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Speciation and Dispersal of *Tribolodon* Species (Pisces, Cyprinidae) around the Sea of Japan

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ABSTRACT—Gene products of 22 protein coding loci from 29 populations of a uniquely sea-going cyprinid genus *Tribolodon*, *T. hakonensis* (anadromous and/or river-resident), *T. brandtii* (anadromous), *T. ezoe* (freshwater) and *T. nakamurai* (freshwater), were analyzed in order to infer the genetic divergence and dispersal of *Tribolodon* species around the Sea of Japan. According to the genetic distance, *T. brandtii* is located near the center between *T. hakonensis* and *T. ezoe-T. nakamurai* cluster. The outgroup root (*Pseudaspius leptocephalus, Rhynchocypris lagowskii* and *Leuciscus waleckii*) is attached between *T. hakonensis-T. brandtii* and the *T. ezoe-T. nakamurai* clusters. The origin of the genus *Tribolodon* and its salinity tolerance may date back to the Miocene Sea-of-Japan Lake and the Pliocene Paleo-Sea-of-Japan, respectively, as judged from the divergence time between *Tribolodon* and the outgroups. The genetic relationships and distribution pattern of *Tribolodon* species suggest they speciated on the Sakhalin-Japan side, and the continental populations of *T. hakonensis* and *T. brandtii* originated from anadromous colonizers. A new faunal element of Japanese freshwater fishes, the Sakhalin-Japan sub-element, is proposed for *Tribolodon* and some other fishes.

Key words: allozyme, anadromy, dace, genetic divergence, biogeography

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INTRODUCTION

Fishes have currently the highest species diversity among vertebrates (about 30000 species), suggesting that they have experienced the most frequent speciation events (Nelson, 1994). Freshwater fishes such as cyprinids, cobitids and bagrids are among the ideal animal groups for research on speciation, because of the restricted gene flow between geographically isolated populations, which facilitates the evolution of reproductive isolation. The areas abutting the Sea of Japan have been thought to form one of such speciation center for freshwater as well as marine fishes throughout the Tertiary to Quaternary (e.g. Lindberg, 1972; Nishimura, 1974; Nakajima, 1986, 1987).

The Far Eastern daces, *Tribolodon*, comprise a unique group in the large family Cyprinidae not only because of their variation in life-history type from freshwater to anadromous mode of life, but also because of their geographic restriction to areas around the Sea of Japan (Nakamura, 1969; Nishimura, 1974). Therefore, the derivation of the genus, especially its anadromy, is thought to have involved the history of the Sea of Japan (Nishimura, 1974).

Tribolodon includes four species, “Ugui” *T. hakonensis* (Günter), “Maruta” *T. brandtii* (Dybowsky), “Ezo-ugui” *T. ezoe* Okada and Ikeda and “Ukekuchi-ugui” *T. nakamurai* Doi and Shinzawa. *Tribolodon hakonensis* populations exhibit freshwater as well as anadromous modes of life, *T. brandtii* has an anadromous life history, *T. ezoe* lives entirely freshwater life, and *T. nakamurai* probably has a freshwater life history (Nakamura, 1969; Kurawaka, 1977; Sakai, 1995).

Tribolodon hakonensis is the most widely distributed species, covering nearly the entire *Tribolodon* range around the Sea of Japan, but the freshwater form scarcely occurs in the continental side (Uchida, 1939). On the other hand, *T. brandtii* is absent from the southern half of Japan, and *T. ezoe* is restricted to the northern half of the Sakhalin-Japan part of the range. *Tribolodon nakamurai* is restricted to a narrow part of the southernmost area of the Tohoku District,

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Honshu Island, Japan, and it is syntopic with all three other species (Nakamura, 1969; Kurawaka, 1977; Sakai, 1995).

Due to the above context, it is very interesting and rewarding to probe into the process of their speciation involving an acquisition of anadromy. Hanzawa and Taniguchi (1982) and Sakai (1995) analyzed allozymes of the four species electrophoretically and suggested closer genetic relationships among the three of the species to the exclusion of *T. hakonensis*. However, their samples were obtained from restricted localities in Japan and no outgroup comparison was made in their analysis. Kartavtsev et al. (2001) compared allozymes between *Tribolodon* and two allied genera, but still surveyed only a small number of populations. Therefore, the results unsatisfactorily address the *Tribolodon* divergence and/or speciation problem.

In the present study, the allozyme genetic relationships of the four species of *Tribolodon*, with some outgroups, were studied based on populations from nearly the entire range, including Sakhalin, the Russian Maritime Territory, the Korean Peninsula and Japan, and derivation and speciation

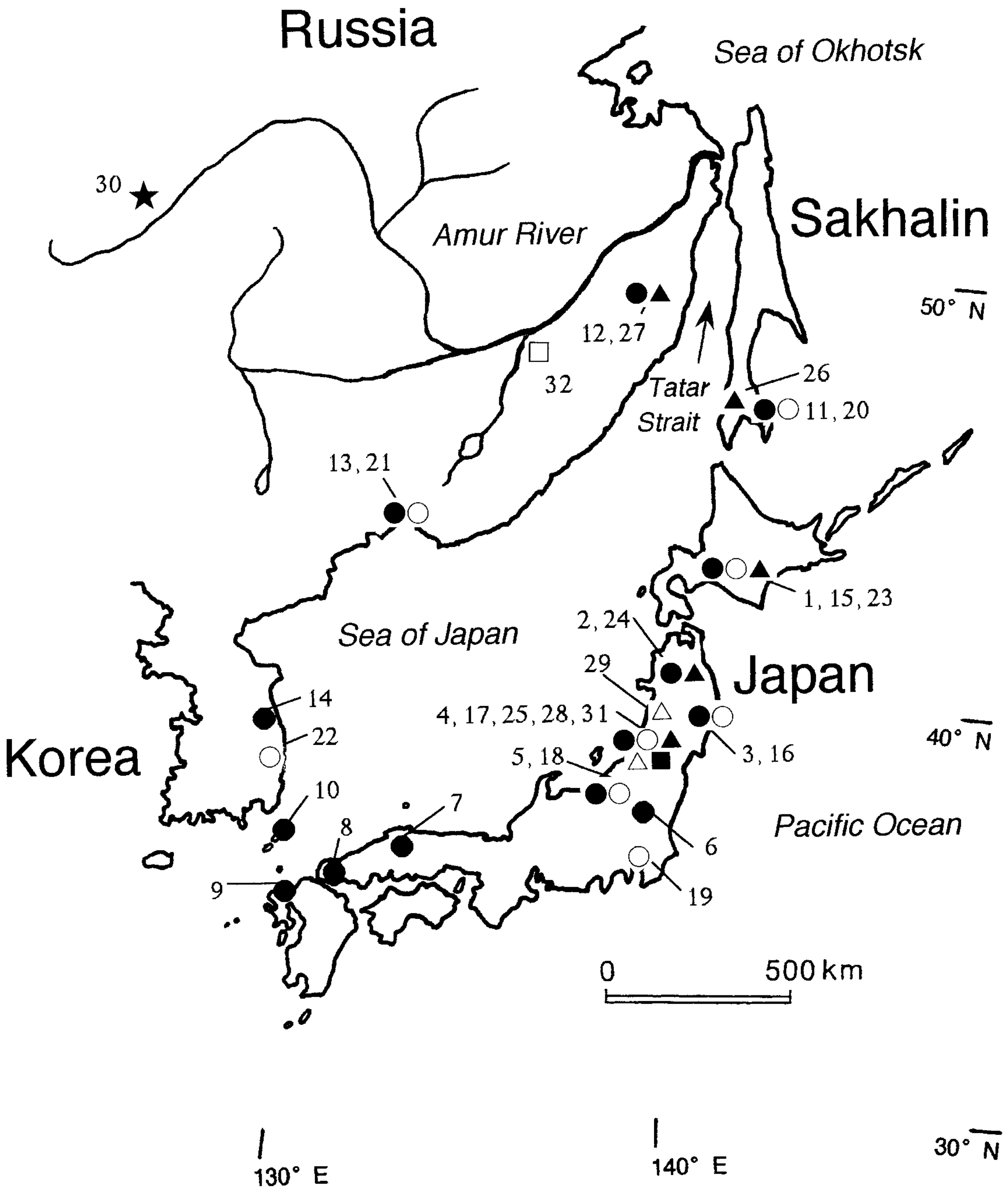

Fig. 1. Sampling localities of *Tribolodon hakonensis* (closed ciecle), *T. brandtii* (open circle), *T. ezoe* (closed triangle), *T. nakamurai* (open triangle), *Pseudaspius leptocephalus* (star), *Rhynchocypris lagowskii* (closed square) and *Leuciscus waleckii* (open circle). Population numbers and other detailed sampling data are shown in Table 1.

of *Tribolodon* along with genetic divergence are discussed.

MATERIALS AND METHODS

A total of 638 individuals from 29 populations of *Tribolodon* were surveyed by allozyme analysis (Fig. 1, Table 1). Two individuals of “Manshu-uo” *Pseudaspius leptocephalus*, 12 individuals of “Abura-haya” *Rhynchocypris lagowskii* and 5 individuals of “Kitano-ugui” *Leuciscus waleckii* were also examined for outgroup comparison in cluster analysis, because the pharyngeal teeth morphology of Amurian *Pseudaspius* has been suggested to be similar to that of *Tribolodon* (Tomoda, 1984, 1998), Far Eastern *Rhynchocypris* has been determined morphologically to be a sister genus of *Tribol-*

Table 1. Twenty-nine *Tribolodon* populations, and *Pseudaspius leptocephalus*, *Rhynchocypris lagowskii* and *Leuciscus waleckii* populations sueveyed

Population	Locality	Date	No. specimen
T. hakonensis			
1. Hokkaido	Mu River, Hokkaido, Japan	1991 Autumn	33
2. Akita	Yoneshiro River, Akita, Japan	1997 Autumn	40
3. Iwate	Sakari River, Iwate, Japan	1996 Winter	11
4. Yamagata	Mogami River, Yamagata, Japan	1997 Autumn	30
5. Niigata	Shinano River, Niigata, Japan	1996 Spring	33
6. Tochigi	Tone River, Tochigi, Japan	1996 Summer	4
7. Shimane	Kando River, Shimane, Japan	1995 Autumn	7
8. Yamaguchi	Awano Harbor, Yamaguchi, Japan	1998 Summer	17
9. Saga	Matsuura River, Saga, Japan	2000 Spring	22
10. Tsushima	Sago River, Tsushima Island, Nagasaki, Japan	1999 Spring	30
11. Sakhalin	Busse Lagoon, Sakhalin, Russia	1994 Aurumn	28
12. Tumnin	Tumnin River, Tuluchi, Russia	1998 Summer	9
13. Vladyvostok	Peter the Great Bay, Vladyvostok, Russia	1995 Autumn	41
14. Korea	Samchokoship River, Korea	1996 Spring	12
T. brandtii			
15. Hokkaido	Mu River, Hokkaido, Japan	1991 Autumn	34
16. Iwate	Oofunato Bay, Iwate, Japan	1998 Winter	8
17. Yamagata	Mogami River, Yamagata, Japan	1997 Autumn	18
18. Niigata	Shinano River, Niigata, Japan	1996 Spring	10
19. Tokyo	Tokyo Bay, Tokyo, Japan	1997 Summer	9
20. Sakhalin	Busse Lagoon, Sakhalin, Russia	1994 Aurumn	10
21. Vladyvostok	Peter the Great Bay, Vladyvostok, Russia	1995 Autumn	51
22. Korea	Songchon River, Korea	1996 Spring	23
T. ezoe			
23. Hokkaido	Mu River, Hokkaido, Japan	1991 Autumn	35
24. Akita	Yoneshiro River, Akita Japan	1997 Autumn	33
25. Yamagata	Mogami River, Yamagata, Japan	1997 Autumn	19
26. Sakhalin	Lyutoga River, Sakhalin, Russia	1994 Autumn	20
27. Tumnin	Tumnin River, Tuluchi, Russia	1998 Summer	27
T. nakamurai			
28. Yamagata	Mogami River, Yamagata, Japan	1995 Spring	23
29. Akita	Koyoshi River, Akita, Japan	1991 Winter	1
P. leptocephalus			
30. Chita	Amur River, Chita, Russia	2001 Summer	3
R. lagowskii			
31. Yamagata	Mogami River, Yamagata, Japan	1997 Autumn	12
L. waleckii			
32. Khabarovsk	Amur River, Khabarovsk, Russia	1995 Summer	5

odon, and Eurasian *Leuciscus* to be an outgroup of the *Tribolodon*-*Rhynchocypris* sister clade (Cavender and Coburn, 1992; Coburn and Cavender, 1992). Among them, *Leuciscus* was set as the outermost-group because it was most distantly related to *Tribolodon* genetically (see the result).

The fish were frozen immediately after collection and stored at –70°C until processed for starch-gel electrophresis (12% gel). The gene products of 22 allozyme or protein coding loci were investigated: aspartate aminotransferase (E.C. 2.6.1.1, *Aat-1**, *Aat-2**), alchohol dehydrogenase (E.C. 1.1.1, *Adh**), fumarate hydratase (E.C. 4.2.1.2, *Fh**), glycerol-6-phosphate isomerase (E.C. 5.3.1.9, *Gpi-1**, *Gpi-2**), L-iditol dehydrogenase (E.C. 1.1.1.14, *Iddh**), isocitrate dehydrogenase (E.C. 1.1.1.42, *Idhp-2**, *Idhp-3**), L-lactate dehydrogenase (E.C. 1.1.1.27, *Ldh-1**, *Ldh-2**, *Ldh-3**), malate dehydrogenase (E.C. 1.1.1.37, *Mdh-1**, *Mdh-2**, *Mdh-3**), phosphogluconate dehydrogenase (E.C. 1.1.1.44, *Pgdh**), phosphoglucomutase (E.C. 5.4.2.2, *Pgm**), superoxide dismutase (E.C. 1.15.1.1, *Sod**), xanthine dehydrogenase (E.C. 1.1.1.204, *Xdh**) and general protein (*Prot-2**, *Prot-3**). Locus and gene nomenclature follows Shaklee *et al.* (1990). The most common allele at a locus of *T. hakonensis* is designated as **100*, the others being named according to their relative mobility to the allele **100*.

A neighbor-joining (NJ) dendrogram (Saito and Nei, 1987) based on Nei's (1972) genetic distance (*D*) between pairs of all populations was constructed with 1000 bootstrap replications using the computer package PHYLIP (version 3.57c, Felsenstein, 1995).

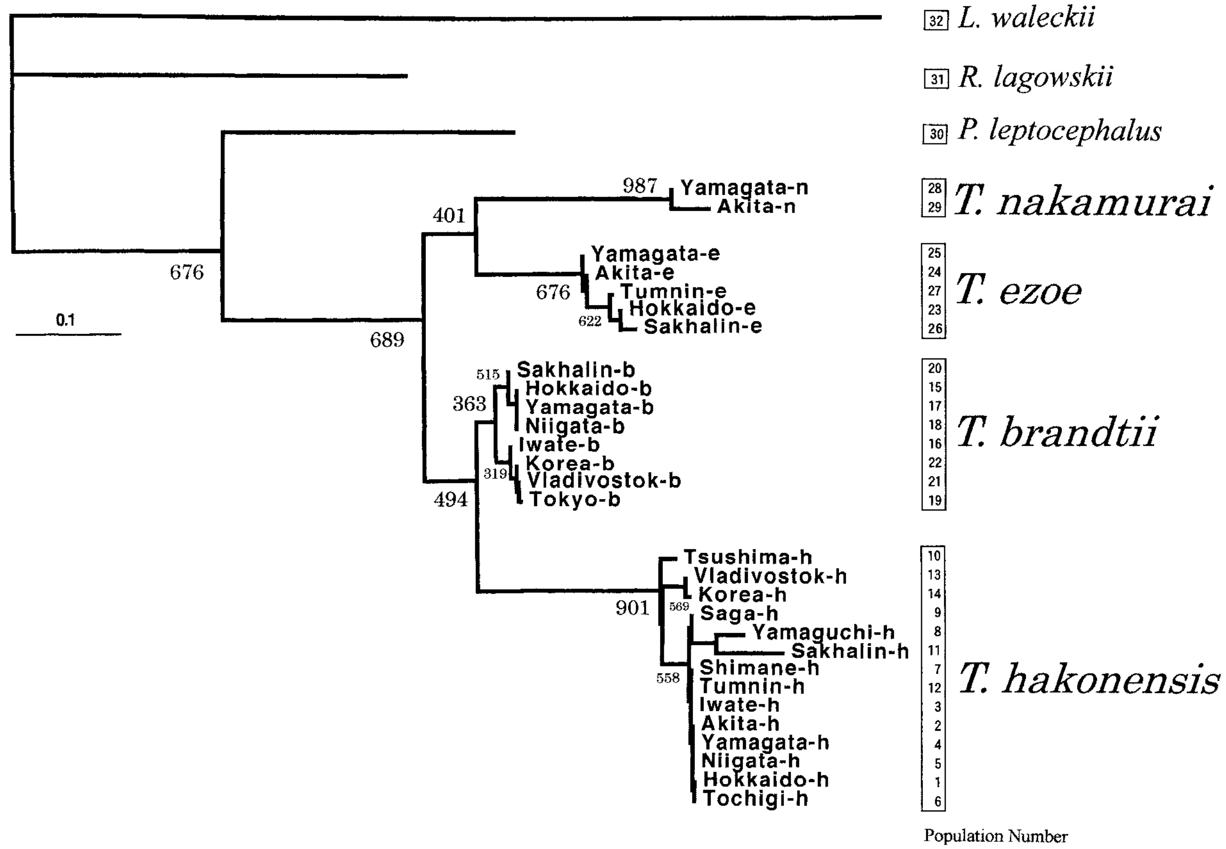

Fig. 2. Neighbor-joining dendrogram of 29 populations of four *Tribolodon* species based on Nei's (1972) genetic distance with *Pseudaspius leptocephalus*, *Rhynchicypris lagowskii* and *Leuciscus waleckii* as outgroups. Bootstrap values for 1000 replicates are indicated at major nodes. Population numbers are shown in Table 1.

Table 2. Number of diagnostic loci (above diagonal) and inter-specific Nei's (1972) genetic distance (mean±SD) (below diagonal) between pairs of *Tribolodon hakonensis, T. brandtii, T. ezoe, T. nakamurai, Pseudaspius leptocephalus, Rhynchocypris lagowskii* and *Leuciscus waleckii*, and intra-specific genetic distance (on diagonal)

	T. h.	*T. b.*	*T. e.*	*T. n.*	*P. l.*	*R. l.*	*L. w.*
T. hakonensis	0.028±0.028	3	6	6	11	14	14
T. brandtii	0.236±0.035	0.025±0.021	3	4	10	13	14
T. ezoe	0.464±0.045	0.241±0.028	0.021±0.011	4	6	12	15
T. nakamurai	0.509±0.046	0.338±0.046	0.296±0.034	0.157	11	13	15
P. leptocephalus	0.774±0.025	0.561±0.040	0.450±0.018	0.895±0.063	–	12	17
R. lagowskii	1.006±0.018	0.979±0.006	0.782±0.005	1.087±0.029	0.905	–	14
L. waleckii	1.425±0.085	1.347±0.069	1.541±0.041	1.446±0.101	1.285	1.222	–

Inter- and intra-generic divergence time was calculated with three calibrations; 1*D*=5 million years (Myr) (traditional one; Nei, 1975), 1*D*=12.5 Myr (for Tanganyikan cichlids; Nishida, 1997) and 1*D*=19.3 Myr (for Greek *Leuciscus*, Cyprinidae; Doadrio and Carmona, 1998).

RESULTS

Allelic frequencies are presented in the Appendix, all populations in all polymorphic loci (most common allele does not exceed 0.95) exhibiting no significant departure from the Hardy-Weinberg expectation.

Tribolodon hakonensis (at *Pgm** and *Prot-2**), *T. ezoe* (at *Ldh-2** and *Pgm**), and *T. nakamurai* (at *Idhp-2**) have their own characteristic alleles. On the other hand, *T. brandtii* has no such diagnostic locus, at least one allele being common to another species at every locus.

Mean *D* with standard deviation and numbers of diagnostic loci between pairs of species are shown in Table 2. The distances between *Tribolodon* species and the outgroups, *P. leptocephalus, R. lagowskii* and *L. waleckii*, are *D*=0.450–0.895 (alleles being displaced at 6–11 loci), 0.782–1.087 (12–14 loci) and 1.347–1.541 (14–15 loci),

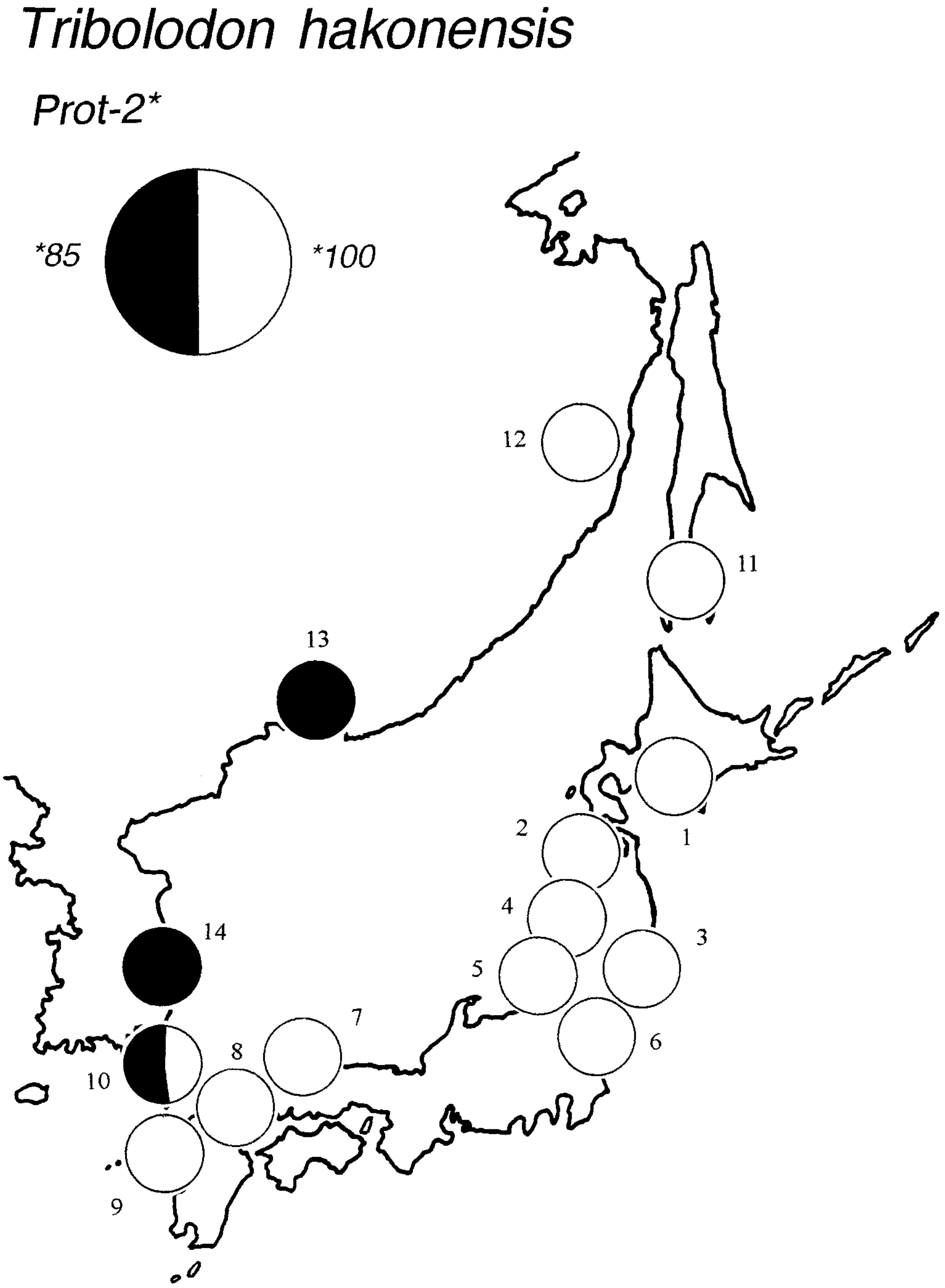

Fig. 3. Distribution of *Prot-2** alleles (**100, *85*) in *Tribolodon hakonensis*. Population numbers are shown in Table 1.

respectively. Among *Tribolodon* species, all species other than *T. brandtii* are closely related to *T. brandtii* at similar distances (*D*=0.236–0.338, alleles being displaced at 3–4 loci). *Tribolodon hakonensis* is distantly related to the other species except for *T. brandtii* (*D*=0.464–0.509, alleles being displaced at 6 loci), the distances being larger than that between *T. ezoe* and *P. leptocephalus* (*D*=0.450).

The relationships among them are exhibited in the NJ dendrogram (Fig. 2). Bootstrap probabilities (*p*) of each *Tribolodon* species cluster other than *T. brandtii* (*p*=36.3%) are fairly high (*p*=67.6–98.7%). *Tribolodon hakonensis* with *T. brandtii* (*p*=49.4%), and *T. ezoe* with *T. nakamurai* (*p*=40.1%) form two pairs of neighbors and the two clusters are rooted by the outgroups (*p*=68.9%). *Tribolodon* cluster is connected first with *P. leptocephalus* (*p*=67.6%).

Populations of *T. hakonensis* from Japan, Sakhalin and the northern Maritime Territory (the Tumnin River population) are connected in a cluster (*p*=55.8%, the locus *Prot-2** is fixed for allele **100*), populations from the southern Maritime Territory and Korean Peninsula form another cluster (*p*=56.9%, *Prot-2** is fixed for **85*), and the Tsushima population is intermediate between them (both alleles exist in

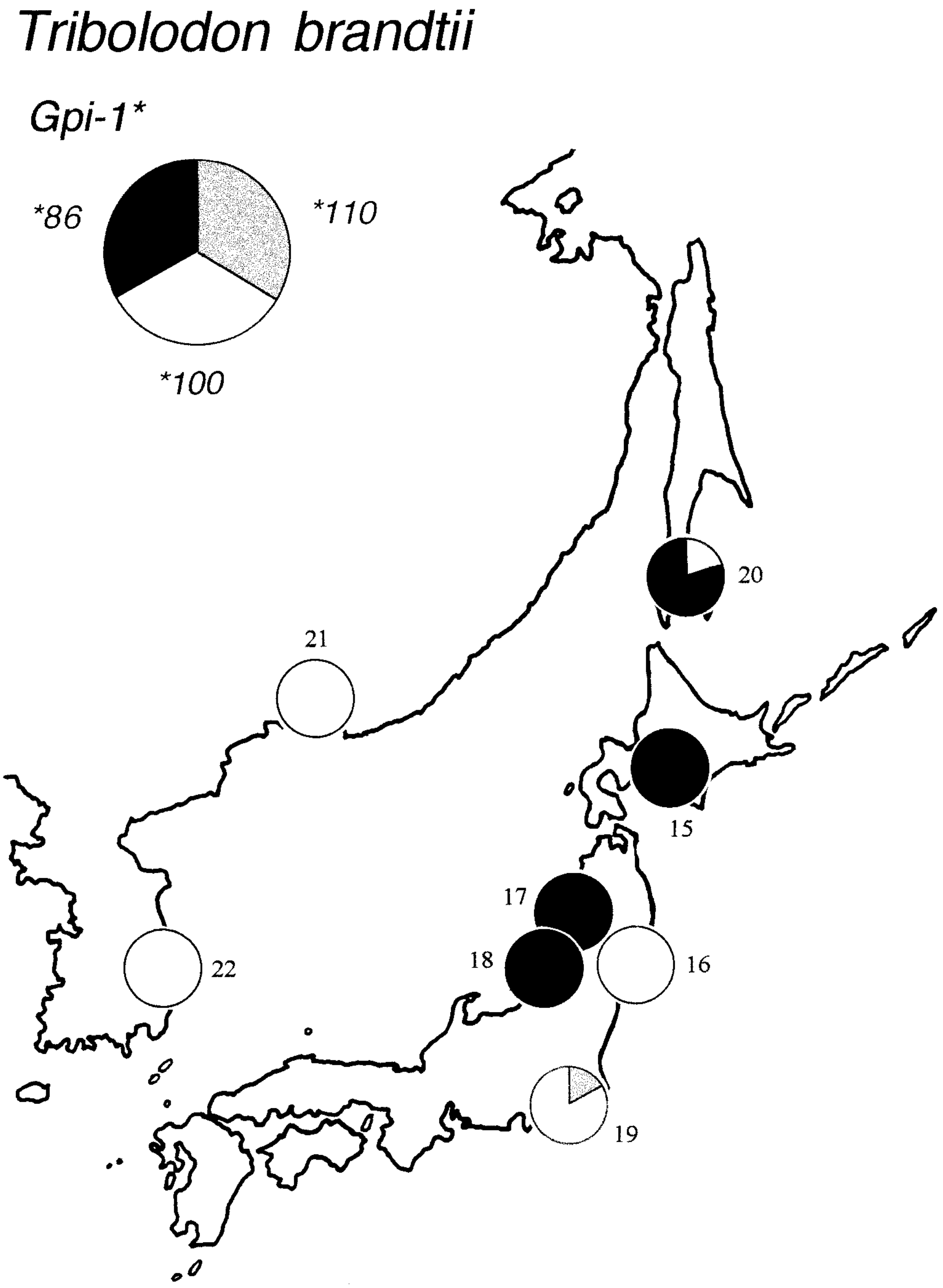

Fig. 4. Distribution of *Gpi-1** alleles (**110*, **100*, **86*) in *Tribolodon brandtii.* Population numbers are shown in Table 1.

*Prot-2**) (Figs. 2 and 3).

Hokkaido, Yamagata and Niigata populations of *T. brandtii* are fixed for the same allele **86* at *Gpi-1** with *T. ezoe*, and form a cluster with the Sakhalin population, which also possesses the allele **86* (*p*=51.5%, Figs. 2 and 4). The other populations have allele **100* in common with *T. hakonensis* at *Gpi-1**, and form a cluster (*p*=31.9%, Figs. 2 and 4).

Tribolodon ezoe are roughly divided into two groups, Tumnin, Sakhalin and Hokkaido cluster (*p*=62.2%) and the Tohoku District populations (Fig. 2). Tohoku populations from Yamagata and Akita Prefectures are fixed or nearly fixed for the allele **–100* at the locus *Gpi-2** (Fig. 5).

Tribolodon nakamurai from Akita Prefecture differ slightly from the Yamagata population in having allele **170* at the locus *Pgdh** (Fig. 2, Appendix).

Mean inter-generic distances, *D*=0.670 (vs. *Pseudaspius*), *D*=0.964 (vs. *Rhynchocypris*) and *D*=1.440 (vs. *Leuciscus*), are calculated to be about 3.4, 4.8 and 7.2 Myr ago by Nei's (1975), 8.4, 12.0 and 18.0 Myr ago by Nishida's (1997), or 12.9, 18.6 and 27.8 Myr ago by Doadrio and Carmona's (1998) calibrations, respectively. Mean inter-specific distance among *Tribolodon* species (*D*=0.347) is

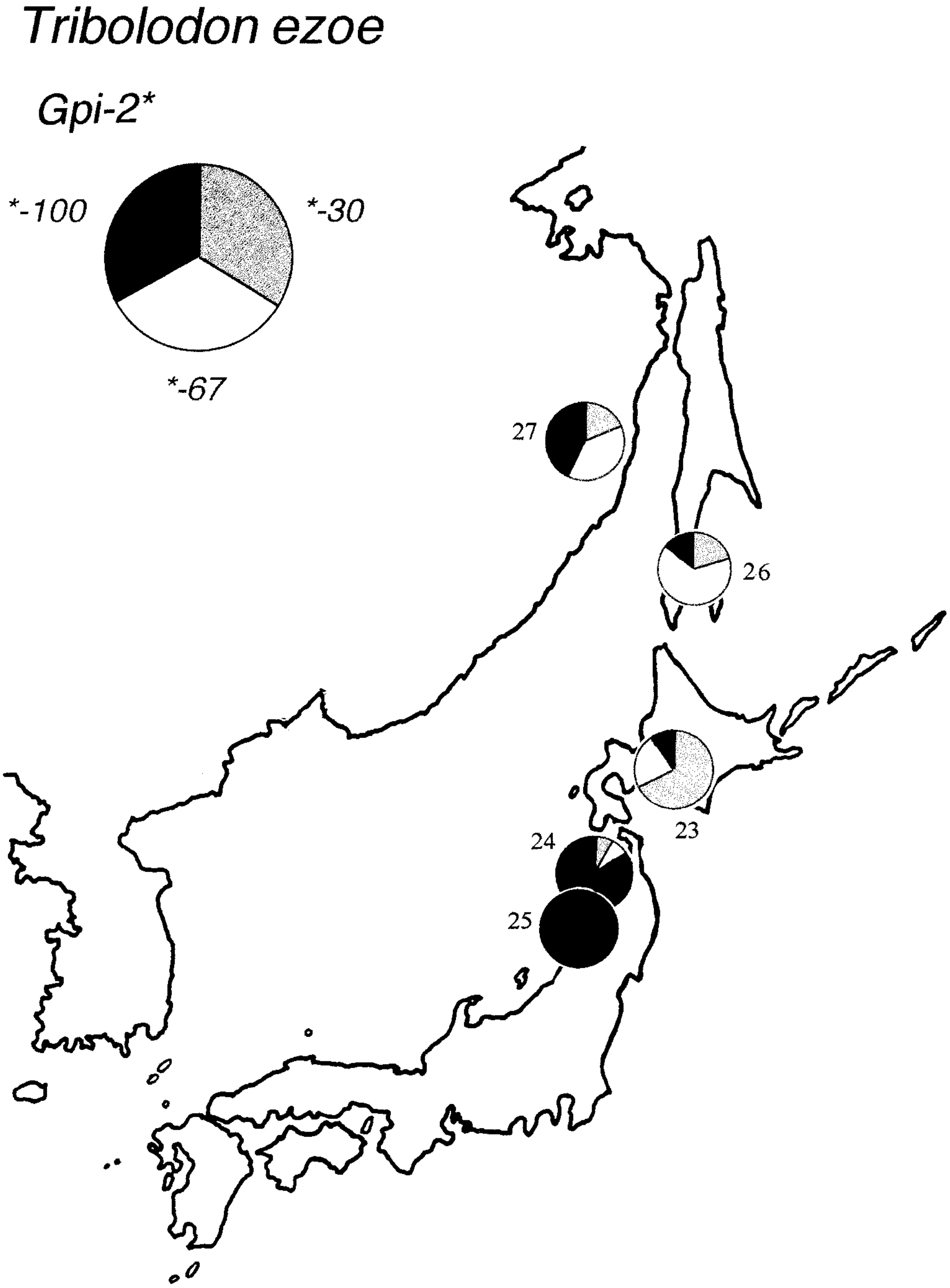

Fig. 5. Distribution of *Gpi-2** alleles (**–30*, **–67*, **–100*) in *Tribolodon ezoe*. Population numbers are shown in Table 1.

about 1.7 Myr (Nei, 1975), 4.3 Myr (Nishida, 1997) or 6.7 Myr ago (Doadrio and Carmona, 1998).

DISCUSSION

Inter-specific relationships

In the resulting dendrogram (Fig. 2), *Tribolodon brandtii* is located near the center between *T. hakonensis* and the *T. ezoe*-*T. nakamurai* cluster with very short branch length. According to Sakai and Hamada (1985) and Sakai (1995), *T. hakonensis* and *T. ezoe* frequently hybridize with *T. brandtii* in Hokkaido (10–20% of wild offspring are hybrids), usually *T. brandtii* being the mother parent in these hybridization events (85%), and most of the hybrid offspring backcross also with *T. brandtii*. In such hybridization, allozyme alleles may have introgressed from *T. hakonensis* and/or *T. ezoe* to *T. brandtii*, and mtDNA introgression may be less likely than allozyme allelic introgression. For example, some *Gpi-1** alleles of *T. brandtii* are assumed to have introgressed from *T. hakonensis* and/or *T. ezoe* as discussed later. *Tribolodon brandtii* does not have its any own diagnostic loci, but has at least one allele in common with other species at every examined locus (Appendix). The allozyme allelic variation in *T. brandtii* may be due to the above-mentioned introgression. Such introgression may cause the genetic intermediacy of *T. brandtii* in the genus, may make the evolutionary rate of allozyme genes of *T. brandtii* slower, and may shorten the branch length of the *T. brandtii* cluster.

In the dendrogram, the outgroups are all freshwater dwellers, and *Tribolodon* is divided into two clusters, freshwater (*T. ezoe*-*T. nakamurai*) and anadromous (*T. brandtii*-*T. hakonensis*) clusters. Therefore, it seems reasonable to infer that the anadromous *Tribolodon* species had been derived from the genus founder which had the same primitive non-anadromous life history as the outgroups, the first anadromous species probably having two modes of life, anadromous and river-resident, like *T. hakonensis*.

Genetic similarity between *Tribolodon* and *Pseudaspius*

Pseudaspius leptocephalus is the nearest neighbor of *Tribolodon* in the dendrogram. Kartavtsev *et al*. (2001) have reported a similar result. Genetic distances between them, D=0.450–0.895 (mean 0.670), correspond to the intergeneric level applied to American cyprinids, D=0.11–1.35 (mean 0.73) (Buth *et al*., 1991). However, the genetic distance between *P. leptocephalus* and *T. ezoe* (D=0.450) is smaller than those between *T. hakonensis* and *T. ezoe* or *T. nakamurai*, fitting also into the inter-specific range determined for American cyprinid genera, D=0.04–0.97 (mean 0.20) (Buth *et al*., 1991). Taking the similarity of pharyngeal teeth morphology between *Tribolodon* and *Pseudaspius* (Tomoda, 1984, 1998) into consideration, it is highly probable that *Pseudaspius* is the nearest sister group of *Tribolodon*. Further analyses on morphology and DNA should be performed.

Intra-specific differentiation of *Tribolodon hakonensis*

In *Tribolodon hakonensis*, only the anadromous form was found in the Maritime Territory and Korean Peninsula before (Uchida, 1939; Gavrenkov and Ivankov, 1979; Dai *et al*., 1982; Jeon and Sakai, 1984). This presents a striking contrast with Western Japan, neighboring the Korean Peninsula, where the river-resident form prevails (Nakamura, 1969). The Samchokoship River population from southern Korea examined in the present study, however, is thought not to have been exposed to seawater based on its otolith Sr/Ca analysis (unpubl. data). The Tumnin River population from the northern Maritime Territory may also be river-resident because the collection site is located more than 100 km from the river mouth and some semi-adult fish larger than 20 cm SL were collected in non-spawning season. These may be the first records of the river-resident form of *T. hakonensis* on the continental side of its range.

The Korean river-resident population of *T. hakonensis* does not share the *Prot-2*100* allele with the river-resident Tumnin and Japanese populations, but does the same allele (**85*) with the anadromous Vladivostok population (Fig. 3). This suggests that the populations from the southern Maritime Territory to Korea originated in the anadromous colonizer, being different from Sakhalin-Japan side populations (plus population from northern Maritime Territory), and the Korean river-resident population was secondarily landlocked in the southernmost range.

The Tsushima population of *T. hakonensis* has both *Prot-2** alleles (**85* and **100*), probably caused by allelic introgression between the Japanese and continental populations. No further introgression is seen beyond the Tsushima population, indicating that the secondary population contact between the two regions might have occurred recently (maybe in the last glacial epoch), and the land connection or freshwater habitat connection at that time was so restricted as to minimize introgression between the two regions even in the anadromous *T. hakonensis*. In fact, based on geological evidences, Ohshima (1991) insisted there was no land connection between Japan and Korea in the last glacial epoch.

Intra-specific differentiation of *Tribolodon brandtii*

Japanese *Tribolodon brandtii* has been divided into two types, “Maruta” type from Tokyo Bay and “Jusan-ugui” type from Aomori Prefecture, based on their mean lateral-line scale number, 83 or less (mean 80, range 76–83) for the former and 83 or more (mean 88, range 83 -95) for the latter (Nakamura, 1969). Although both live an anadromous life, the “Jusan-ugui” type is slightly less tolerant to salinity than the “Maruta” type (Nakamura, 1969). The reported lateral-line scale numbers indicate that the Sendai Bay population (Iwate population in the present study) is included in the “Maruta” type (Zama, 1999), and the continental populations (Gavrenkov and Ivankov, 1979; Dai *et al*., 1982; Jeon and Sakai, 1984), Sakhalin populations (Gritsenko, 1974), Hokkaido populations (Sakai and Hamada, 1985) and Sea of

Japan populations from the Tohoku District (Onodera and Honma, 1976) all fall in the "Jusan-ugui" type.

However, in the present study (Fig. 2), the continental populations ("Jusan-ugui" type) form a cluster with the Iwate and Tokyo populations ("Maruta" type), not with the Sakhalin, Hokkaido and Tohoku populations ("Jusan-ugui" type). This is due to *Gpi-1*100* (in common with *T. hakonensis*) and **110* alleles (in common with *T. ezoe*) (Appendix, Fig. 4) connecting the continental, Iwate and Tokyo populations and the Sakhalin, Hokkaido and Tohoku populations into two different clusters, respectively (Fig. 2). As already mentioned, some male *T. hakonensis* and *T. ezoe* individuals hybridize with female *T. brandtii* in Hokkaido (Sakai and Hamada, 1985; Sakai, 1995). Through such hybridization events, inter-specific allelic introgression of *Gpi-1** between *T. brandtii* and *T. hakonensis* or *T. ezoe* might have caused the complex relationships. The fact that the Sakhalin, Hokkaido and Tohoku populations of *T. brandtii* exhibit a similar distribution range to *T. ezoe* (Figs. 4 and 5) may also support the introgression.

In any way, taking the morphological and physiological differences (Nakamura, 1969) into consideration, the "Maruta" and "Jusan-ugui" types must be two natural intra-specific entities of *T. brandtii*. Mitochondrial DNA data (Sanada *et al*., in prep.) also support the existence of these two types. The continental populations and the Sakhalin, Hokkaido and Tohoku populations probably represent two colonizers of the "Jusan-ugui" type along the continental coast and along the Sakhalin-Japan coast, respectively. The divergence of the "Maruta" and "Jusan-ugui" types would have occurred geographically between the Pacific and Sea-of-Japan slopes at an earlier time than the above-mentioned "Jusan-ugui" dispersals.

Intra-specific differentiation of *Tribolodon ezoe* and *T. nakamurai*

The two recognized groups of *Tribolodon ezoe* perhaps resulted from a loss in genetic diversity in southern populations, especially in *Gpi-2** (Fig. 5). Divergence of *T. nakamurai* between the Akita and Yamagata populations is unclear because only one individual represented the Akita sample.

Time of *Tribolodon* derivation

Lindberg (1972) and Nishimura (1974) hypothesized that *Tribolodon*, which is distributed around the Sea of Japan, originated in the middle to late Pliocene at a hypothesized freshwater lake, the Lake Sea-of-Japan, and acquired their salinity tolerance in the following epoch when sea water began to enter the Lake Sea-of-Japan. However, geological studies, such as lithologic and planktonic foraminiferal studies of piston cores, do not support the existence of such a freshwater lake in the Pliocene (Taira, 1977; Ujiie and Ichikura, 1977; Kaseno, 1989). Reliable evidence of such a freshwater lake dates back to the early Miocene or older time (*e.g.* Kaseno, 1989; Fujita, 1990; Taira, 1990). The early Miocene lake is supposed to have been a center of cyprinid diversity (Tomoda *et al*., 1977; Nakajima, 1986, 1987), and *Tribolodon* has possibly originated there.

In the present study, the divergence time between *Tribolodon* and the outgroups are estimated as 3.4–27.8 Myr ago, but only the estimations adopting the time scale for the divergence data of *Leuciscu*s (Doadrio and Carmona, 1998), one of the outgroups used in the present study, can date back the early Miocene, 12.9 (vs. *Pseudaspius*), 18.6 (vs. *Rhynchocypris*) or 27.8 (vs. *Leuciscus*) Myr ago. Accordingly, it is hypothesized that the *Tribolodon* ancestor itself or a common ancestor of Far Eastern *Tribolodon* and Amurian *Pseudaspius* or Far Eastern *Rhynchocypris* originated in the early Miocene Sea-of-Japan Lake, after which *Tribolodon* derived and acquired salinity tolerance in the middle to late Miocene Sea of Japan, having overcome the entry of seawater.

Place of *Tribolodon* speciation

Adopting the cyprinid calibration (Doadrio and Carmona, 1998), the mean divergence time among *Tribolodon* species is calculated to be 6.7 (range 4.7–10.0) Myr ago, when the Paleo-Sea-of-Japan is thought to have been a semi-closed cool sea (Kaseno, 1989). Therefore, the genus might have speciated through the late Miocene to Pliocene, and intra-specific entities might have derived in the Pleistocene with repeating inter-glacial and glacial epochs. Early Pleistocene *Tribolodon* fossils from Tochigi Prefecture, Japan (Uyeno, 1967) do not contradict this hypothesis.

Although a river-resident *T. hakonensis* population is found in Korea, all of the remaining river-resident populations are distributed in the Sakhalin-Japan side of the range including the northern Maritime Territory facing to the Tatar Strait. Freshwater *T. ezoe* and *T. nakamurai* are also restricted to the same side. *Tribolodon hakonensis* from the southern Maritime Territory to Korea are inferred to have originated from the anadromous colonizer. Continental *T. brandtii* is also thought to represent a colonizer along the continental coast after the "Maruta" vs. "Jusan-ugui" divergence. All these things suggest that most of *Tribolodon* speciation events occurred on the Sakhalin-Japan side.

In focusing on the origin of the freshwater fish fauna of the Japanese Archipelago, many authors have included *Tribolodon* among so-called Siberian element (*e.g.* Aoyagi, 1957; Nishimura, 1974; Goto *et al*., 1978; Goto and Nakano, 1993). However, this designation is probably incorrect for the above-mentioned reasons as well as the fact that no *Tribolodon* species inhabit the Amur River except for anadromous *T. hakonensis* and *T. brandtii* entering the river mouth (Berg, 1948–1949).

Therefore, it might be better to call *Tribolodon* a "Sakhalin-Japan sub-element". There also exist some fish groups present on the Sakhalin-Japan side but absent from Siberia (except for the region facing to the Tatar Strait in some species), which should constitute the same sub-elements: "Itou" *Hucho perryi*, "Ezo-tomiyo" *Pugitius tymensis*, "Hana-kajika" *Cottus nozawae*, "Ezo-hana-kajika" *C.*

amblystomopsis, etc. (unpubl. data).

Aoyagi (1957) and Goto *et al.* (1978) stated that “Yachiugui” *Rhynchocypris percnurus sakhalinensis*, “Fuku-dojou” *Noemacheilus barbatulus toni* and “Ezo-hotoke” *Lefua nikkonis* typify the freshwater ichthyofauna of Hokkaido. These three each have sister species or subspecies in Siberia, and represent true Siberian element. The Sakhalin-Japan sub-element proposed above would also typify northern Japan freshwater ichthyofauna with the real Siberian element.

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Appendix Allele frequencies at 22 loci in 29 *Tribolodon* populations, and *Pseudaspius leptocephalus* (*P. l.*), *Rynchocypris lagowskii* (*R. l.*), and *Leuciscus waleckii* (*L. w.*) populations

		T. hakonensis														*T. brandtii*	
Locus	Allele	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Aat-1*	*140	0.015	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*105	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*100	0.985	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
	*90	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Aat-2*	*–80	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–85	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–90	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	0.875
	*–100	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	–	0.125
Adh*	*158	–	–	–	–	–	–	–	0.794	–	–	–	–	–	–	–	–
	*100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.206	1.000	1.000	1.000	1.000	0.988	0.938	1.000	1.000
	*62	–	–	–	–	–	–	–	–	–	–	–	–	0.012	0.062	–	–
	*54	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fh*	*100	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
	*82	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
G3pdh*	*–40	0.030	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–70	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–94	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–100	0.970	1.000	1.000	1.000	1.000	0.985	1.000	0.353	0.909	0.983	1.000	1.000	0.963	0.708	1.000	1.000
	*–130	–	–	–	–	–	0.015	–	0.647	0.091	0.017	–	–	0.037	0.292	–	–
Gpi-1*	*110	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*100	1.000	1.000	1.000	1.000	1.000	0.985	1.000	1.000	1.000	0.217	1.000	1.000	1.000	1.000	–	1.000
	*92	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*86	–	–	–	–	–	0.015	–	–	–	0.783	–	–	–	–	1.000	–
	*72	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Gpi-2*	*25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–30	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–67	0.288	0.213	0.045	0.250	0.375	0.288	–	0.147	0.068	–	0.268	0.056	0.061	–	0.015	–
	*–100	0.712	0.787	0.955	0.750	0.625	0.712	1.000	0.853	0.932	1.000	0.732	0.944	0.939	1.000	0.882	1.000
	*–120	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.103	–
Iddh*	*–100	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
	*–1830	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–2840	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Idhp-2*	*115	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*100	0.985	0.962	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
	*82	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*53	0.015	0.038	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Idhp-3*	*100	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
Ldh-1*	*113	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*100	0.985	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
	*65	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*54	0.015	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ldh-2*	*60	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–43	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–80	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–100	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
Ldh-3*	*–100	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	–	–
	*–155	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000
	*–190	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mdh-1*	*100	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
Mdh-2*	*100	1.000	1.000	1.000	1.000	1.000	0.958	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*64	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*9	–	–	–	–	–	0.042	–	–	–	–	–	–	–	–	–	–
Mdh-3*	*–100	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
	*–153	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pgdh*	*170	–	–	–	–	–	–	–	0.588	–	–	–	–	–	–	–	–
	*100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.412	1.000	1.000	1.000	1.000	1.000	1.000	0.926	0.875
	*73	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*53	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.074	0.125
Pgm*	*114	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*107	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000
	*100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.976	0.792	–	–
	*90	–	–	–	–	–	–	–	–	–	–	–	–	0.024	0.208	–	–
Sod*	*205	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*105	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*100	0.955	0.775	0.864	0.900	1.000	0.864	1.000	0.442	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	*–30	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–67	0.045	0.225	0.136	0.100	–	0.136	–	0.558	–	–	–	–	–	–	–	–
Prot-2*	*130	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*117	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000
	*100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.467	1.000	1.000	–	–	–	–
	*85	–	–	–	–	–	–	–	–	–	0.533	–	–	1.000	1.000	–	–
Prot-3*	*330	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*325	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*100	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
Xdh*	*105	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*100	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
	*95	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Appendix Continued

		T. brandtii						*T. ezoe*					*T. nakamurai*		*P. l.*	*R. l.*	*L. W.*
Locus	Allele	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Aat-1*	*140	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*105	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	–
	*100	0.985	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	–	–
	*90	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000
Aat-2*	*–80	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	–
	*–85	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000
	*–90	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	–	–
	*–100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Adh*	*158	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*100	1.000	1.000	1.000	1.000	0.980	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	–	1.000	–
	*62	–	–	–	–	0.020	–	–	–	–	–	–	–	–	–	–	–
	*54	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	–	1.000
Fh*	*100	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	–
	*82	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000
G3pdh*	*–40	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*–70	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000
	*–94	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000	–
	*–100	0.970	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	–	–	–
	*–130	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Gpi-1*	*110	–	–	0.167	–	–	–	–	–	–	–	–	–	–	–	–	0.200
	*100	–	–	0.833	0.200	1.000	1.000	–	0.394	0.316	–	–	–	–	–	–	0.800
	*92	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	–
	*86	1.000	1.000	–	0.800	–	–	1.000	0.606	0.684	1.000	0.574	1.000	1.000	1.000	–	–
	*72	–	–	–	–	–	–	–	–	–	–	0.426	–	–	–	–	–
Gpi-2*	*25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	–
	*–10	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	–	–
	*–30	–	–	–	–	–	–	0.200	0.076	–	0.675	0.185	–	–	–	–	–
	*–67	0.083	0.150	–	0.150	0.029	–	0.657	0.076	–	0.225	0.389	0.935	1.000	–	–	1.000
	*–100	0.917	0.850	0.778	0.850	0.834	1.000	0.143	0.838	1.000	0.100	0.426	0.065	–	–	–	–
	*–120	–	–	0.222	–	0.137	–	–	–	–	–	–	–	–	–	–	–
Iddh*	*–100	1.000	1.000	1.000	1.000	1.000	1.000	0.143	–	–	0.025	–	–	–	1.000	–	–
	*–1830	–	–	–	–	–	–	0.857	1.000	1.000	0.975	1.000	1.000	1.000	–	–	–
	*–2840	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000
Idhp-2*	*115	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000
	*100	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	–
	*82	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000	–	–	–
	*53	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Idhp-3*	*100	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
Ldh-1*	*113	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000
	*100	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	–	–
	*65	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	–
	*54	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ldh-2*	*60	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	–
	*–43	–	–	–	–	–	–	1.000	1.000	1.000	1.000	1.000	–	–	1.000	–	–
	*–80	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000
	*–100	1.000	1.000	1.000	1.000	1.000	1.000	–	–	–	–	–	1.000	1.000	–	–	–
Ldh-3*	*–100	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000	–	–	–
	*–155	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	–	–	1.000	–	–
	*–190	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000
Mdh-1*	*100	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
Mdh-2*	*100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.926	0.283	–	1.000	1.000	1.000
	*64	–	–	–	–	–	–	–	–	–	–	0.074	–	–	–	–	–
	*9	–	–	–	–	–	–	–	–	–	–	–	0.717	1.000	–	–	–
Mdh-3*	*–100	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	–
	*–153	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000
Pgdh*	*170	–	–	–	–	–	–	–	–	–	0.025	–	–	0.500	–	–	–
	*100	0.899	0.900	1.000	0.900	1.000	1.000	1.000	1.000	1.000	0.975	1.000	1.000	0.500	1.000	–	1.000
	*73	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	–
	*53	0.111	0.100	–	0.100	–	–	–	–	–	–	–	–	–	–	–	–
Pgm*	*114	–	–	–	–	–	–	1.000	1.000	1.000	1.000	1.000	–	–	1.000	0.850	–
	*107	1.000	1.000	1.000	1.000	1.000	1.000	–	–	–	–	–	1.000	1.000	–	0.150	1.000
	*100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*90	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sod*	*205	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.800
	*105	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	–	–
	*100	1.000	1.000	1.000	1.000	0.941	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	–	1.000	0.200
	*–30	–	–	–	–	0.059	–	–	–	–	–	–	–	–	–	–	–
	*–67	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Prot-2*	*130	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000	–
	*117	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	–	–	–
	*100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	*85	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000
Prot-3*	*330	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000
	*325	–	–	–	–	–	–	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	–
	*100	1.000	1.000	1.000	1.000	1.000	1.000	–	–	–	–	–	–	–	–	–	–
Xdh*	*105	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	1.000
	*100	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	–	–	–
	*95	–	–	–	–	–	–	–	–	–	–	–	–	–	1.000	–	–