange genetic material with each other if the present geographic barriers were removed. Although there were some differences in the frequency of major alleles at some loci between groups 2 and 3, complete allelic substitution between

them was not seen. This result suggests that the degree of genetic isolation between them may be weak, even though they have diverged sufficiently, as to be distinguishable in terms of allele constitution at some loci. In contrast, complete allelic replacement was seen among the populations in group 5, and between group 5 and the other groups, including their isolation for long periods. Group 4 is also considered to have been isolated from the others for a considerable time, having complete allelic replacement at the locus *ME-2**.

Judging from the mean D values among the groups, their genetic divergence is at the species or subspecies level. This is particularly so for groups 4 and 5. However, it is inappropriate to suggest that each group represents a biologically distinct species, owing to a lack of morphological or ecological information. Under experimental conditions, individuals from groups 2, 3 and 5 have interbred successfully (Yasuno, pers. comm.), suggesting that reproductive isolating mechanisms are weak or absent. No meristic differences were evident among the groups (Shimizu, unpubl. data).

Relationship between geographic features and the distribution of *R. flumineus*. The distribution of *R. flumineus* conforms closely to the "Southwestern Region" (Aoyagi, 1957), the designation of which indicated the distribution of primary freshwater fishes considered to have originated from the Asian continent and dispersed in a northerly direction in Japan. The dispersion of fluvial *R. flumineus* seems to have been very similar to that of primary freshwater fishes in Japan.

The northern limit of the distribution range of *R. flumineus* is near the Itoigawa-Shizuoka Line that geologically separates the Japanese Archipelago into the southwestern and northeastern parts. Except for those in the Hokkaido region, almost all primary freshwater fishes living exclusively in mountain streams, as does the fluvial, land-locked *R. flumineus*, are presently limited to south of the line (Mizuno, 1963a), with no relictual populations of them or *R. flumineus* being known in northeastern Japan. Therefore, the possibility that *R. flumineus* might once have spread to the north, far beyond the line, appears unlikely.

The distribution of genetically differentiated populations of *R. flumineus* falls into three major regions: 1) near the Seto Inland Sea slope (group 3); 2) around the Noubi Plains (group 2); and 3) at the eastern edges of the Noubi Plains (group 5) (Fig. 4). Each of these groups, hereafter termed a "Regional Group," comprises many genetically uniform, river populations that are geographically close to, but isolated from, one another.

The mean D values for the populations of Regional Groups 2 and 3 are 0.02 and 0.04, the mean time at which evolutionary divergence occurred, by Nei's (1975) formula, being 0.12 and 0.18 million years B.P., respectively. These times are included in the period from the Riss to the Wülm Glacial Epochs, in the middle or late Pleistocene. It has been considered that some lowering of sea levels in the Seto Inland Sea and Ise Gulf in the Noubi Plains occurred in the Pleistocene glacial stages (Minato et al., 1965). It has also been reported that these events occurred about 0.02 million years ago, as recently as the zenith of the Würm Glacial Epoch (Fujita, 1985), when only two large water systems would have existed around the Seto Inland Sea slope (Sakaizumi, 1986). The higher degree of genetic identity among populations of *R. flumineus* in these regions would have resulted from the wider dispersion of and greater genetic exchange between adjacent river populations, than seen in other regions.

In Regional Group 3, genetic similarities among populations distributed on the Japan Sea slope are lower in all respects than those on the Seto Inland Sea slope (Fig. 3). Some populations on the Japan Sea slope are genetically similar to the populations on the Seto Inland Sea slope, suggesting that the former originated independently from populations in some river systems on the Seto Inland Sea slope, with a corresponding lack of gene exchange on the Japan Sea slope. Evidence for watershed migration between the Seto Inland Sea slope and the Japan Sea slope has been given by Kimizuka and Kobayashi (1983).

Genetic differentiation between Regional Groups 2 and 3 seems to coincide with the geographic boundary formed by the Suzuka Mountains (Fig. 4). A number of genetic or morphological differences among populations of some freshwater fishes bounded by this watershed have been reported (Sakaizumi et al., 1983; Mori, 1987; Taniguchi et al., 1990; Okazaki et al., 1991). Regional Groups 2 and 3 are thought to have become isolated some 0.62 million years ago, close to the time of upheaval of the Suzuka Mountains and other nearby north-south mountain ranges (0.5–0.6 m.y. B.P., Fujita, 1983).

The estimated mean evolutionary time between the most divergent Regional Group (5) and the others is 0.9 million years, the maximum evolution-

ary time for the overall population of *R. flumineus* being 1.7 million years. Thus, the divergence of populations in this species appears to have occurred initially in the middle Pleistocene.

Origin of Regional Group 5 populations. The group 4 and Regional Group 5 populations have unique allelic characteristics relative to one another. The degree of genetic difference among them is larger than that of other regions, when geographic distances are considered. For example, the Okitsu River population (Locality 33) lives near the Fuji River system, that, geographically speaking, includes the Kamanashi River population (Locality 34) (some 10 km distant). However, complete allelic replacement was seen between them. Notwithstanding, the populations in Regional Group 5 also had common alleles at some loci, that were not seen in other populations. A few populations may have dispersed eastward from the region of the Noubi Plains with concomitant genetic drift, and entered adjacent rivers. *Oryzias latipes* and *Tribolodon hakonensis*, which live mainly in lowland to hill streams, showed fewer genetic differences between adjacent populations in the regions north of the Suzuka Mountains than did *R. flumineus* (Sakaizumi et al., 1983; Hanzawa et al., 1988). These differences might reflect differences in the extent of their respective movements.

Mizuno (1963a) hypothesized polypatric evolution within *R. flumineus.* While it is possible that the populations of Regional Group 5 and others have evolved independently from an amphidromous ancestor, given the large genetic differences between them, there is a lack of further evidence supporting such an evolutionary mode.

Acknowledgments

We wish to thank Dr. Y. Yanagisawa, Ehime University, for his critical reading of the manuscript. We also express our thanks to Dr. M. Azuma, Nagasaki University, and Mr. Y. Suzawa, Nishinihon Technological Institute, for their support. Assistance in sampling by Messrs. T. Takakusu, Y. Ikeda and T. Yasuno, Ehime University, and M. Hitomi, Osaka Kyoiku University, is also greatly appreciated. This work was partly supported by a Grant-in-Aid (No. 02304002) from the Japan Ministry of Education, Science and Culture.

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(Received February 14, 1992; accepted December 15, 1992)

カワヨシノボリ (*Rhinogobius flumineus*) の遺伝的分化

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河川陸封性魚種であるカワヨシノボリの地理的変異を，アイソザイム分析を用いて23遺伝子座について検証した．各河川の集団間の遺伝的分化は回遊性魚種や周縁性魚種のそれと比べると大きな値をとり，この差は本種の生態学的特性を反映しているものと考えられる．遺伝子組成の相違により，本種の集団は

5つのグループに大別された．21集団から成る最も大きなグループ（グループ3）は瀬戸内海を中心に分布し，6集団からなる，濃尾平野を中心に分布する第2のグループ（グループ2）とは鈴鹿山脈によって隔てられていた．両グループ内の遺伝的分化の程度は共に小さかった（平均遺伝的距離：0.02–0.04）．本種の分布域の東限に分布する第3のグループ（グループ5）は他のグループとの間だけでなく，グループ内においても遺伝的に最も大きく分化していた．残る2つのグループ（グループ1と4）は，各々，単一の集団からなるグループと，遺伝的に類似するが地理的な近似性の無い3集団からなるグループであった．本種の現在の分布は洪積世以降の日本列島の地史を反映しているものと考えられる．

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