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Genetic Differentiation of Freshwater and Anadromous Threespine Sticklebacks (*Gasterosteus aculeatus*) from Northern Japan

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Abstract To investigate the origin of the freshwater form of the threespine stickleback, *Gasterosteus aculeatus*, genetic differentiation among local populations of the species was elucidated from isozyme genetic markers of skeletal muscle and liver tissue by means of an electrophoretic method. The mean value of the genetic distance between the freshwater and anadromous forms of this species was very large (D= 0.6746), almost at the level for congeneric species pairs in both freshwater and marine fishes, whereas the mean values of pairs within the respective forms were very small (D=0.0006–0.0015). The level of genetic variability of the freshwater form was distinctly lower than that of the anadromous form. Results suggest that local populations of the freshwater form from northern Japan have been derived monophyletically from a common ancestor which was established before the recent postglacial colonization.

Throughout the Holarctic region two forms of the threespine stickleback, *Gasterosteus aculeatus* (Linnaeus), are known; the freshwater and anadromous forms (Wootton, 1976). In Japan, due to its discontinuous distribution in the small streams and springs of the high lands far from the northern coastal water habitat of the marine form, the freshwater form of this species is usually described as "landlocked". In addition, a subspecies, *G. aculeatus microcephalus* (Girard), named Hariyo in Japanese, is known from certain freshwater areas in central Japan. The freshwater and anadromous forms show morphological differences, such as body shape and body colour pattern. Furthermore, the F1 hybrid is sterile, suggesting a marked genetic divergence between these two forms (Honma and Tamura, 1984).

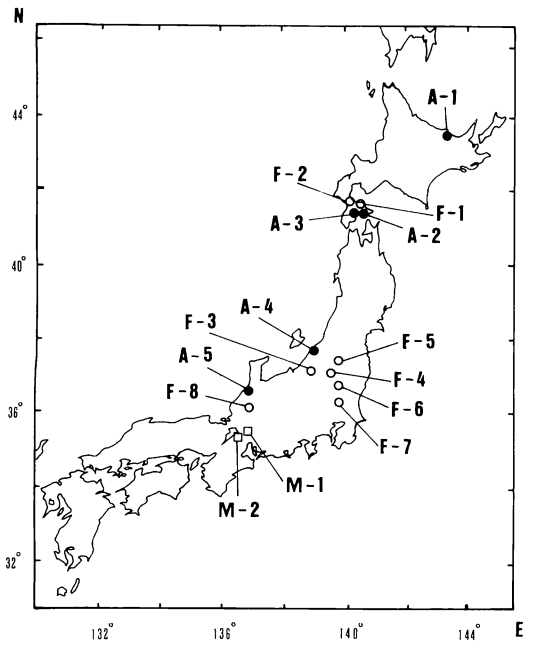

Fig. 1. Sampling locations of threespine sticklebacks *Gasterosteus aculeatus* from northern Japan. A, sampling locations of the anadromous form; F, freshwater form; M, subspecies, *G. a. microcephalus*.

Isozyme gene markers visualized by electrophoresis are generally considered to be very useful for estimation of the genetic divergence between populations. Several researchers have utilized such isozyme markers for evolutionary studies of threespine sticklebacks living in the waters of North America and Europe (Hagen, 1967; Avise, 1976; Hudon and Guderley, 1984; McPhail, 1984; Withler and McPhail, 1985; Baumgarther, 1986; Rafinski et al., 1989; Zietara, 1989). However, with the exception of Hagen (1967) who used information from the genetic marker of protein level to show the gene pools of the two forms in British Columbia of Canada to be independent, no evidence of distinct genetic divergence has emerged. On the other hand, distinct differentiation between the two forms was found in

Japanese threespine sticklebacks by Taniguchi et al. (1985).

In this paper, an attempt is made to estimate the level of genetic differentiation among local populations of both the freshwater and anadromous forms of the Japanese threespine stickleback, using isozyme markers detected by starch-gel electrophoresis, and to relate this to the origin of the freshwater form in northern Japan.

Materials and methods

Specimens were caught with seines from 15 sites shown in Fig. 1 during the period from 1975 to 1986. If the sampling site was distant from the laboratory, samples were immediately frozen in dry ice and stored below −20℃ until electrophoretic analysis. Sample details, such as location, number and size of specimens, scale type etc., are given in Table 1. Among the 15 sample sites, A-4, F-3, F-4 and F-7 were used for the screening of diagnostic isozyme markers, while the remaining 11 samples were used solely to compare allele frequencies among populations. The sampling locations A-4 and F-3 were connected with each other by rivers and drainages in the Niigata plain. Isozyme and protein markers were detected from the tissue of skeletal muscle and liver by the horizontal starch gel electrophoretic method using a citric acid-aminopropyl morpholine buffer system, pH 6.0 (Taniguchi and Numachi, 1978). The analyzed enzymes, their presumed loci, tissue source, and buffer systems are shown in Table 2. The terminology of loci and alleles follows Taniguchi et al. (1983). The distribution of the observed

Table 1. Sample data for threespine sticklebacks, *Gasterosteus aculeatus* used in electrophoretic analysis. *Fork length.

Sample number	Japanese name	Locality	No. of specimens	Total length (mm)	Type of scute
G. a. aculeatus					
Anadromous form					
A-1	itoyo	River Yanbetsu (Near Abashiri City)	68	68–91	trachurus
A-2	itoyo	River Yunokawa (Hakodate City)	31	64–94	trachurus
A-3	itoyo	Off Kamiiso (Hakodate Bay)	146	64–89	trachurus
A-4	itoyo	River Agano (Niigata City)	34	71–89*	trachurus
A-5	itoyo	River Ohno (Kanazawa City)	45	77–93	trachurus
Freshwater form					
F-1	itoyo	Lake Ohnuma (Near Hakodate City)	96	48–68	trachurus
F-2	itoyo	Mori-cho	111	52–80	trachurus
F-3	itoyo	River Uono (Muikamachi)	29	29–58*	trachurus
F-4	itoyo	Aizuwakamatsu City	30	14–58*	trachurus
F-5	itoyo	Takayoshi brook (Kitagawa City)	35	41–62	trachurus
F-6	itoyo	Fukagawa brook (Ohtawara City)	29	39–57	trachurus and semiarmatus
F-7	itoyo	River Kinu	32	60–88	trachurus
F-8	itoyo	Boke creek (Ohno City)	42	44–67	trachurus
G. a. microcephalus					
M-1	hariyo	River Tsuya (Yohro-cho)	35	46–61	semiarmatus and gymnurus
M-2	hariyo	Jizoh creek (Maibara-cho)	57	46–65	semiarmatus and gymnurus

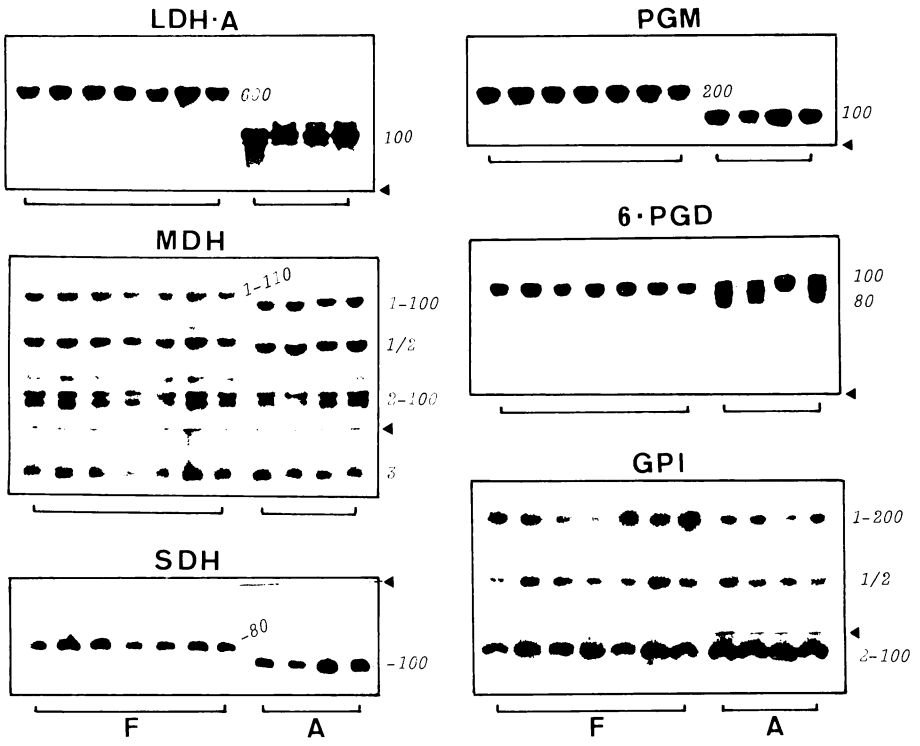

Fig. 2. Examples of electropherograms of isozymes. F, freshwater form from Uono R. (F-3); A, anadromous form from Agano R. (A-4). Closed triangles show the line where the crude isozyme samples were applied.

Table 2. List of enzymes and proteins examined, loci detected and tissues assayed. The buffer system for electrode was citric acid-aminopropyl morpholine, pH 6.0 (Taniguchi and Numachi, 1978).

Enzyme and protein	Locus	Tissue assayed
Aspartate aminotransferase (AAT: 2.6.1.1)	*Aat-1*	liver
	Aat-2	liver
Glucosephosphate isomerase (GPI: 5.3.1.9)	*Gpi-1*	muscle
	Gpi-2	muscle
Isocitrate dehydrogenase (IDH: 1.1.1.42)	*Idh-1*	liver
Lactate dehydrogenase (LDH: 1.1.1.27)	*Ldh-A*	muscle
Malate dehydrogenase (MDH: 1.1.1.37)	*Mdh-1*	muscle
	Mdh-2	muscle
	Mdh-3	liver
6-Phosphogluconate dehydrogenase (6-PGD: 1.1.1.44)	*6-Pgd*	liver
Phosphoglucomutase (PGM: 2.7.5.1)	*Pgm*	liver
Sorbitol dehydrogenase (SDH: 1.1.1.14)	*Sdh*	liver
Superoxide dismutase (SOD: 1.15.1.1)	*Sod*	liver
Sarcoplasmic protein (SP)	*Sp-1*	muscle
	Sp-2	muscle
	Sp-3	muscle

phenotypes of polymorphic loci was compared with that expected from the Hardy-Weinberg equilibrium by using a chi-square test. The genetic distances were quantified by Nei's formula (Nei, 1972).

Results

Genetic variation: The genetic variation was screened using 4 samples, A-4, F-3, F-4 and F-7, as shown in Table 1. The allele frequencies for 16 loci controlling 10 kinds of enzymatic and non-enzymatic proteins were estimated from the phenotypic distribution as shown in Table 3. Of the 16 loci surveyed, four were polymorphic, *Idh, Ldh-A, Mdh-1*, and *6-Pgd*, in the populations of the anadromous form. Of these four polymorphic loci, *Idh* and *6-Pgd* were monomorphic in the samples of the freshwater form. The remaining 12 loci were monomorphic in both the anadromous and freshwater forms. Complete or almost complete allelic substitution between these two forms was observed in 7 loci, *Ldh-A, Mdh-1, 6-Pgd, Pgm, Sdh, Sod* and *Sp-3*, which must therefore be diagnostic markers for the freshwater or anadromous forms of Japanese threespine sticklebacks: This is clearly shown in the examples of electropherograms in Fig. 2.

The genetic variability of the freshwater form was distinctly lower than that of the anadromous form, as shown in Tables 3 (based on 16 loci) and 4 (based on 8 loci). When the criterion for polymorphism

Table 3. Allelic frequencies at 16 biochemical marker loci in one anadromous and three landlocked populations of *Gasterosteus aculeatus aculeatus*. p^1, criterion for polymorphism below 0.99 in major allele frequency; p^2, polymorphysm below 0.95 in major allele frequency.

Loci	Allele	Anadromous A-4 (34 fish)	Landlocked F-3 (29 fish)	Landlocked F-4 (30 fish)	Landlocked F-7 (32 fish)
Aat-1	100	1	1	1	1
Aat-2	100	1	1	1	1
Gpi-1	100	1	1	1	1
Gpi-2	100	1	1	1	1
Idh	100	0.985	1	1	1
	75	0.015	0	0	0
Ldh-A	100	1	0	0	0
	300	0	0	0.217	0
	600	0	1	0.783	1
Mdh-1	115	0.074	0	0	0
	110	0	1	1	0.969
	100	0.926	0	0	0.031
Mdh-2	100	1	1	1	1
Mdh-3	−100	1	1	1	1
6-Pgd	135	0.044	0	0	0
	100	0.838	0	0	0
	95	0	1	1	1
	80	0.118	0	0	0
Pgm	100	1	0	0	0
	200	0	1	1	1
Sdh	−100	1	0	0	0
	−80	0	1	1	1
Sod	−100	1	0	0	0
	−50	0	1	1	1
Sp-1	100	1	1	1	1
Sp-2	−100	1	1	1	1
Sp-3	−100	1	0	0	0
	−50	0	1	1	1
Proportion of p^1		0.188	0	0.063	0.063
p^2		0.126	0	0.063	0
Alleles/locus		1.250	1	1.063	1.063
Heterozygosity		0.028	0	0.019	0.004

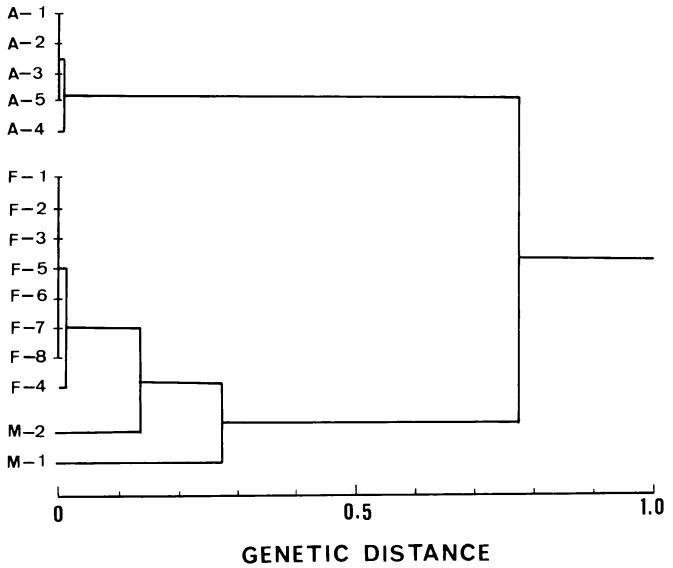

Fig. 3. Dendrogram showing relationships among local populations of threespine sticklebacks based on the genetic distance data in Table 6. The abbreviations are as in Fig. 1.

was placed below 0.99 in major allele frequency, the number of polymorphic loci was significantly lower in the freshwater (P=0–0.125) than in the anadromous form (P=0.125–0.375) (Table 4). The average heterozygosity was also significantly lower in the freshwater form (H=0–0.038) than in the anadromous form (P=0.028–0.052).

Genetic relationships: Table 5 lists the allele frequencies of 8 selected loci which were detected for all samples collected throughout the study period. It is clearly evident that the allele frequencies for all loci were not differentiated within either form. However, distinct differentiations between the anadromous and freshwater forms were observed in 4 loci, *Ldh-A, Mdh-1, Sod* and *Sp-3*. The subspecies, *G. a. microcephalus*, includes one locus, *Mdh-3*, which is divergent from the freshwater form.

Based on the allelic frequencies of 8 loci, estimates of genetic distances (D values) derived from pairwise comparisons of 15 samples and calculated according to Nei's (1972) method, are shown in Table 6. The mean values of the genetic distance between the anadromous and freshwater forms were very large (D=0.6746), whereas the genetic distances between each pair of samples within each form were very small (D=0.0006 and 0.0015). The subspecies, *G. a. microcephalus*, shows a larger genetic distance from the anadromous form, 1.1612, and a smaller distance from the freshwater form 0.2138. The value within the subspecies (M-1 and M-2), was relatively large, 0.1354. These large values between the anadromous and freshwater forms, or between the anadromous form and *G. a. microcephalus*, are comparable with the level of genetic distances observed in congeneric and conspecific populations of freshwater fish species (Buth, 1979, 1980; Hanzawa and Taniguchi, 1982; Hanzawa et al. 1988).

A dendrogram based on D values is illustrated in Fig. 3 to examine genetic relationships among the populations of *G. aculeatus* according to the unweighted pair group method (UPGMA) (Sneath and Sokal, 1973) (Fig. 3). The dendrogram shows the freshwater form to be distantly related to the anadromous form, and closely related to the subspecies, *G. a. microcephalus*. The geographical populations within the respective forms and subspecies show very close genetic relationships.

Discussion

Previous electrophoretic studies on the threespine stickleback revealed freshwater populations to be less polymorphic but more heterogeneous than anadromous populations, in both eastern Europe and North America (Withler and McPhail, 1985; Rafinski et al., 1989). A similar phenomenon was observed in Japanese freshwater populations regarding, but they were less heterogeneous (D=0.0015 on average). It

Table 4. Estimates of genetic variability in anadromous and landlocked forms of *Gasterosteus aculeatus aculeatus*, and *Gasterosteus aculeatus microcephalus* based on 8 biochemical marker loci.

Location	Anadromous form					Freshwater form								*G. a. microcephalus*	
	A-1	A-2	A-3	A-4	A-5	F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8	M-1	M-2
Proportion of polymorphic loci	0.375	0.125	0.250	0.188	0.125	0	0	0	0.125	0.125	0	0.063	0	0.125	0
Average heterozygosity	0.044	0.052	0.043	0.028	0.039	0	0	0	0.038	0.004	0	0.004	0	0.002	0

Table 5. Allelic frequencies at selected loci of isozymes and sarcoplasmic proteins for *Gasterosteus aculeatus microcephalus* and, anadromous and landlocked populations of *Gasterosteus aculeatus aculeatus*. n=no. of populations sampled. *x* includes minor alleles.

Samples	No. of fish	*Ldh-A*			*Mdh-1*				*Mdh-2*		*Mdh-3*		*Sod*		*Sp-1*		*Sp-2*	*Sp-3*	
		100	*300*	*600*	*115*	*110*	*100*	*x*	*100*	*20*	*−100*	*−70*	*−100*	*−50*	*100*	*120*	*−100*	*−100*	*−50*
G. a. aculeatus																			
Anadromous form (n=5)																			
A-1	68	0.993	0	0.007	0.140	0.007	0.816	0.037	0.993	0.007	1	0	1	0	1	0	1	1	0
A-2	31	1	0	0	0.146	0	0.792	0.060	1	0	1	0	1	0	1	0	1	1	0
A-3	146	1	0	0	0.149	0	0.815	0.036	0.994	0.006	1	0	1	0	1	0	1	1	0
A-4	34	1	0	0	0.074	0	0.926	0	1	0	1	0	1	0	1	0	1	1	0
A-5	45	1	0	0	0.178	0	0.822	0	1	0	1	0	1	0	1	0	1	1	0
Freshwater form (n=8)																			
F-1	96	0	0	1	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1
F-2	111	0	0	1	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1
F-3	29	0	0	1	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1
F-4	30	0	0.217	0.783	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1
F-5	35	0	0.014	0.986	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1
F-6	29	0	0	1	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1
F-7	32	0	0	1	0	0.969	0.31	0	1	0	1	0	0	1	1	0	1	0	1
F-8	42	0	0	1	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1
G. a. microcephalus (n=2)																			
M-1	35	0	0.071	0.929	0	1	0	0	1	0	0	1	0	1	0	1	1	0	1
M-2	57	0	0	1	0	1	0	0	1	0	1	0	0	1	0	1	1	0	1

is supposed that the ancestral freshwater populations of the last glacial periods lost their genetic variability owing to bottle-neck effects, and that the resultant level of variation was too low to produce intrapopulational genetic divergence during subsequent post-glacial colonization.

In Europe, only a small genetic divergence was detected between anadromous and freshwater forms (D=0.0079) based on 13 loci (Rafinski et al., 1989). Similarly in North America, only a small genetic divergence was observed, based on 5 polymorphic loci (Withlar and McPhail, 1985). However, in Japan, the genetic distance between the freshwater and anadromous forms is very large, being almost at the level of congeneric species within the Gasterosteidae: *G. aculeatus* and *G. wheatlandi* (Hudon and Guderley, 1984). Since the isozyme loci and samples investigated differed from those described in other papers, a direct comparison between these D values and those of other studies is not possible. However, it seems unequivocal that the data obtained in this study are not consistent with the Rafinski et al.

Table 6. Genetic distances between every pair of populations (above diagonal) and average genetic distances between forms or subspecies (below diagonal) of threespine sticklebacks, *Gasterosteus aculeatus*, on the basis of 8 loci of biochemical markers.

Samples	A-2	A-3	A-4	A-5	F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8	M-1	M-2
G. a. aculeatus														
Anadromous form (n=5)														
A-1	.0001	.0000	.0011	.0002	.6696	.6696	.6696	.9483	.6679	.6696	.6596	.6696	1.3530	.9597
A-2		.0001	.0016	.0004	.6709	.6709	.6709	.6492	.6692	.6709	.6610	.6709	1.3558	.9586
A-3			.0012	.0001	.6740	.6740	.6740	.6523	.6722	.6740	.6639	.6740	1.3603	.9621
A-4	0.0006			.0013	.6845	.6845	.6845	.6628	.6828	.6845	.6736	.6845	1.3693	.9721
A-5					.6745	.6745	.6745	.6528	.5363	.6745	.6644	.6745	1.3593	.9622
Freshwater form (n=8)														
F-1						.0000	.0000	.0058	.0000	.0000	.0001	.0000	.2913	.1335
F-2							.0000	.0058	.0000	.0000	.0001	.0000	.2913	.1335
F-3								.0058	.0000	.0000	.0001	.0000	.2913	.1335
F-4									.0051	.0058	.0060	.0058	.3015	.1433
F-5			0.6746							.0000	.0001	.0000	.2916	.1338
F-6							0.0015				.0001	.0000	.2913	.1335
F-7												.0001	.2927	.1342
F-8													.2913	.1335
G. a. microcephalus (n=2)														
M-1														.1354
M-2			1.1612						0.2138					

Table 7. Summary of characters of freshwater and anadromous forms of *Gasterosteus aculeatus*.

Common name Scientific name*	Anadromous form	Freshwater form		References
	Anadromous Itoyo *G. a. aculeatus*	Hariyo *G. a. microcephalus*	Landlocked Itoyo *G. a. aculeatus*	
Lateral plates (genotype)	trachrus (T/T)	leiurus (t/t)	trachrus (T/T) semiarmatus (T/t)	Wootton (1976)
Habitat	sandy bottom	muddy bottom	muddy bottom	Wootton (1976)
Overwintering	seawater	freshwater	freshwater	Wootton (1976)
Distribution	more northerly	more southerly	more southerly	Wootton (1976)
Salinity tolerance	euhalabous	oligohalabous	oligohalabous	Wootton (1976)
Temperature	psychrophile	mesophile	mesophile	Wootton (1976)
Spawning season	later	earlier	earlier	Wootton (1976)
Electrophoretic markers (alleles)	*Ldh-A*^{100} *Mdh-1*^{100} *Sod*$^{-100}$ *Sp-1*^{100} *Sp-3*$^{-100}$	*Ldh-A*^{600} *Mdh-1*^{110} *Sod*$^{-50}$ *Sp-1*^{100} *Sp-3*$^{-50}$	*Ldh-A*^{600} *Mdh-1*^{110} *Sod*$^{-50}$ *Sp-1*^{120} *Sp-3*$^{-50}$	Present paper

(1989) hypothesis of a postglacial polyphyletic origin for the freshwater populations of eastern Europe. Using Nei's (1975) formula, the evolutionary time was estimated as about 3.4 million years, and it is therefore suggested that the various freshwater populations in Japan may have been derived monophyletically. Furthermore, since the freshwater form clearly differs from the anadromous form in several characters (Table 7), the freshwater form from northern Japan should be separated from the anadromous form of *Gasterosteus aculeatus* at the species level.

In order to consider just why the level of genetic divergence is so different between Japanese threespine stickleback and those of other areas, it is essential to perform a direct comparison of European and North American threespine stickleback populations using our own electrophoretic method. In this manner it should be possible to accurately determine the evolutionary and taxonomic relationships among freshwater and anadromous populations from all over the world.

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日本産イトヨの淡水型と遡上型間の遺伝的分化

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日本産イトヨの淡水型の起源を解明するため，北日本を中心とする各地の淡水型および遡河型集団から採集した標本を用いて電気泳動法によりアイソザイム遺伝標識を検出し，集団間の遺伝的距離を推定した．淡水型と遡河型集団間の遺伝的距離の平均値は0.6746と顕著に大きく，この値は海産魚や淡水魚の同属内種間の遺伝的分化の平均的レベルに匹適した．一方，それぞれの型内での集団間の遺伝的距離の平均値は0.0006–0.0015と著しく小さかった．淡水型と遡河型集団間の遺伝的距離にもとづき推定された進化時間はおよそ340万年と極めて長く，淡水型集団が遡河型集団から隔離された時代は最後の氷河期より遙か以前であって，両集団が顕著な遺伝的分化を遂げた後，現在のような分布状態に致ったことが示唆された．

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