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Comparison of the Gene Frequency between Sympatric Populations of Ninespine Sticklebacks, Genus *Pungitius*, in Hokkaido, Japan

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Abstract Gene frequencies, estimated by electrophoretical analysis, were compared between sympatric populations of ninespine sticklebacks, *Pungitius pungitius*, *P. tymensis* and *P. sinensis*, in Hokkaido, Japan. The loci examined were *Ldh*-E, *Sod*, *Pgm* and *Mp*. Consequently, significant differences were detected between *P. tymensis* and other species in all rivers examined. This result strongly suggests that *P. tymensis* is reproductively isolated from the other species even when they coexist, although a few natural hybrids between *P. pungitius* and *P. tymensis* were found through its heterozygosity in esterase isozyme patterns and in *Pgm*. On the other hand, no significant difference was detected between *P. pungitius* and *P. sinensis* populations in the Biwase River of eastern Hokkaido. Therefore, it is suggested that they possibly belong to a single interbreeding population.

The three species of ninespine sticklebacks, *Pungitius pungitius*, *P. tymensis* and *P. sinensis*, are distributed in Hokkaido, Japan and they often coexist in many rivers in varying combinations. With respect to their classification, there have been two different views: Ikeda (1933, 1950), Ishigaki (1967) and Miyadi et al. (1967) classified them as three independent species, while Okada (1960) and Wootton (1976) classified *P. tymensis* and *P. sinensis* as subspecies of *P. pungitius*. To date, the systematic problem of them as mentioned above has not yet been resolved.

In general, one species maintains its own biological characteristics by some reproductive isolating mechanisms (Mayr, 1963). Therefore, it is important to clarify whether or not reproductive isolation exists among these three populations, especially when they coexist in rivers, in order to judge whether they should be recognized as indepndent species or intraspecific variations. Electrophorectical analysis allows one to estimate gene frequencies of enzymatic loci. If significant differences in gene frequencies are detected between sympatric populations, it is acceptable to conclude that reproductive isolation should exist between the populations and thus they may be classified as independent species.

In the present study, gene frequencies were compared between sympatric populations of the genus *Pungitius* in Hokkaido, and thereby, degrees of reproductive isolation between the populations were estimated.

Materials and methods

The ninespine sticklebacks were captured with a dip net or a casting net at five localities (Fig. 1), where two or three species were distributed sympatrically: *P. pungitius* and *P. tymensis* in the Osatsu and the Rurumappu Rivers, *P. tymensis* and *P. sinensis* in the Onnebetsu River and the upper reaches of the Bettouga River, and all three species in the middle reaches of the Biwase River. The collected samples were conveniently classified by their morphology based on the description by Ikeda (1933). The frequency distribution of lateral plate numbers in a collection consisting of *P. pungitius* and *P. sinensis* from the Biwase River is shown in Fig. 2. The count of lateral plates of the individuals, which were stained with alizarin red S, was carried out after the clearing of the bodies with 4% KOH solution. The distribution exhibited two discontinuous ranges and they corresponded to ranges for *P. pungitius* and *P. sinensis* as reported by Ikeda (1933); the low range in the former and the high range in the latter, respectively. However, some individuals with intermediate lateral plate morphology between *P. pungitius* and *P. sinensis* were collected (Fig. 3). In this study, such intermediates were excluded from

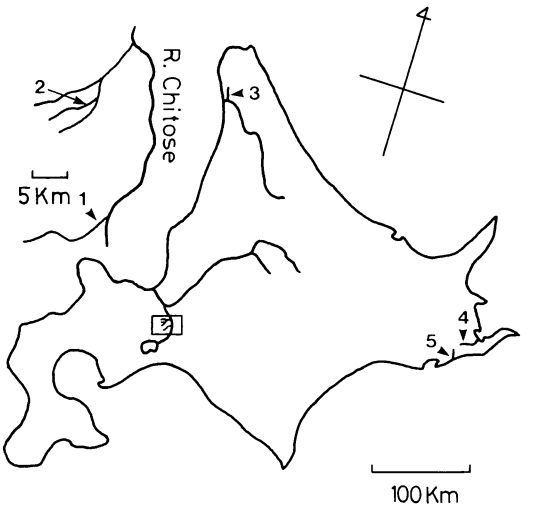

Fig. 1. Location of examined rivers in Hokkaido. 1, R. Osatsu; 2, R. Rurumappu; 3, R. Onnebetsu; 4, R. Bettouga; 5, R. Biwase.

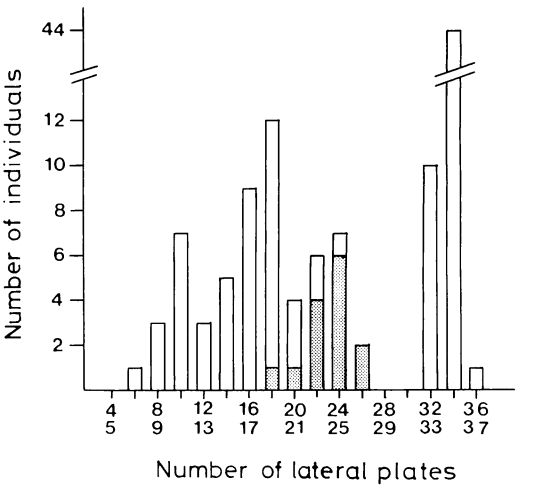

Fig. 2. Frequency distribution of lateral plate numbers in a collection consisted of *P. pungitius* and *P. sinensis* from the Biwase River. Stippled columns indicate morphologically intermediate individuals.

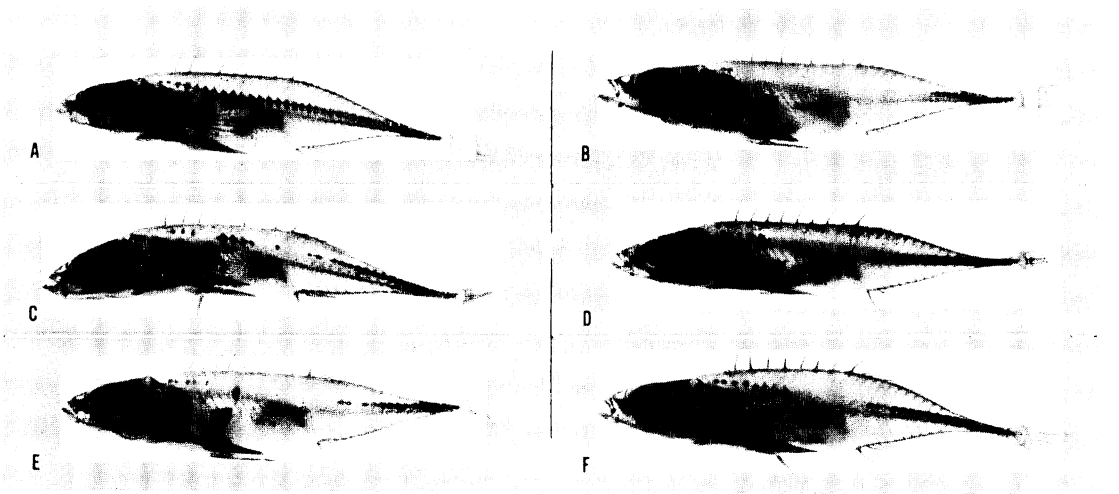

Fig. 3. Lateral plate morphology of individuals collected from the Biwase River. A, typical *P. sinensis*; B, typical *P. pungitius*; C–F, intermediate individuals. Lateral plates were stained with alizarin red S after clearing of bodies by 4% KOH solution.

the electrophoretical examination since they could not be identified as either species. Furthermore, all specimens of *P. pungitius* used corresponded to the freshwater type named by Takata et al. (1987).

The specimens captured were immediately frozen on dry ice and stored in the laboratory at −20°C until electrophoretical analysis. Eyes, brain, heart, liver, kidney, gill and a small piece of the lateral muscle were removed from the frozen fish and then the tissues, to which a small amount of distilled water was added, were minced with a pair of scissiors. The crude extracts from the tissues were absorbed by a filter paper of suitable size and then used as the electrophoretical preparation. Gel plates were prepared by adding hydrolyzed starch of Amylan (Joko Sangyo Co., Ltd.) to the appropriate gel buffer to produce a

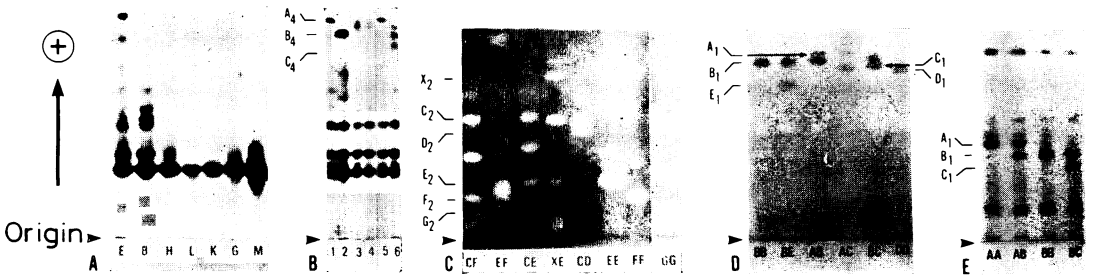

Fig. 4. Electrophoretic patterns in the three species of *Pungitius*. A, tissue distribution of LDH; B, LDH variations; C, SOD variations; D, PGM variations; E, MP variations. For panel A: E, eye; B, brain; H, heart; L, liver; K, kidney; G, gill and M, muscle. For panel B, phenotypes of each sample are AA (1 and 5), AB (3 and 4), BB (2) and AC (6). For panel C, D and E, phenotypes are shown below each sample.

concentration of 12% (W/V). Enzymes and protein examined were lactate dehydrogenase (LDH), phosphoglucomutase (PGM), superoxidedismutase (SOD), esterase (EST) and muscle protein (MP). Two buffer systems were used: citric acid-aminopropyldiethanolamine buffer (Clayton and Tretiac, 1972) for LDH and EST, and Ridgway's buffer (Ridgway et al., 1970) for PGM, SOD and MP. Horizontal electrophoresis was carried out at 4°C for 3 hours (LDH and EST) and 2 hours (PGM, SOD and MP) at 4 mA per cm^2 on the cross section of a gel plate.

Artificial hybrids were producted between *P. pungitius* and *P. tymensis* in order to analyze the isozyme patterns in EST. Adult fish of both species used for hybridization were collected from the Osatsu and the Rurumappu Rivers during the spawning season. Ripe eggs were stripped from females, while testes were removed from males and chopped using scalpel. The eggs from one species were inseminated with the testes homogenate from another species using the dry method. The fertilized egg masses were placed in plastic shalets filled with fresh water and reared at room temperature. After hatching, the larvae fed on nauplii of brine shrimp and then on live tubifex or cladoceran. When the hybrids grew up to be young, they were frozen on dry ice and then used for electrophoretic analysis.

Results

Genetic control of isozymes and protein. LDH: When comparing LDH isozyme patterns of eye, brain, heart, liver, kidney, gill and muscle, eye-specific isozymes (LDH-E) were observed in all three species, as well as in most of the other teleosts (Fig. 4A, B). They migrated more rapidly to the anode than did the isozymes from the other tissues, and are thought to be controlled by three alleles of a locus. The locus was named *Ldh*-E and each allele *a*, *b* and *c*.

SOD: SOD isozymes, which were most active in the liver, appeared in the anodal zone (Fig. 4C). The heterozygous type had three bands and so this enzyme was thought to be dimeric in subunit composition. The one locus (*Sod*) with seven alleles (*a–g*) model was presumed. However, because the frequencies of allele *a* and *b* were very low and the homodimers encoded by the two alleles migrated so closely that it was often difficult to distinguish them, alleles *a* and *b* were conveniently pooled and named *x*.

PGM: High PGM activity was detected in muscle and polymorphism was observed in the anodal zone (Fig. 4D). The heterozygous type had two bands so PGM may be monomeric in subunit composition. The one locus (*Pgm*) with five alleles (*a–e*) model was presumed for this enzyme.

MP: The polymorphism was observed in the middle of the anodal zone (Fig. 4E) and presumed to be controlled by three alleles (*a*, *b* and *c*) of one locus (*Mp*). The heterozygous type had two bands so the protein may be monomeric in subunit composition.

Gene frequency. Significant differences in gene frequencies were detected between *P. tymensis* and other species in all rivers examined (Table 1). Especially, the replacement of the allele between

Table 1. Gene frequencies at 4 loci in 11 populations of ninespine sticklebacks.

	Ldh-E			*Sod*						*Pgm*				*Mp*		
	a	*b*	*c*	*x*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*a*	*b*	*c*
R. Osatsu																
P. pungitius	0.679	0.307	0.014	—	—	1.0	—	—	—	0.007	0.972	0.021	—			
P. tymensis	1.0	—	—	—	—	1.0	—	—	—	—	—	—	1.0			
R. Rurumappu																
P. pungitius	0.649	0.351	—	—	—	1.0	—	—	—	—	1.0	—	—			
P. tymensis	1.0	—	—	—	—	1.0	—	—	—	—	—	—	1.0			
R. Onnebetsu																
P. sinensis	1.0	—	—	—	0.337	0.612	0.013	0.038	—							
P. tymensis	1.0	—	—	—	—	0.414	—	—	0.586							
R. Bettouga																
P. sinensis	1.0	—	—	—	0.179	0.076	0.245	0.50	—					0.490	0.510	—
P. tymensis	—	1.0	—	—	—	—	—	—	1.0					1.0	—	—
R. Biwase																
P. pungitius	0.988	0.012	—	0.069	0.104	0.218	0.218	0.391	—					0.119	0.881	—
P. sinensis	0.989	0.011	—	0.069	0.118	0.240	0.191	0.382	—					0.107	0.887	0.006
P. tymensis	—	1.0	—	—	—	—	—	—	1.0					1.0	—	—

Table 2. Observed and expected numbers of SOD phenotypes in the populations of *P. pungitius* and *P. sinensis* cohabiting in the Biwase River and pooled data of the two populations.

		Phenotype															N	X^2
		XX	XC	XD	XE	XF	CC	CD	CE	CF	DD	DE	DF	EE	EF	FF		
P. pungitius	Obs.	0	2	4	2	4	0	4	3	9	2	13	13	2	16	13	87	8.535
	Exp.	0.4	1.3	2.6	2.6	4.7	0.9	3.9	3.9	7.1	4.1	8.4	14.9	4.1	14.9	13.3		($p<0.5$)
P. sinensis	Obs.	0	5	3	4	8	0	8	10	11	8	12	30	5	19	21	144	9.564
	Exp.	0.7	2.3	4.8	3.8	7.6	2.0	8.2	6.5	12.9	8.3	13.2	26.4	5.3	21.0	21.0		($p<0.25$)
Total	Obs.	0	7	7	6	12	0	12	13	20	10	25	43	7	35	34	231	7.885
	Exp.	1.1	3.6	7.4	6.4	12.3	2.9	12.1	10.5	20.1	12.4	21.5	41.3	9.3	35.8	34.3		($p<0.5$)

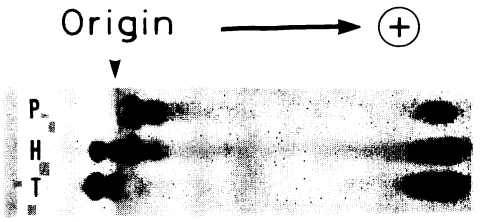

Fig. 5. EST isozyme pattern of an artificial hybrid, *P. pungitius*×*P. tymensis*. P, *P. pungitius*; H, hybrid; T, *P. tymensis*.

them was observed in the Osatsu and the Rurumappu Rivers (*Pgm*), in the Bettouga River (*Ldh*-E and *Sod*) and in the Biwase River (*Sod*). These results show that the *P. tymensis* population is reproductively isolated from both the *P. pungitius* and *P. sinensis* populations, even when they coexist. On the other hand, using the Chi-square test for homogeneity of gene frequencies (Kimura, 1960), no significant differences were detected between the *P. pungitius* population and the *P. sinensis* population coexisting in the Biwase River. Furthermore, when data from the two populations were pooled, the Chi-square values indicated that there was no significant departure of the observed phenotypic proportions from the Hardy-Weinberg expectations (Table 2, for *Sod*).

Hybrids. Unfortunately, the electrophoretical condition used in this study did not allow enough resolution to analyze the genetic controlling system of EST. Even in this condition, however, *P. pungitius* and *P. tymensis* exhibited some bands in the anodal and cathodal zones, respectively. Hybrids between the two species would be expected to exhibit heterozygotic EST patterns demonstrating some bands in both the anodal and cathodal zones. In fact, the artificial hybrids between *P. pungitius* and *P. tymensis* exhibited such heterozygotic EST patterns (Fig. 5). Four out of 131 specimens collected from the Rurumappu River exhibited heterozygosity, not only in the EST patterns, but also in *Pgm*, that allelic replacement had taken place between *P. pungitius* and *P. tymensis* in the river. Therefore, the 4 specimens were considered to be natural hybrids of *P. pungitus* and *P. tymensis*.

Discussion

From the biological species concept of "groups of interbreeding natural populations that are reproductively isolated from other such groups" defined by Mayr (1963), there is no doubt that *P. tymensis* should be classified as an independent species because of the different interbreeding population from the other cohabiting *Pungitius* population, although it rarely hybridizes with them. This view, based on the genetic evidence, agrees with the recent one that *P. tymensis* should be recognized as an independent species from the morphological and ecological point of view (Takata et al., 1984). Kobayashi (1959) suggested that natural interspecific hybridization might rarely occur between *P. pungitius* and *P. tymensis*, because of the findings that the morphologically intermediate characteristics exhibited by a few individuals collected from cohabiting rivers were quite similar to those of hybrids produced artificially. From the present results, it was confirmed that such hybridization occurred in a natural stream. In the Rurumappu River, which was a very narrow and shallow stream, and from which 4 natural interspecific hybrids were collected, the two species coexisted very closely. The rare natural hybridization between them may result from such ecological conditions as mentioned above (Hubbs, 1955).

With regard to the relationships between *P. pungitius* and *P. sinensis* populations cohabiting in the Biwase River, however, the present electrophoretic data may indicate two possibilities. First, although the two populations are separate interbreeding populations, genetic differentiation between them was not sufficiently achieved. Second, they belong to a single interbreeding population. In the Biwase River, most individauls could be identified as either species by their lateral plate morphology, except for some intermediates. Furthermore, Tanaka (1982) found that there were some morphological differences between *P. pungitius* and *P. sinensis* populations cohabiting at Hiraga, Akita Prefecture on Honshu, Main Island of Japan, and thus suggested that they might be reproductively isolated from each other. These may support the first possibility that the two cohabiting populations are separate interbreeding populations. On the other hand, the following four findings may support second possibility that they belong to a single interbreeding population: 1) Avice (1976) studied a population of threespine sticklebacks, *Gasterosteus aculeatus*, in Friant, Medera County, California, which was

composed of a great number of individuals with low or high lateral plates and an extremely few individuals with the intermediate morphology. His electrophoretical survey indicated that the low and high plate morphs were in Hardy-Weinberg equilibria when data from the two morphs were pooled, and thus suggested that the two morphs belonged to a single interbreeding population. Furthermore, his results of artificial hybridization between the two morphs showed that the F_1 hybrids were segregated into the low or high plate morphs; 2) Takata et al. (1984) reported that such morphological differences between *P. pungitius* and *P. sinensis*, as pointed out by Tanaka (1982) were not detected in the Biwase River except for the differences in lateral plate morphology; 3) Niwa and Ishigaki (unpublished) found that the adult individuals of *P. pungitius* and *P. sinensis*, collected from the Rurumappu and Shiriuchi Rivers in Hokkaido during the spawing season, crossed freely with each other in experimental aqualia; 4) Allelic frequencies at some loci of *P. sinensis* in the Biwase River were significantly different from those in the Bettouga and Oboro Rivers (Niwa, unpublished data). The three rivers are nearly adjacent, but are still completely isolated from one another. This fact suggests that allelic divergence has already been achieved among the allopatric populations.

At least in the Biwase River, therefore, it is strongly suggested that the populations of *P. pungitius* and *P. sinensis* may belong to a single interbreeding population, and that they may be segregated into the "*pungitius* type" as the low plate morph and the "*sinensis* type." as the high plate morph, as pointed out for *Gasterosteus aculeatus* (Avice, 1976). However, in the Hiraga examined by Tanaka (1982), reproductive isolation appeared to occur between *P. pungitius* and *P. sinensis*, while in the Biwase River, reproductive isolation was considered to have not occured. This inconsistency suggests that there may be geographic variations with respect to the extent of reproductive isolation between the two populations. Further population genetical studies of the two sympatric populations would offer some valuable keys to help make clear the taxonomic relationships between *P. pungitius* and *P. sinensis*.

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北海道産トミヨ属魚類の同所集団間における遺伝子頻度の比較

丹羽卓朗

北海道産トミヨ属魚類 3 種，イバラトミヨ，エゾトミヨおよびトミヨについて，北海道内の 5 河川で混生する 2 種あるいは 3 種間で，電気泳動法により推定された遺伝子頻度を比較した．調べた遺伝子座は *Ldh*-E, *Pgm*, *Sod* および *Mp* である．その結果，エゾトミヨは調査した全ての河川において，混生するイバラトミヨあるいはトミヨとは異った遺伝子頻度を示し，独立の繁殖集団を形成していると推定された．このうち，エゾトミヨとイバラトミヨが混生するルルマップ川では，エステラーゼおよび PGM の電気泳動パターンによって，131 個体中，種間雑種が 4 個体検出された．一方，琵琶瀬川で混生するイバラトミヨとトミヨの間では，遺伝子頻度に違いは見い出されなかった．さらに，両者を同一集団と仮定し計算した場合にも，ハーディ・ワインベルグの平衡にあることが認められた．従って，少なくとも琵琶瀬川においては，イバラトミヨとトミヨは同一の繁殖集団に属する可能性が示唆された．

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