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Electrophoretic Discrimination of *Tribolodon* Species (Cyprinidae) and the Occurrence of Their Hybrids*

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Abstract Electrophoretic discrimination was carried out on three species of the genus *Tribolodon* (Cyprinidae), *T. hakonensis*, *T. brandti* and *T. ezoe*, by examining five loci controlling lactate dehydrogenase (LDH), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), and muscle protein (MP). Consequently, a clear-cut identification of the three species was possible even in 0^+ young which were difficult to distinguish morphologically. Moreover, 1.0–19.9% of fish examined were detected electrophoreticaly as hybrids which had been previously undetected. The existence of such spontaneous hybrids has further complicated the classification of the genus *Tribolodon*. In spite of imperfect reproductive isolation, the electrophoretic data supports the independent nature of the three species of the genus *Tribolodon*.

The genus *Tribolodon* is a unique group in the large family Cyprinidae because of its variation in life type from a fluvial or residual to an anadromous mode of life (Nakamura, 1969). Not only are the members of the genus morphologically very similar, but also each of them exhibits a wide range of variation in meristics. Therefore, they can not be easily distinguisned and various opinions have been presented about their classification (e.g. Jordan and Fowler, 1903; Tanaka, 1931; Ikeda, 1936, 1938; Okada and Ikeda, 1937; Kanoh, 1949; Nakamura and Mochizuki, 1953; Onodera and Honma, 1976).

Recently, nominal species have been reclassified into four species, *T. hakonensis* (Günther), *T. brandti* (Dybowsky) (species name see Jeon and Sakai, 1984), *T. ezoe* Okada et Ikeda and *T.* sp. This has been done by using some qualitative characteristics such as the cephalic lateral-line system (Nakamura, 1963; Kurawaka, 1977), morphology of the gas-bladder (Kahata, 1981; Churikov and Sabitov, 1982), or the spawning color (Nakamura, 1969; Gritsenko, 1974).

Nevertheless, identification of *Tribolodon* species is accomplished with some difficulty. Much overlapping in meristics and intermediate character states in qualitative characters are seen among these species. This is particulary true of juveniles in which the key characters are incompletely developed. Moreover, the classification would be further complicated if the previously unknown hybrids were taken into consideration.

Many authors have suggested the great advantages of adopting the biochemical method to distinguish such morphologically similar species; e.g. making use of electrophoreticaly distinct isozymes. It has been also emphasized that such isozymes are highly useful to identify hybrids (e.g. Asspinwall and Tsuyuki, 1968; Nyman, 1970; Brassington and Ferguson, 1976; Fujio, 1977). In this method, one reliable allelic displacement among species can make a clear-cut identification of hybrids as clearly as species. By examining plural loci bearing allelic displacement, it is even more possible to distinguish F_1 hybrids and later filial generations (Brassington and Ferguson, 1976; Fujio, 1977).

Also, in the genus *Tribolodon*, the availability of allelic displacement in some enzyme and protein loci has been suggested to identify particular species (Hanzawa and Taniguchi, 1982a, b; Bushuyev *et al.*, 1980; Gavrenkov *et al.*, 1984).

With this context in mind, we tried to classify biochemically three species of *Tribolodon* from Hokkaido, *T. hakonensis*, *T. brandti* and *T. ezoe*, by using isozymes and protein alleles detected through starch gel electrophoresis. Consequently, the advantage of this method was reconfirmed in identifying such morphologically similar species as those of *Tribolodon*. Many hybrid fish previously undistinguished were clearly discriminated by biochemical markers. Then the intermediacy of these hybrids was reexamined in terms of meristic characteristics.

* Studies on the Freshwater Fishes in Hokkaido, Japan—V.

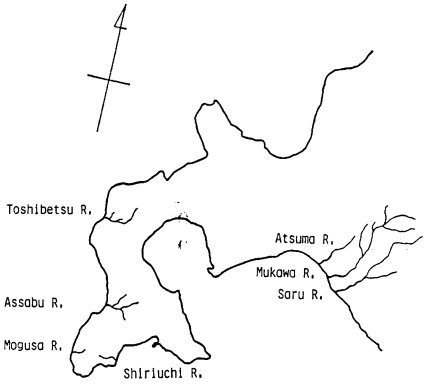

Fig. 1. Map showing surveyed rivers in southern Hokkaido, Japan.

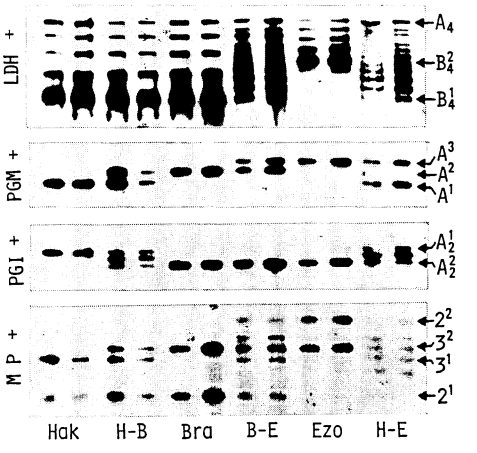

Fig. 2. Electrophoretic patterns of lactate dehydrogenase (LDH), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI) and muscle protein (MP) in *Tribolodon hakonensis* (Hak), *T. hakonensis* × *T. brandti* (H-B), *T. brandti* (Bra), *T. brandti* × *T. ezoe* (B-H), *T. ezoe* (Ezo), and *T. hakonensis* × *T. ezoe* (H-E). The hybrids were artificially made.

Materials and methods

Seven rivers were surveyed; the Mukawa, Saru, Atsuma, Toshibetsu, Assabu, Mogusa and Shiriuchi (Fig. 1). More intense samplings were performed on the Mukawa River because it contained a large stock of the three species of *Tribolodon*. The sampling dates in this river were as follows; 0^+ young in Oct., 1982, Apr. and Oct., 1983, spawning adults in Jun., 1983, and parental fish for artificial hybrids in Jun., 1982. The other rivers were surveyed only in Oct. of 1983 and from them we collected 0^+ young.

The fish were frozen with dry ice immediately after being caught and were carried to the laboratory. A small piece of lateral muscle was removed and stocked at −20°C for electrophoretic analysis, and the remaining body was fixed with 10% formalin for morphological examination.

At the first electrophoretic inspection, the allelic displacement was checked by examining the specimens of three species from the Mukawa River identified beforehand by the spawning color. *T. hakonensis* from the Mogusa River, and *T. ezoe* from the upper stream of the Atsuma River were also used to check the allelic displacement because only one species is isolated in these rivers. Secondly, the artificial hybrids were examined to check for heterozygotic isozyme patterns. Then the other fish were electrophoreticaly analyzed and it was determined to which species or hybrid they belonged. Individuals were identified by species-specific bands. Those which demonstrated heterozygotic patterns in all marker loci were identified as F_1 hybrids. Fishes demonstrating heterozygousity in a part of all marker loci were judged as backcross, F_1 hybrids, or later filial generations.

Clearly identified fishes were then used for morphological analysis. The connection of canals between postocular commisure (POC) and preoperculomandibular canal (POM) was observed in adult specimens. The number of scales were counted on a lateral-line series and on a predorsal series for countable larger specimens. However, those hybrids of *T. hakonensis* × *T. ezoe* were not counted because of a scanty sample of small fish.

Electrophoreticaly analyzed loci were restricted to those controlling lactate dehydrogenase (LDH), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI) and muscle protein (MP). Other than the locus controlling PGI, the same allelic displacement as in this study was reported on the *Tribolodon* species from Fukushima Prefecture (Hanzawa and Taniguchi, 1982a, b). Horizontal starch gel electrophoresis was carried out at 4°C, 4 mA/cm² for about 2 hours in a 12% gel of Amylan (Joko Industry Co.), Starch-Hydrolyzed (Connaught Laboratory Ltd.) or Hydrolyzed starch (Toyo Scientific Industry Co.) through the discontinuous buffer system described by Ridgway *et al.* (1970).

Table 1. Observed genotypic frequencies for five loci diagnostic of *Tribolodon hakonensis* (H), *T. brandti* (B), *T. ezoe* (E) and their hybrids (HB, HE and BE), in the Mukawa (Muk), Saru (Sar), Atsuma (Ats), Toshibetsu (Tos), Assabu (Ass), Shiriuchi (Shi) and Mogusa (Mog) rivers.

	N	*Ldh*-A			*Pgm*-A					
		H, B	E	HE, BE	H	B	E	HB	HE	BE
1982 10, Muk	207	0.599	0.280	0.121	0.126	0.420	0.285	0.053		0.116
1983 5, Muk	349	0.562	0.407	0.031	0.239	0.241	0.410	0.074	0.003	0.034
1983 6, Muk	191	0.429	0.560	0.011	0.277	0.115	0.560	0.037		0.011
1983 10, Muk	390	0.815	0.177	0.008	0.408	0.341	0.174	0.064		0.013
Sar	110	0.655	0.327	0.018	0.645		0.318	0.009		0.027
Ats	101		1.000				1.000			
Tos	110	0.427	0.518	0.055	0.391	0.027	0.518	0.009	0.055	
Ass	98	0.316	0.684		0.269	0.010	0.684		0.010	
Shi	100	0.670	0.310	0.020	0.670		0.310		0.020	
Mog	99	1.000			1.000					

	N	*Pgi*-A			*Mp*-2			*Mp*-3		
		H	B, E	HB, HE	H, B	E	HE, BE	H	B, E	HB, HE
1982 10, Muk	207	0.125	0.828	0.047	0.604	0.275	0.121	0.126	0.831	0.043
1983 5, Muk	349	0.238	0.702	0.060	0.544	0.404	0.052	0.238	0.699	0.063
1983 6, Muk	191	0.280	0.691	0.029	0.429	0.560	0.011	0.277	0.697	0.026
1983 10, Muk	390	0.415	0.510	0.075	0.813	0.174	0.013	0.408	0.513	0.079
Sar	110	0.627	0.346	0.027	0.655	0.336	0.009	0.628	0.345	0.027
Ats	101		1.000			1.000			1.000	
Tos	110	0.400	0.555	0.045	0.427	0.518	0.055	0.391	0.554	0.055
Ass	98	0.306	0.684	0.010	0.306	0.684	0.010	0.296	0.694	0.010
Shi	100	0.670	0.310	0.020	0.670	0.310	0.020	0.670	0.310	0.020
Mog	99	1.000			1.000			1.000		

The staining procedure followed the method of Shaw and Prasad (1970), with slight modifications.

Results

Genetic control of isozyme and protein. LDH: Tetrameric isozymes formed by random association of LDH-A and -B subunits were observed (Fig. 2). The allele on the *Ldh*-A locus controlling the LDH-A system was monomorphic and ubiquitous in the three species, in comparison to that on the *Ldh*-B locus controlling the LDH-B system which was displaced between *T. ezoe* (*Ldh*-b_2) and the other species (*Ldh*-b_1). Homo-tetrameric LDH-B_4^2 isozyme in *T. ezoe* moved more anodaly than LDH-B_4^1 in the other species. Presumed hybrids between *T. ezoe* and the other species were expected to exhibit 15 bands of random association among three subunits, LDH-A, -B_1 and -B_2. Artificial hybrids expressed about 12 bands which split incompletely.

PGM: The monomeric PGM-A system was only demonstrated in the muscles. The allele on the *Pgm*-A locus was interspecifically displaced in all three species, *Pgm*-a_1 in *T. hakonensis*, -a_2 in *T. brandti*, and -a_3 in *T. ezoe*. Therefore, each species could be easily distinguished. The artificial hybrids demonstrated both paternal and maternal bands, as was expected.

PGI: Dimeric isozymes formed by random association of PGI-A and -B subunits were observed in the muscle. However, only homo-dimeric isozymes of the PGI-A system appears in Fig. 2, because it involved allelic displacement. The PGI-A_2^1 isozyme controlled by the *Pgi*-a_1 allele in *T. hakonensis* moved more anodaly than the PGI-A_2^2 isozyme controlled by the *Pgi*-a_2 allele in the other species. Hanzawa and Taniguchi (1982a) did not observe such displacement in these species from Fukushima Prefecture. The hybrids between *T. hakonensis* and the other species showed three bands formed by random association of PGI-A_1

and -A_2 subunits, as was expected.

MP: Five bands were observed in each species, but only MP-2 and -3 are represented in Fig. 2 as they expressed the allelic displacement. MP-2^2 of *T. ezoe* moved more anodaly than MP-2^1 of the other species. Whereas in MP-3, the band was more cathodaly displaced in *T. hakonensis* (MP-3^1) than that in the other species (MP-3^2). Hybrids between two of the three species were expected to demonstrate both paternal and maternal bands, as did the artificial hybrid betweeen *T. hakonensis* × *T. brandti*. In the other two combinations, two bands were also found on the expected pattern. Although the genetic background of these additional bands is obscure, it is possible to identify these hybrids as clearly as their parental species.

Occurrence pattern of species and hybrids. Observed genotypic frequencies for examined loci are given in Table 1. Table 2 shows the number of 0^+ young classified to each species and hybrids in all surveyed rivers. Table 3 denotes the change of the occurrence pattern by year and/or between 0^+ young and adult in the Mukawa River.

The frequencies of genotypes indicating diagnosis of each species were high compared to those of hybrids in all loci of all samples (Table 1). Pooling the three species and hybrids, the population was so deviated from the Hardy-Weinberg equilibria that no statistical analysis was necessary. One hundred and sixty-six of 1,755 individuals had one to five heterozygotic loci (Fig. 3). About half of them (82 individuals) indicated the hybrid pattern in all displaced loci (three or five) between species, and they were judged as F_1 hybrids. Individuals with no heterozygotic loci (1,589 individuals) were identified with each species. The other individuals were grouped as backcross hybrids because they showed heterozygous in only one or two loci (84 individuals).

The occurrence proportion of the three species varied from river to river. Only one species was detected from the Mogusa River and the upper stream of the Atsuma River, as mentioned in the materials and methods. *T. brandti* was not detected from the Saru and Shiriuchi rivers, but it definitely existed in the Saru River because several hybrid individuals between *T. brandti* and the other species were collected. In the other three rivers, all three species were distributed in various proportions. The Mukawa River had a much larger stock of *T. brandti* than the other rivers.

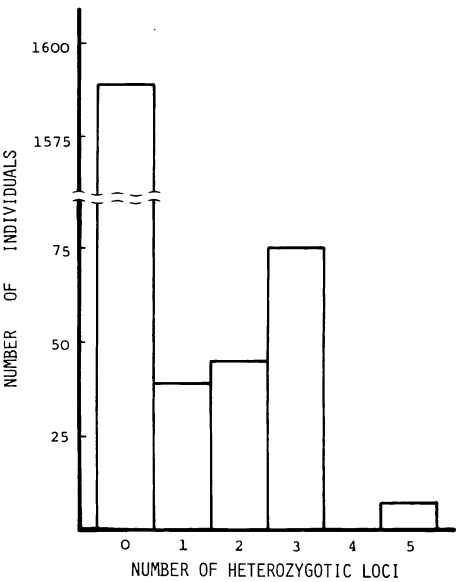

Fig. 3. Distribution of individuals having heterozygotic loci in all *Tribolodon* species examined (1,755 individuals).

The hybridization rate was also different among the rivers. The lowest recorded was 1.0% in the Assabu River (excepting the monospecific rivers, 0%), whereas 19.9% of the sample from the Mukawa River in Oct., 1982 were judged as hybrids. In the Mukawa River, the rate was always high (13.1–19.9%) in 0^+ young, but only 4.7% of the spawning adult were hybrids, which is much lower than that of the 0^+ young.

Finally, the parental combination of hybrids appeared to differ according to rivers. In the Mukawa River, no hybrid of *T. hakonensis* × *T. ezoe* was found from a total of 148 hybrids out of 1,137 individuals examined. Five of 7 hybrids out of 110 individuals from the Toshibetsu River and 2 hybrids out of 100 individuals from the Shiriuchi River were those of this combination.

Morphology. Cephalic lateral-line system: *T. brandti* can be distinguished from the other two species by the connection of canals, POC and POM (Kurawaka, 1977). Concerning this characteristic, some hybrid individuals showed an intermediate state between that of their parents (Fig. 4). In spawning adults, 20 indviduals of each species had no variation in this characteristic, whereas 3 out of 9 hybrid individuals exhibited the intermediate state of the canal connection at the left or right side (Table 4).

Table 2. Occurrence pattern of three species of *Tribolodon* and their hybrids in the seven rivers in Oct., 1983 (for abbreviations of rivers, see legend of Table 1). In the hybrids, for example, *h-b* shows those of *T. hakonensis* × *T. brandti* and *h-b-b* denotes their backcross hybrids.

	Sar	Muk	Ats	Ass	Tos	Shi	Mog
hakonensis	69	154		43	29	67	99
brandti		118		3	1		
ezoe	35	67	101	57	67	31	
h-b	1	12					
b-e	1	3					
h-e				5		2	
h-b-h	2	9		1			
h-b-b		19					
b-e-b		3					
b-e-e	2	2					
h-e-h					1		
h-e-e							
the others		3		1			
Total	110	390	101	110	98	100	99
Hybrid %	5.5	13.1	0	6.4	1.0	2.0	0

Table 3. Change in occurrence pattern of three species of *Tribolodon* and their hybrids in the Mukawa River. For abbreviations, see legend of Table 2.

	0^+ young			Spawning adult
	'82 Oct.	'83 May	'83 Oct.	'83 Jun.
hakonensis	26	83	154	53
brandti	85	76	118	22
ezoe	55	141	67	107
h-b	7	14	12	5
b-e	18	9	3	2
h-b-h			9	
h-b-b	5	17	19	2
b-e-b	2	4	3	
b-e-e	8	3	2	
the others	1	2	3	
Total	207	349	390	191
Hybrid %	19.9	14.0	13.1	4.7

Number of scales: The hybrids were intermediate in mean number of scales between their parents, and the ranges of the latter were widely related to that of the former (Table 5). The range of lateral-line scales was 72-83 (mean 76.0) in *T. hakonenesis*, 83–97 (90.6) in *T. brandti* and 70–82 (75.2) in *T. ezoe*, while that of hybrids was counted 77–90 (80.9) in *T. hakonensis* × *T. brandti* and 79–93 (83.7) in *T. brandti* × *T. ezoe*. This was also true of predorsal scales, namely 29–36 (33.1) in *T. hakonensis*, 40–50 (45.1) in *T. brandti* and 38–48 (42.3) in *T. ezoe*, while 35–47 (39.3) in *T. hakonensis* × *T. brandti* and 42–47 (44.4) in *T. brandti* × *T. ezoe*.

Discussion

It must be pointed out first that identifications based on the method used in the present study are quite effective but may involve partial mistakes. For example, some of the F_1 hybrids may have fallen into backcross hybrids. In such mistakenly identified F_1 hybrids, certain unexamined isozymes might have latently exhibited homozygotic patterns which were originated from a backcross hy-

bridization. A brief calculation of such probability indicates that, through examination of five loci, about 3% of the backcross may have been mistakenly included in the F_1 hybrids.

The genus *Tribolodon* is a group in which the members are morphologically very similar and difficult to distinguish from each other. The classification of such members is more perplexing to a degree when they hybridize. The hybrids were intermediate in mean number of scales between their parental species. The range of scales of the parents and their hybrids greatly overlapped. However, the connection of canals, POC and POM, was not always intermediate in hybrids. Such wild hybrids have further complicated the classification of the genus *Tribolodon*.

Intense research on the Mukawa River revealed that as many as 13.2% of a total of 1,137 individuals were hybrids. However, no hybrid of *T. hakonensis* × *T. ezoe* was detected in the Mukawa River, unlike the research results for the Toshibetsu or Shiriuchi rivers. The residual type of *T. hakonensis* is not distributed in the Mukawa River, while perhaps in the other two rivers the type is distributed. In contrast with the residual type, the anadromous type of *T. hakonensis* may not hybridyze with *T. ezoe* which lives an entirely fluvial life.

In the Mukawa River, all the three species often simultaneously spawn on the same rapids in high

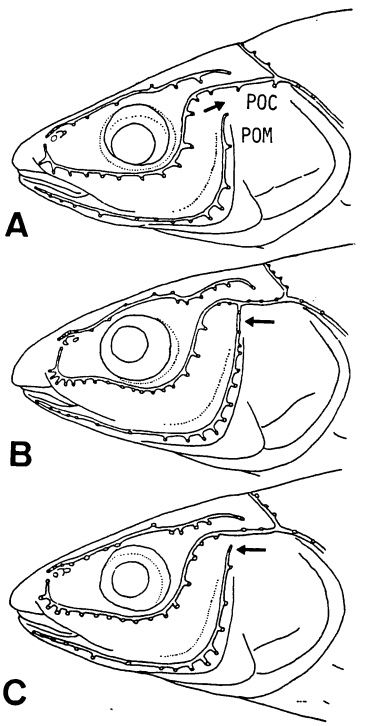

Fig. 4. Diagramatic drawing of the cephalic lateral-line system in *Tribolodon hakonensis* (A), *T. brandti* (B) and that observed in *T. hakonensis* × *T. brandti* or *T. brandti* × *T. ezoe* (C). The arrow indicates the portion of canal connection between postocular commisure (POC) and preoperculomandibular canal (POM).

Table 4. Connection pattern of canals between postocular commisure (POS) and preoperculomandibular canal (POM) seen in 20 individuals of each species, *Tribolodon hakonensis*, *T. brandti* and *T. ezoe*, and 9 individuals of hybrids. Specimens examined were spawning adult caught from the Mukawa River. For abbreviations of hybrids, see legend of Table 2. Yes, no and int. indicate presence, absence and intermediate state of the canal connection, respectively.

	Connection of canals (POC-POM)					
	right			left		
	yes	int.	no	yes	int.	no
hakonensis			20			20
brandti	20			20		
ezoe			20			20
Hybrid 1 *h-b*			+			+
2 *h-b*			+			+
3 *h-b*			+			+
4 *h-b*			+			+
5 *h-b*		+		+		
6 *b-e*	+			+		
7 *b-e*			+		+	
8 *h-b-b*	+				+	
9 *h-b-b*	+			+		

Table 5. Numbers of scales on a lateral-line series and on a predorsal series in three species of *Tribolodon* and their F_1 hybrids. For abbreviations of hybrids, see legend of Table 2.

	Number of scales on lateral line																										
	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98
hakonensis			3	6	10	7	14	10	6	4	2	1		1															
h-b								1	3	1	3	1	3	2								1							
brandti														1			2	2	8	6	7	7	6	5	4	2	1	1	
b-e										2	1	1	2	2	3		1	1		1				1					
ezoe	1	5	4	13	10	9	8	9	6	2	4	1	1																

	Number of predorsal scales																					
	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
hakonensis	1	5	4	12	21	10	9	5														
h-b							1	1	2	2	4	3	1		1				1			
brandti												1	2	4	3	12	8	9	7	4	2	1
b-e														1	5	2	3	2	2			
ezoe										1	1	10	14	21	12	5	6	2	2	1		

concentrations, where a weir prevents the spawning runs from further upstearm migration. Such forced crowding of fish on a limited spawning ground must be one of the causes yielding many natural hybrids (Hubbs, 1955). If successive generations of hybridization had occurred, this would have lead to continuous intergradation of phenotypes and fusion of the participating species. In reality, the scale number was overlapped and somewhat intergradated among the parental species and their hybrids. However, the electrophoretic data does not support the fusion, but rather the independence of the species. The total population pooled of the three species and hybrids was so deviated from the Hardy-Weinberg equilibria that no statistical analysis was necessary. This deviation indicates that reproductive isolation is imperfect but highly functional among species. In the Mukawa River, the isolating mechanism is rather perfect between *T. hakonensis* and *T. ezoe*.

An alternative explanation for this deviation from the Hardy-Weinberg equilibria is that the population may be in the process of approaching equilibria. However, this hypothesis is rejected because the proportion of F_1 to the total hybrids was much more than expected. As many as half of hybrids were judged as F_1 hybrids. This proportion of F_1 hybrids could have been reached in only two generations since the parental species began to hybridyze. In the later generation, the proportion would swiftly decrease and at last attain the level predicted by Hardy-Weinberg equilibria. It is almost impossible to conclude that the population began to hybridyze two generations ago.

The circumstances mentioned above suggest the existence of post-mating isolation between species. The rate of the hybrids among spawning adults was about one-third that in 0^+ young in the Mukawa River. This decrease of hybrid individuals according to growth may contribute to a partial resistance in the genetic introgression between species. Further study is necessary concerning the isolating mechanism functioning among the species of the genus *Tribolodon*.

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電気泳動法によるコイ科ウグイ属魚類の種の判別と雑種の出現

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北海道産ウグイ属魚類3種，ウグイ，マルタおよびエゾウグイについて，電気泳動法による種の判別を試みた．調査した遺伝子座は乳酸脱，水素酵素 (LDH), フォスフォグルコムターゼ (PGM), フォスフォグルコースイソメラーゼ (PGI) および筋肉蛋白 (MP) を支配する遺伝子座である．その結果，形態学的には識別の困難な当才魚についても，明確な種の同定が可能であった．その上，最高 19.9% の割合で雑種個体が検出され，これらの存在がウグイ属魚類の分類を一層困難にしているものと思われた．雑種を考慮に入れて，3 種を一つの集団と考えると，この集団がハーディ・ワインベルグの平衡から大きく逸脱していること，およびこれが移入交雑の過程にある集団と仮定した場合でも，雑種に占める第一代の割合が多すぎることの2点から，本属魚類では，種間の生殖的隔離は不完全であるが，各々の種の維持が可能であると考えられた．

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