Japanese Journal of Ichthyology
Vol. 34, No. 2 1987

魚類学雑誌
34巻2号 1987年

Biochemical Identification of a Brackish Water Type of *Pungitius pungitius*, and its Morphological and Ecological Features in Hokkaido, Japan

Keisuke Takata, Akira Goto and Fumio Yamazaki

(Received August 15, 1986)

Abstract A brackish water type of *Pungitius pungitius* (Gasterosteidae) is newly reported from Japan. Genetic differences, based on 13 loci of 6 enzymes and muscle protein, were examined between fresh and brackish water types in the Biwase River in the eastern part of Hokkaido. The alleles on *Gpd*, *Pgi* and *Pgm* were displaced between them, even though their distributions partially overlapped in the river. The brackish water type was also statistically different from the fresh water type on the basis of three morphological characters, such as number of dorsal spines, gill-rakers and vertebrae, although ranges overlapped. Segregation of spawning sites and differing nuptial colors of the male were found between fresh and brackish water types. These facts suggest that the brackish water type is reproductively isolated from the fresh water type and that both types should be regarded as independent species.

Pungitius pungitius has an almost continuous circumpolar distribution along the Arctic Ocean (McPhail, 1963; Münzing, 1969; Wootton, 1976). This species is also widely distributed in Hokkaido and northern part of Honshu islands, Japan. *Pungitius sinensis* closely related to *P. pungitius* is distributed in this area. *P. sinensis* was distinguished from *P. pungitius* by its complete row of lateral plates. Since Ikeda (1933) first regarded *P. pungitius* and *P. sinensis* as independent species by virtue of geographic variations in number of dorsal spines and lateral plates in Sakhalin and northern Japan, various opinions have been presented concerning their taxonomic relationship but a common agreement has not been reached (Okada, 1960; Münzing, 1969; Tanaka, 1982; Takata et al., 1984).

Because many authors concentrated attention on the taxonomic relationship between *P. pungitius* and *P. sinensis*, which exhibit a wide range of variation in morphology, little attention has been paid to intraspecific heterogeneity of *P. pungitius*, which is believed to be homogeneous in Japan. We found conspicuous populations of *P. pungitius* which were distributed in the estuary or coastal waters of the eastern part of Hokkaido. Populations living in such habitat were previously unknown in Japan. Before comparisons of genetical, morphological and ecological features were performed, this brackish water type was, for convenience, first distinguished from common fresh water *P. pungitius* populations by body color and/or habitat.

Many authors have noted the great advantages of adopting biochemical methods to distinguish such morphologically similar populations or types; e.g. making use of electrophoreticaly distinct isozymes (Hagen, 1967; Turner and Gross, 1980; Dowling and Moore, 1984; Stein et al., 1985). In this method, allelic displacement even on one locus between sympatric populations can reveal reproductive isolation. Even if partial hybridization has occurred, it is possible to discriminate F_1 hybrids and filial generations by several combinations of allelic displacement.

In the present study, in order to avoid any taxonomic confusion caused by identification depending only on morphology, we employed isozyme analysis to determine the degree of reproductive isolation between fresh and brackish water types of *P. pungitius* in a cohabited river. We furthermore examined whether these types should be regarded as independent species based on not only the extent of reproductive isolation but also comparisons of morphological and ecological features.

Materials and methods

Specimens of both types of *P. pungitius* used in the present study were collected in August 1983

and May 1984 at two stations of the Biwase River (Fig. 1). The Biwase River is a small stream running through the marsh of the Konsen lowlands in the eastern part of Hokkaido to the Pacific Ocean. This river, about 10 km in length, has developed marshes or swamps at its mouth. St. 1 was located in the mouth of the river and affected by the tides. St. 2 was located about 4 km upstream from St. 1 and scarcely affected by tides.

Before analyzing isozymes, specimens were divided into fresh and brackish water types for convenience sake. The brackish water type was distinguished from the fresh water type by its silver complexion.

All specimens were captured with a dip net and carried to the laboratory packed with dry ice in an insulated container and stored at −40°C until use for electrophoretic analysis. Livers and skeletal muscles were removed from frozen fish and minced with distilled water. Cell-lysates or drip component were directly soaked by filter paper wick (Toyo Roshi Co. No. 51A) of 3×4.5 mm in size. Thirteen loci, controlling phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), 6 phosphogluconate dehydrogenase (PGD), α-glycerophosphate dehydrogenase (GPD), malate dehydrogenase (MDH), superoxide dismutase (SOD) and muscle protein (MP), were analyzed by electrophoresis. Two buffer systems described by Ridgway et al. (1970) for PGM, PGD, SOD and MP and by Clayton and Tretiak (1972) for PGI, MDH and GPD were used in the course of the present study. Starch gel plates were prepared by adding 12% Amylan (Jookoo Industry Co. Lot No. 1207003) to the appropriate gel buffer. Horizontal electrophoresis was carried out at 4°C, 4 mA/cm^2 for about 2 hours for enzymes using the buffer system of Ridgway et al. (1970), and for 3 hours for enzymes using the buffer system of Clayton and Tretiak (1972). The staining procedure followed Shaw and Prasad (1970) with slight modifications. The zygotic frequencies in each of the types were compared to those expected from Hardy-Weinberg equilibrium by G test and differences of genotypic frequency on polymorphic loci between the types were examined by chi square test (Sokal and Rolf, 1981).

All specimens used were preserved in 10% formalin for morphological analysis. Counts were made based on Hubbs and Lagler (1947), after staining with Alizarin Red S and clearing with 2% KOH and 0.1% H_2O_2. Differences of mean in each character of the both types were compared by t-test (Sokal and Rolf, 1981).

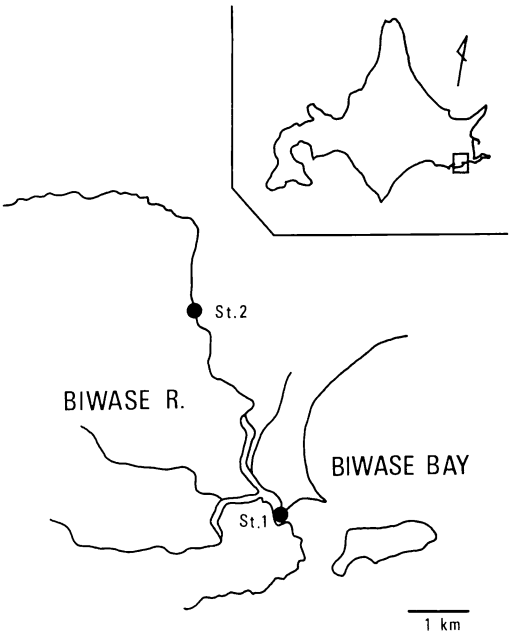

Fig. 1. Map of the Biwase River and collection sites of two types of *Pungitius pungitius*.

Ecological surveys of fresh water and brackish water populations were performed during the breeding season (late May 1984) and non-breeding season (August 1983) in the Biwase River. Geographic distribution of two types of *P. pungitius* was surveyed on the samples collected throughout Hokkaido.

Results

In the Biwase River, the brackish water type of *P. pungitius*, accounting for 47 of a total of 50 specimens, was predominant in St. 1. On the other hand, the fresh water type, accounting for 38 of 39 specimens, was dominant in St. 2.

Genetic features. Six enzymes and muscle proteins examined in the present study are described briefly below. The assumption of loci and their alleles was consistent with the known subunit structure of each enzyme (Harris and Hopkinson, 1976).

MDH: The products of the muscle-specific locus *Mdh*-I were found to move rapidly toward the

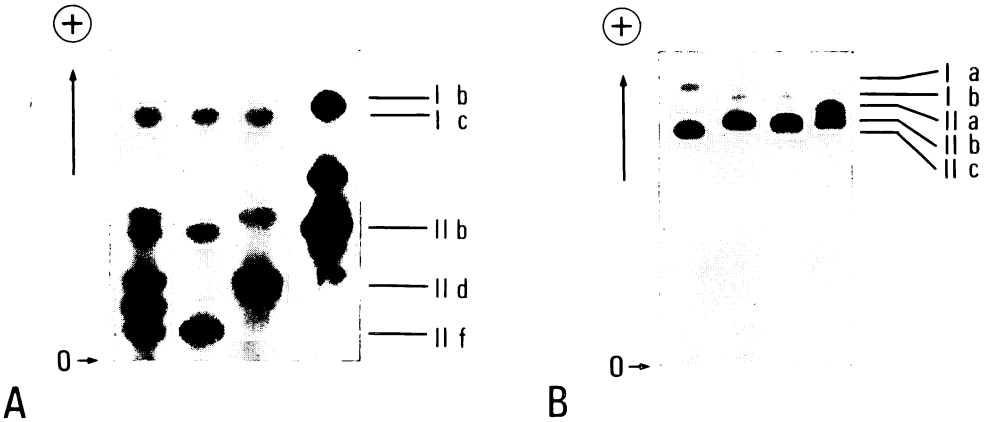

Fig. 2. Electrophoretic patterns of PGI in muscle (A) and PGM in liver (B) of *Pungitius pungitius*.

anode, and those of the liver-predominant loci *Mdh*-II and *Mdh*-III migrated more slowly. The *Mdh*-I and *Mdh*-II were represented by a single invariant band, whereas, *Mdh*-III locus was polymorphic with two alleles (a and c) in the present populations.

GPD: This dimeric enzyme had low anodal mobility and was detected in muscle extracts. The extracts of all specimens examined showed only one band but displacement of alleles (c and d) was observed in the present populations.

PGD: The activity of this enzyme was observed in both liver and muscle extracts. Heterozygotes on the *Pgd* locus had three bands, indicating the dimeric structure of this enzyme, and two alleles (a and c) were assumed from the zygograms.

PGI: The activity of this dimeric enzyme was found in muscle extracts (Fig. 2). The products

Table 1. Frequency of allele on 13 loci of fresh and brackish water types of *Pungitius pungitius* in the Biwase River.

Locus		Fresh water type (N=38)	Brackish water type (N=47)	Locus		Fresh water type (N=38)	Brackish water type (N=47)
Mdh-I	a	1.000	1.000	*Pgm*-I	a	1.000	0.000
					b	0.000	1.000
Mdh-II	a	1.000	1.000				
				Pgm-II	a	0.000	0.220
Mdh-III	a	1.000	0.872		b	0.015	0.700
	c	0.000	0.128		c	0.970	0.080
					e	0.015	0.000
Gpd	c	1.000	0.000				
	d	0.000	1.000	*Sod*	a	0.017	0.000
					b	0.117	0.000
Pgd	a	1.000	0.900		c	0.416	0.970
	c	0.000	0.100		d	0.117	0.030
					e	0.333	0.000
Pgi-I	b	1.000	0.000				
	c	0.000	1.000	*Mp*-I	a	1.000	0.080
					b	0.000	0.920
Pgi-II	b	0.985	0.090				
	d	0.015	0.610	*Mp*-II	b	0.045	0.920
	f	0.000	0.300		c	0.955	0.080
				Mp-III	a	0.182	0.000
					b	0.818	1.000

of *Pgi*-I moved rapidly toward the anode, and those of *Pgi*-II migrated more slowly. *Pgi*-I was represented by a single band, but, displacement of alleles (b and c) was observed in the present populations. Heterozygotes on the *Pgi*-II locus showed highly polymorphic and three alleles (b, d and f) were supposed on the locus.

PGM: This enzyme was detected in extracts of liver (Fig. 2). The heterozygotes exhibited a two band pattern, consistent with the known monomorphic structure of this enzyme. Two alleles (a and b) and four alleles (a, b, c and e) were assumed on *Pgm*-I and II respectively.

SOD: The activity of this enzyme was found in liver extracts. The locus *Sod* exhibited highly polymorphic and heterozygotes showed three-band patterns indicating dimeric enzyme structure. Five alleles (a, b, c, d and e) were supposed on the locus from their electrophoretic patterns.

MP: Muscle proteins were assumed to be controlled by three loci, *Mp*-I, *Mp*-II and *Mp*-III. The products of *Mp*-I had highest mobility toward the anode, and those of the other two loci migrated more slowly. Since heterozygotes showed two bands pattern on these three loci, muscle protein structure was assumed to be monomeric. Two alleles (a and b or b and c) were assumed in each locus.

Observed frequencies of alleles for 13 examined loci are given in Table 1. The phenotypic patterns for 5 out of 13 loci were monomorphic in the present populations: *Mdh*-I, *Mdh*-II, *Gpd*, *Pgi*-I and *Pgm*-I. The other 8 loci showed polymorphism. Comparison of observed genotypic frequencies with Hardy-Weinberg expectations showed no statistically significant deviations in any loci of either population.

As for the loci *Gpd*, *Pgi*-I and *Pgm*-I, displacement of alleles were observed between the fresh water and brackish water types. The fresh water type, predominant at St. 2, had *Gpd*-c, *Pgi*-I-b and *Pgm*-I-a, whereas the brackish water type, predominant at St. 1, had different alleles *Gpd*-d, *Pgi*-I-c and *Pgm*-I-b respectively. Significant differences in allelic frequency of the loci *Mdh*-III ($P<0.01$), *Pgi*-II, *Pgm*-II, *Sod*, *Mp*-I, *Mp*-II and *Mp*-III ($P<0.001$), were also detected. Out of 89 individuals examined, no hybrid between the two types was confirmed by isozyme analysis.

Morphological features. Morphological com-

Table 2. Number of lateral plates, dorsal spines, gill-rakers and caudal and total vertebrae of fresh and brackish water types of *Pungitius pungitius* in the Biwase River.

	N	No. of lateral plates																											M	SE
		7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33		
Fresh water type	38	1						1	1	4	4	1	2	3	3	3	3	5	1	1		1			1		2	1	19.53	0.93
Brackish water type	47										1	1	3	7	10	6	9	6		2	2								19.94	0.32

	N	No. of dorsal spines				M	SE	No. of gill-rakers					M	SE
		8	9	10	11			11	12	13	14	15		
Fresh water type	38	3	31	4		9.03	0.07	4	25	7	2		12.18	0.11
Brackish water type	47		10	28	9	9.98	0.09	3	16	19	7	2	12.77	0.14

	N	No. of caudal vertebrae				M	SE	No. of total vertebrae					M	SE
		16	17	18	19	20		32	33	34	35			
Fresh water type	38			2	25	11	19.24	0.09		6	27	5	33.97	0.09
Backish water type	47	3	12	26	6		17.74	0.11	16	23	7	1	32.85	0.11

Fig. 3. Male of brackish water type of *Pungitius pungitius* (52 mm SL), with nuptial color just turning to black on the ventral side.

parisons between fresh and brackish water types in the Biwase River are shown in Table 2.

The number of lateral plates ranged from 7 to 33 in the fresh water type and 15 to 25 in the brackish water type. No significant difference was found between the averages of fresh (19.53) and brackish (19.94) water types.

The number of dorsal spines, 8 to 10, in the fresh water type partly overlapped with that of brackish water type, 9 to 11. The average number of dorsal spines of the brackish water type (9.98) was significantly larger than that of the fresh water type (9.03, $P<0.05$).

On the other hand, the number of dorsal, anal, and pectoral rays of both types overlapped in their ranges with no significant difference in their averages.

Number of gill-rakers, 11 to 14, in fresh water type fell within the range of the brackish water type, 11 to 15. A significant difference in average number of gill-rakers was detected between fresh (12.18) and brackish (12.77) water types ($P<0.05$).

Number of abdominal vertebrae ranged from 14 to 15 in the fresh water type, similar to that of the brackish water type (14 to 16). No significant difference was found between averages for fresh (14.71) and brackish (15.11) water types. Number of caudal vertebrae ranged from 18 to 20 in the fresh water type, partly overlapping the range of the brackish water type, 16 to 19. The average for the brackish water type (17.74), was however, significantly less than that of fresh water type (19.24, $P<0.05$). Total number of vertebrae, 33 to 35, in fresh water type fell within the range of brackish water type, 32 to 35. But, reflecting the smaller average number of caudal vertebrae, the average of total number of vertebrae in the brackish water type (32.85) was significantly smaller than that of the fresh water type (33.97, $P<0.05$).

In spite of such differences in the calculated averages, it was quite difficult to discriminate fresh and brackish water types individually on the basis of meristic characters alone.

Ecological features. Two ecological features, male nuptial color and distribution of mature individuals in the Biwase River were compared between fresh and brackish water types. Males of the brackish water type possessed quite different nuptial color compared with other *Pungitius* in Japan. Although the entire body of males of the fresh water type and other *Pungitius* species in Japan turns to black, males of the brackish water type maintain their usual silver coloration, the ventral side only turning to black during the breeding season (Fig. 3).

Both females with ripe eggs and males with nuptial color of the brackish water type were captured exclusively in the estuary of the Biwase River during the breeding season. On the other hand, mature males and females of the fresh water type were restricted to fresh water. This habitat difference was also maintained outside the breeding season.

Geographic distribution. Genetic discrimination between fresh and brackish water types was without exception coincident with the conventional one of body coloration. So, on the basis of body color, we tried reexamining specimens which were

captured in Hokkaido and identified as *P. pungitius* by Takata et al. (1984), if they included the brackish water type. In addition, further field surveys on the rivers adjacent to the Biwase River were performed in order to obtain more accurate information on the distribution range of the brackish water type. Fish of the brackish water type was found only in the Bekanbeushi, Charo, Onbetsu and Atsunai Rivers and the Notsuke and Akkeshi Bays. Their distribution was completely restricted to the estuary and coastal waters of the eastern part of Hokkaido (Fig. 4). This is the first record of *Pungitius* inhabiting coastal waters of Japan.

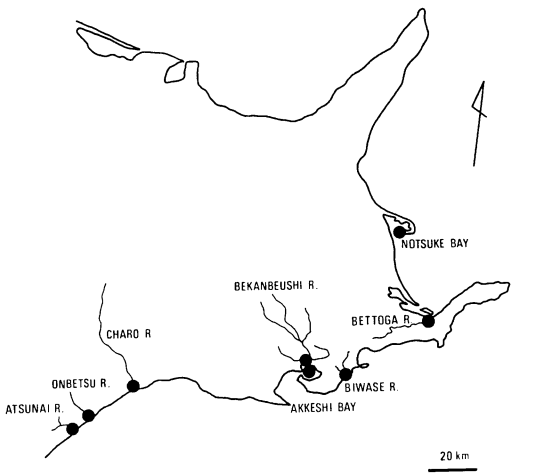

Fig. 4. Geographic distribution of the brackish water type of *Pungitius pungitius* in Hokkaido. Solid circles indicate the localities where this type of fish was caught.

Discussion

In the Biwase River, evident displacement of alleles and no hybrids were found between fresh and brackish water types which should be identified as *Pungitius pungitius* by the lateral plate morphology (Ikeda, 1933; Miyadi et al., 1976). It is supposed that a marked or complete deficiency of heterozygotes between two sympatric populations means that they lack the ability for exchange of genes through interbreeding, and thus they are isolated reproductively (Dowling and Moore, 1984). So, it is reasonable to consider that in the Biwase River the fresh water and brackish water types are different interbreeding populations and there should be some reproductive isolation mechanisms between them.

The brackish water type appears to build nests and spawn only in brackish waters, whereas the fresh water type spawns in fresh water in the Biwase River. But, some individuals of both types, though they were not mature, were found in the habitat of the other. This meaningful overlap of distribution suggests that reproductive isolation between fresh and brackish water types is maintained not only by the difference of habitat but also by some mechanisms acting in the same habitat. Especially, the difference in nuptial coloration between the two types suggests that ethological mechanisms might maintain reproductive isolation between them even if there is a chance to encounter each other during the breeding season.

According to the biological species concept as presented by Mayr (1963), such observations as not only reproductive isolation but also conspicuous morphological and ecological differences between sympatric populations may suggest that they should be regarded not as variations within a same species but as independent species. It is acceptable, therefore, that fresh and brackish water types of *P. pungitius* should be distinguished as different species.

Concerning the taxonomic status of these two types of *P. pungitius*, the fresh water type corresponds to common *P. pungitius*, which has been known as "ibaratomiyo" in Japanese name, because its distribution in fresh water and blotched yellowish pale green lateral color pattern are coincident with descriptions of common *P. pungitius* reported from Japan (Okada, 1960; Miyadi et al. 1976). Furthermore, in the Biwase River, some allelic displacement was detected between brackish water type and other cohabiting species of sticklebacks, *P. sinensis* and *P. tymensis* (Takata, unpublished data). Therefore, the brackish water type should be regarded as a new independent species of *Pungitius* in Japan.

One of the most noticeable results concerning the distribution of the brackish water type is the discovery of coastal populations in the Notsuke and Akkeshi Bays. Artificially fertilized egges of the brackish water type are able to hatch in sea water (Takata, nupublished data). So, these findings suggest that the brackish water type is highly tolerant of sea water and therefore can alter their distribution ranges by emigration through

the sea.

Tidal and fresh water populations of *P. pungitius*, called Bering form and Mississippi form respectively, were also reported from North America (McPhail, 1963). The relationships between the two forms in habitat are similar to that of the brackish and fresh water types in Hokkaido. One should, therefore, consider whether the Bering form and brackish water type belong to the same group, since both are able to disperse to other regions through the sea.

Comparing morphological and ecological features, the following differences are found. Modes of number of lateral plates of the Bering form only varied from 3 to 5 in North America, whereas the mode of the brackish water type in the Biwase River is 19. Bering and Mississippi forms easily hybridize in natural cohabiting rivers (Narita, 1970), but the brackish water type and fresh water type rarely mate. Such differences in morphological variations and the degree of reproductive isolation with fresh water type and inland form suggest that the brackish water type might be distinguishable from the Bering form. In order to make clearer the taxonomic relationship, further investigations concerning the distribution and genetic relationship between the brackish water type and Bering form from the Kuril Islands to the Aleutian Islands are needed.

Acknowledgments

We are grateful to Dr. Keikichi Hamada, former Professor of Hokkaido University, Dr. Hiroshi Onozato of the Inland Station, National Research Institute of Aquaculture and Dr. Kenkichi Ishigaki of Tomakomai Experiment Forest, Hokkaido University, who provided many valuable suggestions. We express thanks to Mr. John Goodier who corrected the manuscript. We are indebted to Dr. Kazuya Nagasawa of Hokkaido Fisheries Experimental Station and Messrs. Toshihiro Mizushima of the same station, Akihisa Iwata of the Crown Prince's Palace and Makoto Matsuoka of our laboratory for their help in collecting materials. The field work in the present study was partly supported by a grant-in-aid (No. 58121004) from the Ministry of Education, Science and Culture, Japan.

Literature cited

Clayton, J. W. and D. N. Tretiak. 1972. Amine-citrate buffer for pH control in starch gel electrophoresis. J. Fish. Res. Bd. Can., 29(8): 1167–1172.

Dowling, T. E. and W. S. Moore. 1984. Level of reproductive isolation between two cyprinid fishes, *Notropis cornutus* and *N. chrysocephalus*. Copeia, 1984(3): 617–628.

Hagen, D. W. 1967. Isolating mechanisms in three-spine sticklebacks (*Gasterosteus*). J. Fish. Res. Bd. Can. 24(8): 1637–1692.

Harris, H. and D. A. Hopkinson. 1976. Handbook of enzyme electrophoresis in human genetics. North Holland Publ. Co., Amsterdam.

Hubbs, C. L. and K. F. Lagler. 1947. Fishes of the Great Lakes region. Bull. Cranbrook Inst. Sci., 26: xi+186 pp., 26 pls.

Ikeda, K. 1933. The distribution and variation of Gasterosteidae in Japan. Zool. Mag., Tokyo, 45: 141–173. (In Japanese.)

Mayr, E. 1963. Animal species and evolution. Harvard Univ. Press, Cambridge, 797 pp.

McPhail, J. D. 1963. Geographic variation in North American ninespine sticklebacks, *Pungitius pungitius*. J. Fish. Res. Bd. Can., 20(1): 27–40.

Miyadi, D., H. Kawanabe and N. Mizuno. 1976. Coloured illustrations of the freshwater fishes of Japan. Hoikusha, Osaka, 462 pp.

Münzing, J. 1969. Variabilität, Verbreitung und Systematik der Arten und Unterarten in der Gattung *Pungitius* Coste, 1848 (Pisces, Gasterosteidae). Z. Zool. Syst. Evol. Forsch., 7: 208–233.

Narita, T. 1970. Physiological, ecological and morphological differences between two forms of nine-spine stickleback *Pungitius pungitius*, in North America. Ph. D. Thesis., Univ. of Manitoba, Winnipeg, vii+90 pp.

Okada, Y. 1960. Studies on the freshwater fishes of Japan II. Special Part. J. Fac. Fish., Pref. Univ. Mie, 4(3): 589–860.

Ridgway, G. J., S. W. Sherburne and R. D. Lewis. 1970. Polymorphism in the esterases of Atlantic herring. Trans. Amer. Fish. Soc., 99(1): 147–151.

Shaw, C. R. and R. Prasad. 1970. Starch gel electrophoresis of enzymes—a compilation of recipes. Biochem. Genet., 4: 297–320.

Sokal, R. and F. J. Rolf. 1981. Biometry. Second edition. W. H. Freeman Co., San Francisco, xviii+859 pp.

Stein, D. W., J. S. Rogers and R. C. Cashner. 1985. Biochemical systematics of the *Notropis roseipinnis* complex (Cyprinidae: Subgenus *Lythrurus*). Copeia, 1985(1): 154–163.

Takata, K., A. Goto and K. Hamada. 1984. Geographic distribution and variation of three species of

ninespine sticklebacks (*Pungitius tymensis*, *P. pungitius* and *P. sinensis*) in Hokkaido. Japan. J. Ichthyol., 31(3): 312–326. (In Japanese with English abstract.)

Tanaka, S. 1982. Variations in ninespine sticklebacks, *Pungitius pungitius* and *P. sinensis*, in Honshu, Japan. Japan. J. Ichthyol., 29(2): 203–212. (In Japanese with English abstract.)

Turner, B. J. and D. J. Gross. 1980. Trophic differentiation in *Ilyodon*, a genus of stream-dwelling goodeid fishes: speciation versus ecological polymorphism. Evolution, 34(2): 259–270.

Wootton, R. J. 1976. The biology of the sticklebacks. Academic Press, London, x+387 pp.

(Laboratory of Embryology and Genetics, Faculty of Fisheries, Hokkaido University, Hakodate 041, Japan; KT, present address: Department of Biology, Faculty of Science, Shinshu University, Matsumoto 390, Japan)

イバラトミヨ *Pungitius pungitius* の汽水型個体群の生化学的同定，およびその形態的，生態的特徴

髙田啓介・後藤　晃・山崎文雄

北海道東部の琵琶瀬川において，主に淡水域に分布する通常の体色を有するイバラトミヨ（淡水型）の他に，体側が銀白色を呈する個体（汽水型）が河口域から多数見いだされた．これらの汽水型は鱗板形態から鑑別するとイバラトミヨに同定される．しかし，アイソザイムを用いた集団遺伝学的解析の結果，調査した 13 遺伝子座のうち，*Pgi*-I, *Pgm*-I および *Gpd* の 3 座において，淡水型との間に対立遺伝子の置換が認められた．このことは両型が同一河川において遺伝的交流を欠き，その間に生殖的隔離が存在することを示している．そして，汽水型は，従来日本でイバラトミヨと呼ばれてきた集団（淡水型）とだけでなく，北米に分布する Bering form との間でも形態的・生態的特徴が異なることから，これらとは別の独立種である可能性が示唆された．汽水型の日本における分布域は，北海道東部の太平洋から根室海峡に面した河川や内湾に限られている．

(041 函館市港町 3-1-1　北海道大学水産学部発生学・遺伝学講座；髙田，現住所：390 松本市旭 3-1-1 信州大学理学部生物学教室)