LIFE-HISTORIES AND GENETIC DIVERGENCE IN THREE SPECIES OF *TRIBOLODON* (CYPRINIDAE)[1)]

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Contents

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I. Introduction

The Far Eastern dace 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). The members of the genus are morphologically very similar, and also each of them exhibits a wide range of meristic variation. Therefore, they can not be easily distinguished and various opinions have been presented concerning 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, *Tribolodon hakonensis* (Günther), *T. brandti* (Dybowsky), *T. ezoe* Okada et Ikada and *T.* sp. (see Nakamura, 1963) (Fig. 1). This has been done utilizing qualitative characteristics such as; cephalic lateral-line system (Nakamura, 1963; Kurawaka, 1977), gas bladder morphology (Kahata, 1981; Churikov and Sabitov, 1982), and spawning color (Nakamura, 1969; Gritsenko, 1974). *T. brandti* exhibits an anadromous life history, *T. ezoe* passes an entirely fluvial life and *T. hakonensis* populations experience both modes (Nakamura, 1969; Kurawaka, 1977; Sakai, 1987). The last species *T.* sp. distribution is restricted to Akita, Yamagata, Niigata and Fukushima prefectures and its exact mode of life still remains unclear (Sakai et al., 1991b).

Due to the unique life histories of *Tribolodon*, it is very interesting and rewarding to solve the central problem of why they had evolved the sea-run life stage and how their course of speciation had developed.

After the reclassification of *Tribolodon* spp., several comparative studies have been published on certain aspects of their life histories. Ito (1975) and Gritsenko

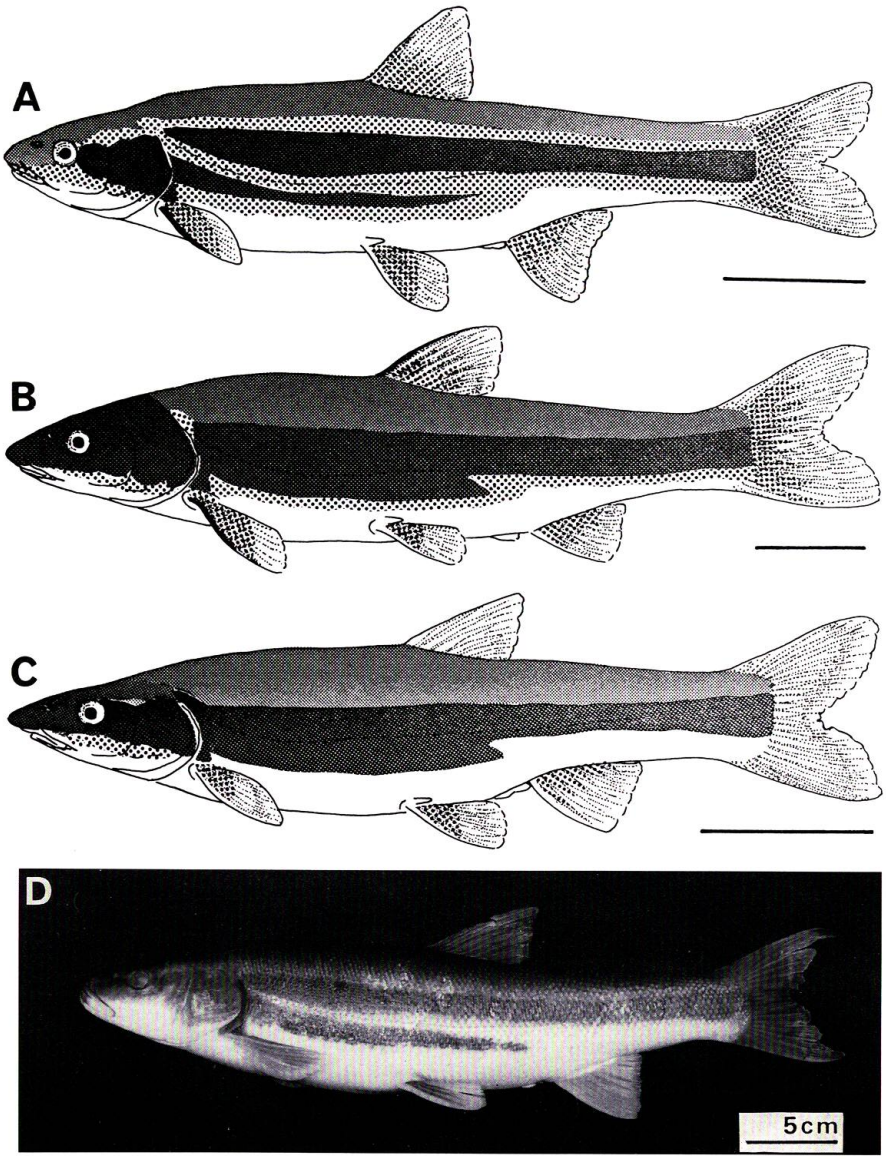

Fig. 1. Four species of *Tribolodon*, all express the spawning color. Largely dotted parts are tinted red. A, *T. hakonensis*; B, *T. brandti*: C, *T. ezoe*; D, *T.* sp.

(1982) made brief observations of spawning habits of three species, *T. hakonensis*, *T. brandti* and *T. ezoe*, from Hokkaido and Sakhalin, and reported differences in spawning seasons, spawning sites, etc., with large overlappings among the three species. Kurawaka (1977) clarified morphological differences and presented geographical distribution maps of the four species. Naito (1992) examined jaws and

suspensorium of *T. hakonensis*, *T. brandti* and *T. ezoe* and pointed out character state similarities in the latter two species. Hanzawa and Taniguchi (1982a, 1982b) compared electrophoreticaly analyzed allozymes of the four species from Fukushima Prefecture and suggested a little closer genetic relationship between three species other than *T. hakonensis*. Sakai and Hamada (1985) and Sakai (1987) reported the existence of natural hybrids among *T. hakonensis*, *T. brandti* and *T. ezoe* and discussed the population genetics of hybridization in *Tribolodon* briefly.

The literatures examined individual topics or fragments of *Tribolodon* biology. Nakamura's (1969) life history study on cyprinid fishes which included detailed descriptions of juvenile developments and ecological information concerning *Tribolodon* spp. may be an exception. However, Nakamura's study was descriptive and not comparative. Therefore, there has been no study which attempts to solve the problem of *Tribolodon* speciation from comprehensive comparative viewpoints.

The present study, the life histories and the degree of the reproductive isolation of three species, *T. hakonensis*, *T. brandti* and *T. ezoe* from Hokkaido were surveyed and compared, and the genetic relationships and the geographic distribution patterns of the four species of *Tribolodon* (including *T.* sp.) were researched. An hypothesis was formulated as to how and why the difference of *Tribolodon* life histories had developed along with their genetic differentiation. The life history of the last species *T.* sp. was not surveyed due to its endemic and rare nature.

II. Materials

Detailed data from 4,415 preserved individuals are shown in Table 1. The life historical traits were ascertained from a selection of the total preserved samples. Large number of eggs squeezed artificially or collected from rivers were utilized to determine egg distribution in rivers. 583 larval individuals were sampled for determining larval developmental intervals, 830 live juveniles were examined for space preference and salinity tolerance, and 3,603 frozen samples were analyzed for allozyme and mtDNA markers. Of them, 2 frozen samples were *T.* sp. from the Mogami River, Yamagata Prefecture, and all the other were collected in Hokkaido mainly from the Mu River (Figs. 21, and 66). Additionally, many formalin fixed samples deposited to the Laboratory of Embryology and Genetics, Faculty of Fisheries, Hokkaido University, collected from various quarters of Hokkaido from 1978-1982 were also examined to determine the distribution patterns in Hokkaido and/or in rivers. The details of each material and method are described in each chapter.

III. Larval and juvenile developmental intervals

Although the larval characteristics and metamorphic changes of fish are usually subtler than in amphibians, many fishes do undergo a very abrupt ecological and morphological transformation, for example, from a pelagic larva to a bottom inhabiting juvenile (Orton, 1953). Numerous studies have been focused on the larval development and systematics of fishes (see American Society of Ichthyologists and Herpetologists, 1984). However, the classification and terminology of develop-

Table 1. Formalin samples used in this study. Asterisks indicate checked items.

species	Locarity	Date	N	Standard length (mm)	Length composition	Gonad weight	No. eggs	Scale reading	Note
T. hakonensis									
	Mu River	Oct. 6, '80	114	31.5-107.9	*				
	〃	Nov. 18, '80	91	29.7-124.9	*				
	〃	Dec. 17, '80	89	25.4- 98.5	*				
	〃	Mar. 23, '81	76	34.0-102.1	*				
	〃	Apr. 24, '81	104	29.9-124.5	*			*	
	〃	May 28, '81	94	30.4- 84.1	*				
	〃	〃	22	228.7-313.0		*		*	spawners
	〃	May 31, '81	14	266.0-319.1		*			〃
	〃	Jun. 1, '81	76	38.2-141.2	*				
	〃	〃	23	224.2-310.7		*	*	*	spawners
	〃	Jun. 15, '81	28	215.0-307.8		*	*	*	〃
	〃	Jun. 20, '81	61	12.1- 52.8	*				
	〃	Jul. 7, '81	31	11.0- 56.3	*				
	〃	Jul. 9. '81	32	213.1-291.6		*			spawners
	〃	Aug. 21, '81	17	50.0- 63.2	*				
	〃	Sep. 18, '81	131	34.6-108.0	*				
	〃	Oct. 27, '81	123	25.9- 85.1	*				
	Utonai lake	Oct. 16, '80	6	191.5-320.8		*			wintering fish
	〃	Nov. 16, '80	29	204.0-341.9		*	*	*	〃
	〃	Mar. 27, '81	11	251.0-322.0		*	*	*	〃
	〃	Apr. 20, '81	9	282.0-336.0		*	*	*	〃
	Yufutsu River	Nov. 13, '80	16	196.3-231.8				*	〃
	〃	Dec. 18, '80	64	90.1-276.8		*		*	〃
	〃	Jan. 29, '81	27	190.1-229.7				*	〃
	〃	Mar. 19, '81	30	195.9-227.9				*	〃
	〃	Mur. 20, '81	33	111.2-214.3				*	〃

Table 1. Continued

species	Locarity	Date	N	Standard length (mm)	Length composition	Gonad weight	No. eggs	Scale reading	Note
	〃	Apr. 25, '81	30	201.7-249.6				*	〃
	〃	May 17, '81	67	49.7-206.0				*	〃
	Sea (set net)	Oct. 6, '80	18	214.9-321.5		*			Mukawa Town
	〃	May 2, '81	35	233.5-287.6		*	*	*	〃
	〃	Oct. 30, '81	36	153.2-255.2		*			〃
	Hime River	Nov. 1, '82	76	28.1-143.0	*	*	*	*	fluvial fish
	〃	Mar. 24, '83	117	22.1-143.0	*	*		*	〃
	〃	Apr. 17, '83	83	25.9-164.0	*	*	*	*	〃
	〃	May 20, '83	61	23.9-199.5	*	*	*		〃
	〃	Jun. 29, '83	97	28.9-189.5	*	*	*		〃
	〃	Jul. 27, '83	231	10.2-123.1	*	*	*		〃
	〃	Aug. 27, '83	41	45.8-106.2	*	*			〃
	〃	Sep. 30, '83	113	22.5-125.0	*	*		*	〃
T. brandti									
	Mu River	Nov. 18, '80	11	32.6-148.7	*				
	〃	May 28, '81	3	36.3- 68.8	*				
	〃	〃	5	312.0-389.5		*		*	spawners
	〃	May 31, '81	5	330.7-391.7		*		*	〃
	〃	Jun. 1, '81	19	291.8-372.4		*	*	*	〃
	〃	Jun. 15, '81	8	284.5-380.2		*	*	*	〃
	〃	Jun. 20, '81	12	34.0- 45.1	*				
	〃	Jul. 7, '81	4	40.1- 52.3	*				
	〃	Jul. 9, '81	2	302.4-308.7		*		spawners	
	〃	Oct. 27, '81	83	22.9- 88.3	*				
	Utonai Lake	Nov. 16, '80	9	304.1-399.0		*	*	*	wintering fish
	〃	Mar. 27, '81	12	288.0-400.0		*	*	*	〃
	〃	Apr. 20, '81	17	293.0-354.0		*	*	*	〃

	Yufutsu River	Jan.	20,	'81	1	199.4				*	〃
	〃	Mar.	19,	'81	7	201.8-221.0				*	〃
	〃	Mar.	20,	'81	5	183.5-220.1				*	〃
	〃	Apr.	25,	'81	9	201.4-222.8				*	〃
	〃	May	17,	'81	5	114.7-186.5				*	〃
	Sea (set net)	Oct.	6,	'80	11	235.1-401.0		*		*	Mukawa Town
	〃	Oct.	30,	'81	7	171.4-237.1				*	〃
T. ezoe											
	Mu River	May	31,	'81	26	116.9-166.6		*			spawners
	〃	Jun.	1,	'81	71	88.5-223.2		*	*	*	〃
	Atsuma River	Oct.	7,	'80	64	28.2-104.9	*				
	〃	Nov.	10,	'80	93	18.4- 44.1	*				
	〃	Dec.	8,	'80	154	16.4-103.6	*				
	〃	Mar.	13,	'81	56	19.2- 92.1	*				
	〃	Apr.	22,	'81	266	19.3-120.2	*			*	
	〃	May	15,	'81	128	20.7-161.0	*	*	*	*	
	〃	Jun.	21,	'81	180	23.2-336.0	*	*			
	〃	Jul.	15,	'81	200	9.3-135.0	*				
	〃	Sep.	14,	'81	214	17.7-146.8	*	*			
	〃	Oct.	22,	'81	156	22.3-163.2	*				
	Utonai Lake	Nov.	16,	'80	4	195.8-215.5		*			
	Yufutsu River	Feb.	25,	'81	3	164.0-193.8		*			
	〃	Mar.	30,	'81	59	62.9-230.4		*	*	*	
	〃	Apr.	25,	'81	45	86.4-298.0		*		*	
	〃	May.	1,	'81	31	108.6-247.2		*	*		
	〃	Jun.	26,	'81	23	183.4-253.3		*	*		
	〃	Jul.	20,	'81	22	183.0-238.9		*	*		
	〃	Sep.	22,	'81	19	153.7-216.2		*			

mental intervals are different among authors (reviewed by Snyder, 1976 ; Okiyama, 1979 ; Kendall et al., 1984 ; see also Richards, 1976 ; Balon, 1976) and the objective groups researched (Balon, 1975b).

Recently, Balon (1979, 1985) and Balon and Goto (1989) advanced the theory of saltatory ontogeny. In that theory, the ontogeny is grasped as a sequence of longer homeorhetic or steady states (developmental intervals or steps) interrupted by rapid changes in form and function (thresholds) (Balon, 1985 ; Balon and Goto, 1989). However, the recognition of thresholds is often difficult (McElman and Balon, 1979 ; Balon, 1979, 1985), and it does not always seem clear how we can recognize a threshold between two developmental intervals.

Sakai (1990) investigated the larval developmental intervals of fluvial type of *T. hakonensis* from the Kotoh River, Yamaguchi Pref., Japan, and recommended using the peaks in histograms of developmental event numbers as thresholds for establishing intervals on morphological development related to feeding and swimming functions. The histogram method is considered useful, as shown by Sakai (1990), for characters related to larval functions (and hence survival), because such characters are hardly subject to individual variatiion of presence or absence (Kohno et al., 1993).

Therefore, in this study, the developmental intervals of three species of *Tribolodon* (including two types of *T. hakonensis*, anadromous and fluvial) from Hokkaido were clarified comparing with those (intervals I to VI) of *T. hakonensis* larvae collected from the Kotoh River by Sakai (1990).

Sakai (1990) described and divided the development of fluvial *T. hakonensis* from the hatched larvae to the metamorphosed juveniles, and pointed out that their cephalic lateral line system, one of the key characteristics in classifying *Tribolodon* spp., would be completed in later stages. Therefore, the development of the cephalic lateral line system was reexamined. In addition, to analyze the developmental intervals after juvenile (interval VI by Sakai, 1990), relative growth patterns of head length, snout length, upper jaw length, eye diameter, suborbital length, and preanus length were investigated. The present research excluded study of the embryonic development because the developmental process is nearly identical in all *Tribolodon* spp. (Kanoh, 1949 ; Nakamura, 1969 ; Dai et al., 1982 ; Katsura et al., 1995).

Materials and methods

1. Three species of *Tribolodon* from Hokkaido

Brood Stock, one ripe female, 324.9 mm SL, and two males, 223.6 mm and 259.1 mm SL of anadromous *T. hakonensis*, one ripe female, 349.5 mm SL, and two males, 381.7 mm and 265.0 mm SL, of *T. brandti*, and one ripe female, 161.1 mm SL, and two males, 173.6 mm and 169.1 mm SL, of *T. ezoe* were collected from fish aggregated in the Mu River spawning ground (Figs. 21 and 66), Hokkaido, Japan on 7, 9, and 12 June 1982, respectively. Brood stock of fluvial *T. hakonensis*, one Ripe female, 147.3 mm SL, and two males, 93.5 mm and 91.5 mm SL, were collected from fish in the Hime River spawning ground (Fig. 66), Hokkaido, Japan, on 1 July, 1983.

Approximately 500 eggs for each species and type fertilized by the dry method

were scattered on gravel in each closed circulating aquarium (63*l*) for incubating and rearing. The larvae were reared at 14.5-27.5°C (anadromous *T. hakonensis*, *T. brandti* and *T. ezoe*) and 15.4-29.5°C (fluvial *T. hakonensis*) until they reached the juvenile stage in the same aquarium, fed on commercially prepared Tetramin. Ten to 40 larval and juvenile specimens were sampled at two- to three-day intervals and fixed in 5% formalin. The external morphology and gut development were sketched from a representative specimen of every larval developmental intervals in each species and type.

2. Development of cephalic lateral line system

Development of the cephalic lateral line system was observed from selected samples utilized in larval development analysis of each type and species. Observations followed published methods (Kurawaka, 1977) using Suminol Cyanine 5R. Nomenclature of canals of the cephalic lateral line system followed Reno (1969).

3. Relative growth

Relative growth of head, snout, upper jaw, suborbital, preanus length in% of body length and eye diameter were analyzed using various-sized 80, 51, 82 and 71 individuals of anadromous *T. hakonensis*, fluvial *T. hakonensis*, *T. brandti* and *T. ezoe*, respectively, selecting from samples listed in Table 1 and samples used in observing larval development. Measurements followed Matsubara (1955).

Results

1. Developmental intervals of *Tribolodon* from Hokkaido

Developments of two types of *T. hakonensis*, *T. brandti* and *T. ezoe* are presented in Figs. 2, 3, 4 and 5 and their gut developments are illustrated in Figs. 6, 7, 8 and 9, respectively. As they experienced similar developmental intervals with *T. hakonensis* from the Kotoh River (Sakai, 1990), representatives of the developmental intervals are explained in the following descriptions. Some subtle heterochronic differences (Gould, 1977) seen in some character developments are noted in the intervals concerned comparing to the development of *T. hakonensis* from the Kotoh River.

Anadromous *T. hakonensis* from the Mu River. Interval I (earliest stage, Figs. 2A, 6A): Immediately after hatching, 5.1-6.9(mean 6.3) mm TL (20 individuals). Tadpole-shaped. Pigmentation absent. Intestine straight and filamentous, liver located on the left side of the intestine. Fish laying on the bottom.

Interval II (early stage, Figs. 2B, 6B): Eight days after hatching, 9.6-10.8 (mean 10.3) mm TL (14 individuals). Mouth opened. Eyes pigmented silver. Melanophores appeared on head, thorax, dorsal, lateral midline and ventral sides of the trunk. Vertical finfolds developed higher. Branchial blood circulation observed. Lower caudal vein in anal finfold and segmental respiratory vessels (Balon, 1975a) developed. Pancreas on the right side and posterior chamber of gas bladder on the dorso-dextral side of intestine appeared. Notochord flexion began. Intense negative phototaxis.

Interval II (last stage, Figs. 2C, 6C): 12 days after hatching, 10.7-11.7 (mean

11.2) mm TL (31 individuals). Six principal rays appeared in caudal fin. Mesenchyme concentration in dorsal fin. Gall bladder and anterior chamber of gas bladder appeared. Temporary respiratory organs gradually reduced. Negative phototaxis.

Interval III (early stage, Figs. 2D, 6D) : 15 days after hatching, 11.2-12.1 (mean 11.7) mm TL (18 individuals). Peritoneum and breast tinted silver. Dorsal rays began to develop. Principal caudal rays numbered 16. Posterior chamber of gas bladder inflated. Emerging out of the gravel, feeding on small suspended particles was initiated, but yolk still evident.

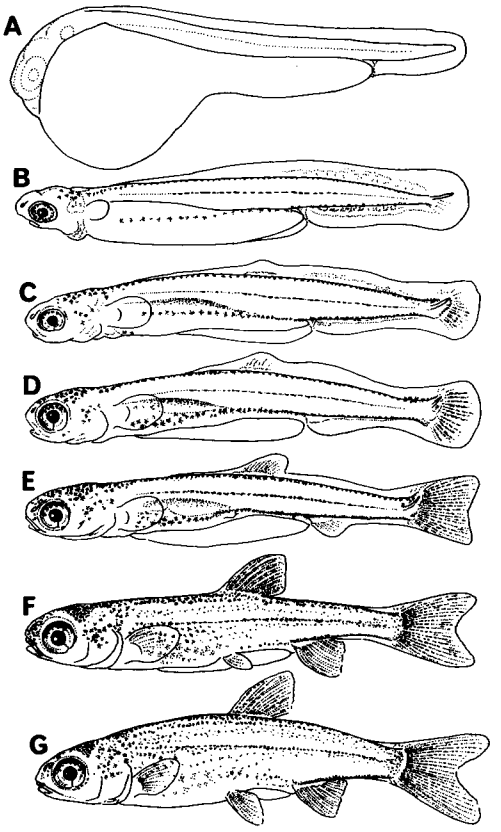

Fig. 2. Development of larvae of anadromous *Tribolodon hakonensis* from the Mu River, Hokkaido, A, immediately after hatching, 6.4 mm TL (6.3 mm SL) ; B, 8 days, 10.3 mm (10.0 mm) ; C, 12 days, 11.3 mm (10.6 mm) ; D, 15 days, 11.8 mm (10.8 mm) ; E, 23 days, 12.7 mm (11.1 mm) ; F, 41 days, 16.9 mm (13.9 mm) ; G, 48 days, 20.8 mm (16.6 mm).

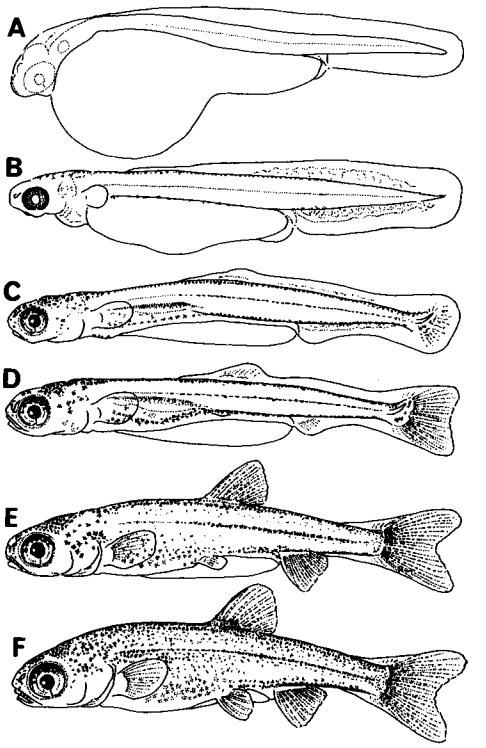

Fig. 3. Development of larvae of fluvial *Tribolodon hakonensis* from the Hime River, Hokkaido. A, immediately after hatching, 7.3 mm TL (7.1 mm SL) ; B, 3 days, 9.6 mm (9.2 mm) ; C, 10 days, 11.1 mm (10.5 mm) ; D, 13 days, 11.6 mm (10.4 mm) ; E, 25 days, 13.9 mm (11.7 mm) ; F, 35 days, 16.2 mm (13.6 mm).

Interval IV (early stage, Figs. 2E, 6E): 23 days after hatching, 12.6–14.0 (mean 13.3) mm TL (30 individuals). Preanus finfold development maximized. Pelvic finfolds appeared. Gas bladder's anterior chamber inflated.

Interval V (Figs. 2F, 6F): 41 days after hatching, 15.6–17.5 (mean 16.4) mm TL (25 individuals). Pectoral fins became triangular. Dorsal and anal fins development completed. Caudal fin notched. Preanal finfold gradually degenerated. Initiation of intestine folding.

Interval V (late stage, Figs. 2G, 6G): 48 days after hatching, 16.1–20.5 (mean 17.7) mm TL (30 individuals). Pectoral and anal fins development completed. Small preanal finfold remaining. Intestine deeply folded. Dorsal and anal finfolds

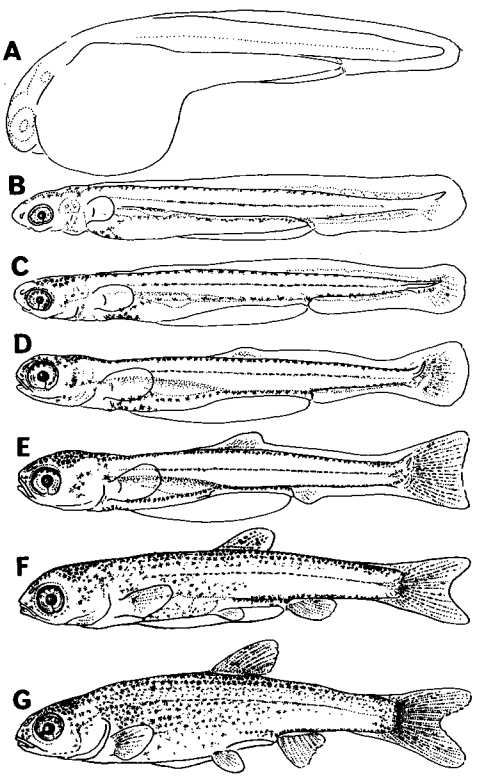

Fig. 4. Development of larvae of *Tribolodon brandti* from the Mu River, Hokkaido. A, immediately after hatching, 6.0 mm TL (5.8 mm SL); B, 7 days, 10.1 mm (9.7 mm); C, 14 days, 10.5 mm (10.0 mm); D, 21 days, 11.2 mm (10.2 mm); E, 25 days, 12.7 mm (11.2 mm); F, 43 days, 16.2 mm (13.7 mm); G, 50 days, 20.1 mm (17.0 mm).

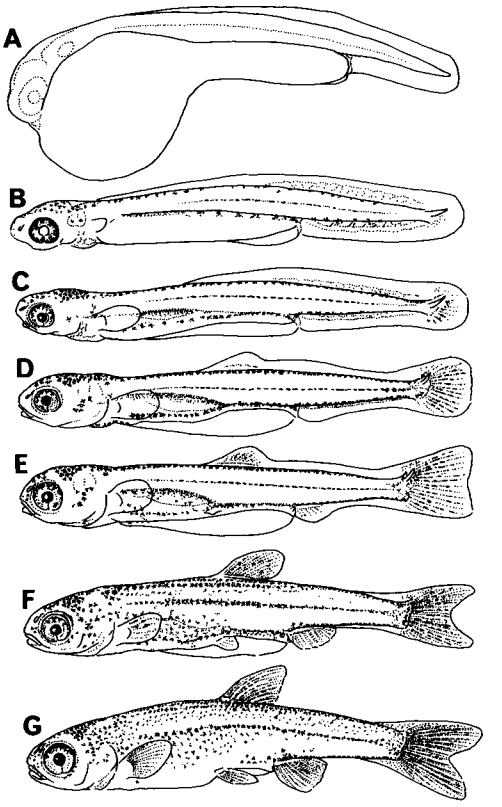

Fig. 5. Development of larvae of *Tribolodon ezoe* from the Mu River, Hokkaido. A, immediately after hatching, 5.0 mm TL (5.9 mm SL); B, 6 days, 8.3 mm (8.0 mm); C, 13 days, 8.8 mm (8.4 mm); D, 20 days, 10.1 mm (9.2 mm); E, 24 days, 11.3 mm (9.9 mm); F, 42 days, 13.5 mm (11.6 mm); G, 49 days, 17.1 mm (14.3 mm).

distinct.

Fluvial *T. hakonensis* from the Hime River. Interval I (earliest stage, Figs. 3A, 7A): Immediately after hatching, 6.8–8.3 (mean 7.7) mm TL (12 individuals). Tadpole-shaped and pigmentation absent. Intestine straight and filamentous, liver seen on the left side of the intestine. Fish restricted to laying on the bottom.

Interval II (earliest stage, Figs. 3B, 7B): 3 days after hatching, 8.8–10.0 (mean 9.6) mm TL (13 Individuals). Mouth opened and eyes pigmented black. Melanophores appeared on head, thorax, dorsal and ventral sides of the trunk. Progressive development of vertical finfolds. Branchial blood circulation was visible. Lower caudal vein in anal finfold and segmented respiratory vessels (Balon, 1975a) developed. Pancreas on the right side and posterior chamber of gas bladder on the dorso-dextral side of intestine visible. Intense negative phototaxis.

Interval III (early stage, Figs. 3C, 7C): 10 days after hatching, 9.5–11.4 (mean 10.8) mm TL (11 individuals). Peritoneum and breast tinted silver. Concentration of mesenchyme in dorsal fin observed. Caudal rays began to develop. Gall bladder and anterior chamber of gas bladder visible. Posterior chamber of gas bladder inflated. Emergence from gravel, feeding on small suspended particles initiated, but yolk still apparent.

Interval IV (early stage, Figs. 3D, 7D): 13 days after hatching, 10.3–11.5 (mean 11.0) mm TL (11 individuals). Preanus finfold development maximized. Pelvic finfolds appeared and anterior chamber of gas bladder inflated.

Interval V (early stage, Figs. 3E, 7E): 25 days after hatching, 10.3–14.0 (mean

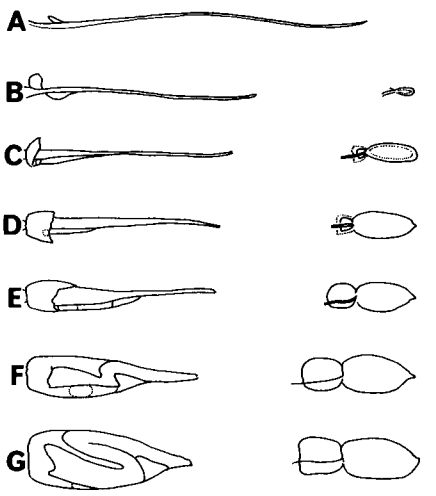

Fig. 6. Gut development of anadromous *Tribolodon hakonensis* (ventral view) from the Mu River, Hokkaido. Left, digestive gut; right, gas bladder (removed from behind the intestine). Alphabetical nomenclature and magnifications correspond to Fig. 2.

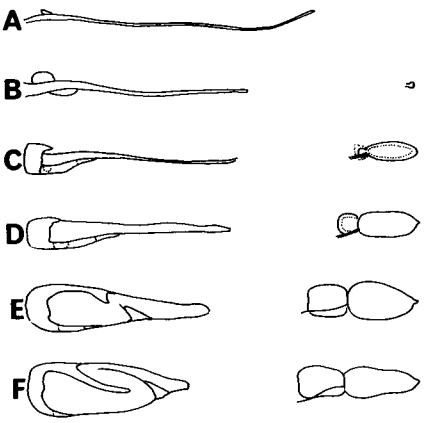

Fig. 7. Gut development of fluvial *Tribolodon hakonensis* (ventral view) from the Hime River, Hokkaido. Left, digestive gut; right, gas bladder (removed from behind the intestine). Alphabetical nomenclature and magnifications correspond to Fig. 3.

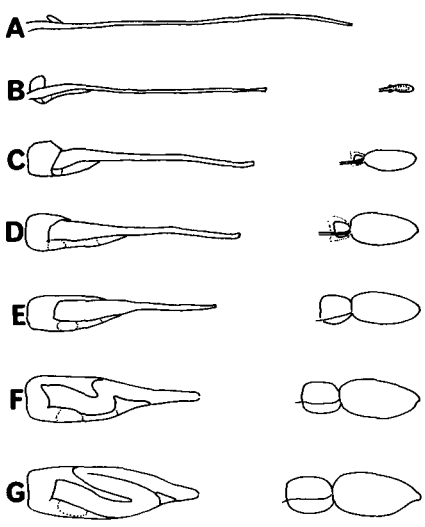

Fig. 8. Gut development of *Tribolodon brandti* (ventral view) from the Mu River, Hokkaido. Left, digestive gut; right, gas bladder (removed from behind the intestine). Alphabetical nomenclature and magnifications correspond to Fig. 4.

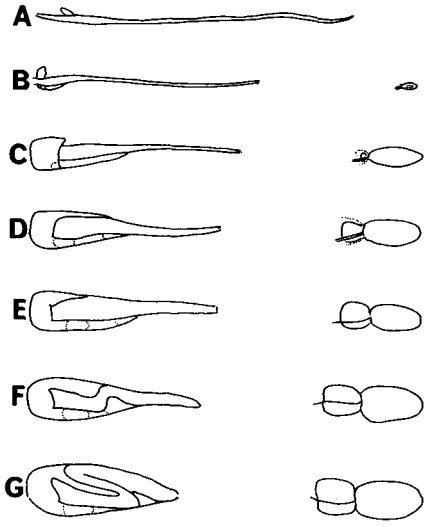

Fig. 9. Gut development of *Tribolodon ezoe* (ventral view) from the Mu River, Hokkaido. Left, digestive gut; right, gas bladder (removed from behind the intestine). Alphabetical nomenclature and magnifications correspond to Fig. 5.

12.0) mm TL (11 individuals). Pectoral fins became triangular. Dorsal and anal fins completed. Caudal fin notched. Preanal finfold gradually degenerated. Intestine folding initiated.

Interval V (late stage, Figs. 3F, 7F): 35 days after hatching, 11.1-16.3 (mean 14.8) mm TL (16 individuals). Pectoral and anal fins completed. Only a small preanus finfold remained. Dorsal and anal finfolds were distinct.

***T. brandti* from the Mu River.** Interval I (earliest stage, Figs. 4A, 8A): immediately after hatching, 5.3-6.3 (mean 5.8) mm TL (10 individuals). Tadpole-shaped with no pigmentation. Intestine straight and filamentous, liver seen on the left side of the intestine. Fish restricted to laying on the bottom.

Interval II (Figs. 4B, 8B): 7 days after hatching, 9.8-10.5 (mean 10.2) mm TL (24 individuals). Mouth opened and eyes pigmented silver. Melanophores appeared on head, thorax, dorsal, lateral midline and ventral sides of the trunk. Continued development of vertical finfolds. Branchial blood circulation observed. Lower caudal vein in anal finfold and segmentary respiratory vessels (Balon, 1975a) developed. Appearance of pancreas on the right side, posterior chamber of gas bladder on the dorso-dextral side of intestine, and gall bladder. Notochord flexion initiated. Intense negative phototaxis observed.

Interval II (last stage, Figs. 4C, 8C): 14 days after hatching, 9.9-11.4 (mean 10.5) mm TL (27 individuals). Initial development of caudal rays. Anterior chamber of gas bladder observed and posterior chamber inflated. Temporary respi-

ratory organs gradually reduced. Emerging from the gravel, feeding on small suspended particles initiated, but yolk still apparent. Acceleration of gas bladder development and/or retardation of fin development were recognized as compared to similar larval stage of *T. hakonensis* (Figs. 2, 3, 6 and 7).

Interval III (early stage, Figs. 4D, 8D) : 21 days after hatching, 10.9-11.7 (mean 11.3) mm TL (25 individuals). Peritoneum and breast tinted silver. Concentration of mesenchyme seen in dorsal fin and notochord end flexed.

Interval IV (early stage, Figs. 4E, 8E) : 25 days after hatching, 11.5-13.1 (mean 12.3) mm TL (42 individuals). Maximum development of preanus finfold. Pelvic finfolds observed and anterior chamber of gas bladder inflated.

Interval V (Figs. 4F, 8F) : 43 days after hatching, 14.0-18.0 (mean 15.2) mm TL (15 individuals). Pectoral fins became triangular. Dorsal and anal fins completed. Caudal fin notched. Preanal finfold gradually degenerated. Intestine folding initiated.

Interval V (late stage, Figs. 4G, 8G) : 50 days after hatching, 14.0-20.7 (mean 16.0) mm TL (20 individuals). Pectoral and anal fins completed. Small preanal finfold remained. Intestine deeply folded. Dorsal and anal finfolds were distinct.

***T. ezoe* from the Mu River.** Interval I (earliest stage, Figs. 5A, 9A) : immediately after hatching, 4.0-5.4 (mean 4.8) mm TL (27 individuals). Tadpole-shaped and pigmentation absent. Intestine straight and filamentous, and liver seen on the left side of the intestine. Fish restricted to laying on the bottom.

Interval II (early stage, Figs. 5B, 9B) : 6 days after hatching, 7.3-8.6 (mean 8.1) mm TL (30 individuals). Mouth opened and eyes slightly pigmented silver. Melanophores appeared on thorax, dorsal, lateral midline and ventral sides of the trunk. Maximum development of vertical finfolds achieved. Branchial blood circulation visible. Lower caudal vein in anal finfold and segmentary respiratory vessels (Balon, 1975a) developed. Pancreas on the right side and posterior chamber of gas bladder on the dorso-dextral side of intestine appeared. Notochord flexion initiated. Intense negative phototaxis apparent.

Interval II (last stage, Figs. 5C, 9C) : 13 days after hatching, 8.6-9.2 (mean 8.9) mm TL (19 individuals). Caudal ray development initiated. Anterior chamber of gas bladder appeared and posterior chamber inflated. Temporary respiratory organs gradually reduced. Emergence from gravel, feeding on small suspended particles initiated, but yolk still apparent. Acceleration of gas bladder and/or retardation of fin development were recognized as compared to similar larval stages of *T. hakonensis* (Figs. 2, 3, 6 and 7).

Interval III (early stage, Figs. 5D, 9D) : 20 days after hatching, 9.3-10.8 (mean 10.0) mm TL (31 individuals). Peritoneum and breast tinted silver and concentration of mesenchyme observed in dorsal fin.

Interval IV (early stage, Figs. 5E, 9E) : 24 days after hatching, 10.3-12.8 (mean 11.2) mm TL (38 individuals). Preanus finfold development maximized. Pelvic finfolds observed and anterior chamber of gas bladder inflated.

Interval V (Figs. 5F, 9F) : 42 days after hatching, 13.3-18.0 (mean 14.7) mm TL (31 individuals). Pectoral fins became triangular and dorsal and anal fins completed. Caudal fin notched. Preanal finfold gradually degenerated. Intestine folding initiated.

Interval V (late stage, Figs. 5G, 9G): 49 days after hatching, 13.7-17.4 (mean 15.8) mm TL (40 individuals). Development of pectoral and anal fins complete. Small preanal finfold remained. Intestine deeply folded. Dorsal and anal finfolds were distinct.

2. Development of cephalic lateral line system

The cephalic lateral line system of *Tribolodon* (Fig. 10) is composed of the supraorbital canal (SO), infraorbital cancal (IO), postocular commisure (POC), supratemporal canal (ST), preoperculomandibular canal (POM), and cephalic lateralis (CL) (Nakamura, 1963, 1969; Kurawaka, 1977). The development of ST and CL were not included in the following observations.

In all types and species (Figs. 11-14), pit lines existed as the cephalic lateral line system during the final larval stage. Canalization started at SO and POM in early juvenile stage, and initially completed at POC, SO and POM in approximately 50 mm SL young, IO and POC were connected and the system nearly completed in

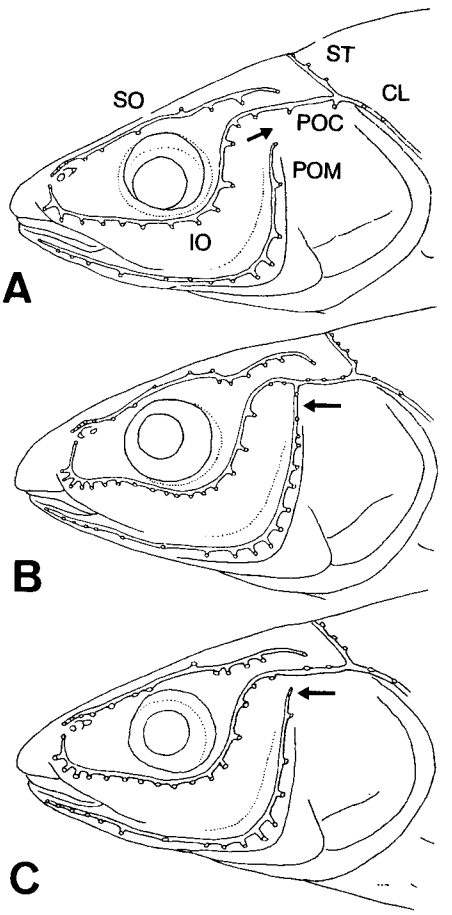

Fig. 10. Diagrammatic drawing of the cephalic lateral-line system in *Tribolodon hakonensis* (A), *T. brandti* (B), and the intermadiate state observed in hybrid between *T. hakonensis* and *T. brandti* (C). SO, supraorbital canal; IO, infraorbital canal; POC, postocular commisure; POM, preoperculomandibular canal; ST, supratemporal canal; CL, cephalic lateralis; arrow, portion of canal connection between POC and POM.

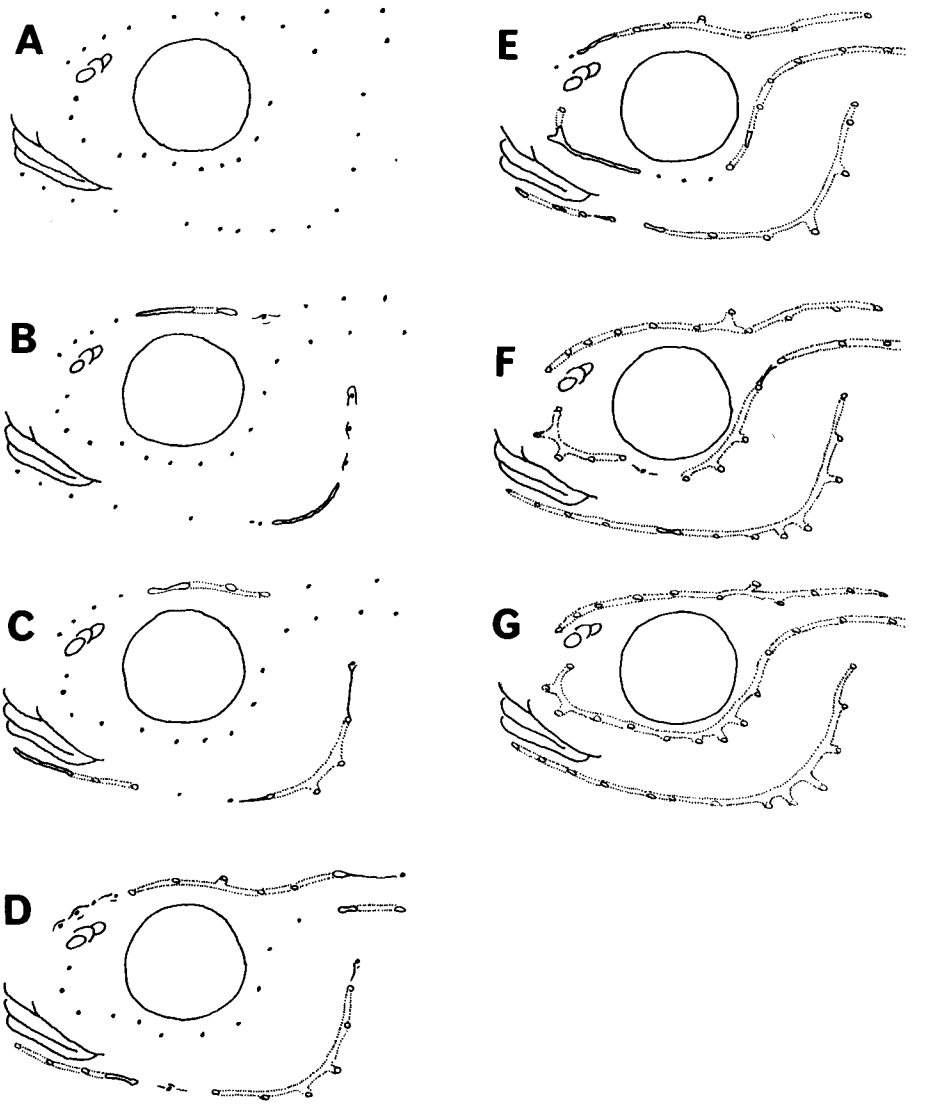

Fig. 11. Development of cephalic lateral-line system in anadromous *Tribolodon hakonensis* from the Mu River, Hokkaido. A, 16.7 mm SL; B, 18.2 mm; C, 22.0 mm; D, 24.1 mm; E, 25.6 mm; F, 45.0 mm; G, 58.2 mm.

about 60 mm SL young with the exception of *T. brandti*. In *T. brandti*, the connection between POC and POM was not completed during this stage, which is a distinguishing characteristic of *T. brandti*. Canals are usually completed in the young of 80 mm SL (Kurawaka, 1977).

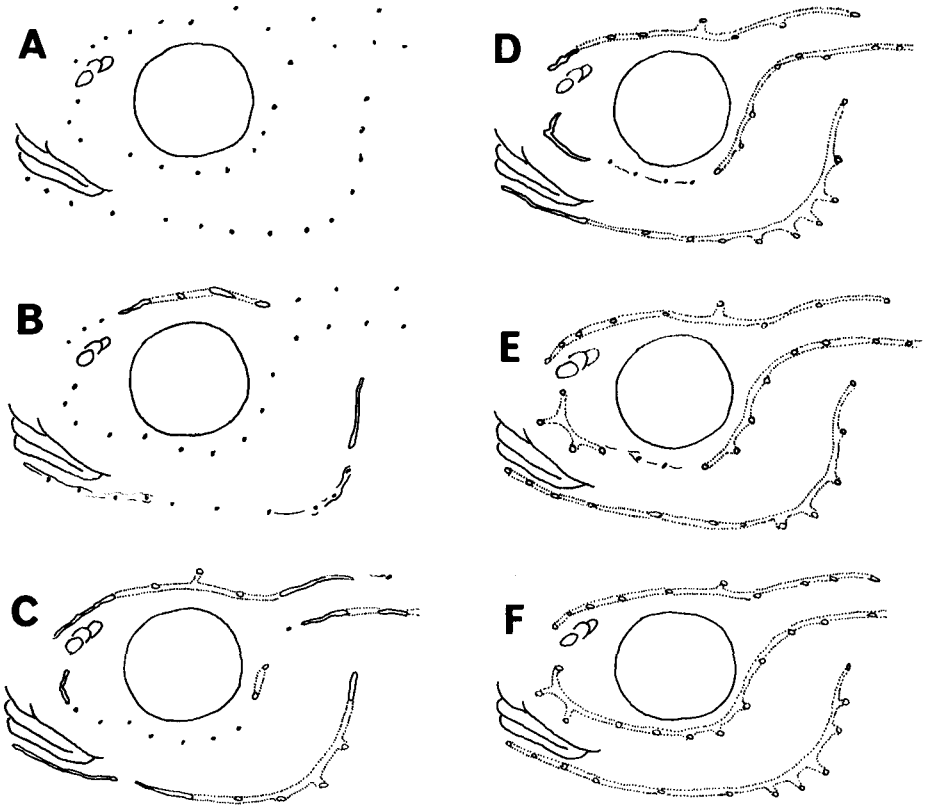

Fig. 12. Development of cephalic lateral-line system in fluvial *Tribolodon hakonensis* from the Hime River, Hokkaido. A, 16.5 mm SL; B, 18.9 mm; C, 23.7 mm; D, 28.9 mm; E, 40.1 mm; F, 56.6 mm.

3. Relative growth

Head length (Fig. 15): The rapid growth of the relative head size from hatching until reaching approximately 20 mm SL was observed, and then it became fixed at approximately 25-30% of SL. The largest head size was realized in *T. brandti* (approximately 30% of SL).

Snout length (Fig. 16): The relative snout size in *T. hakonensis* increased until 70 mm SL was realized when it achieved approximately 8-9% of SL. Relative snout length in *T. brandti* and *T. ezoe* advanced slowly until 130 mm SL was achieved and then fixed at approximately 9-10% of SL.

Upper jaw length (Fig. 17): Maximum relative length of the upper jaw reached 8% of SL when fish approached 50 mm SL. Subsequently the length gradually reduced in *T. hakonensis* and *T. ezoe*.

Eye diameter (Fig. 18): The relative size of eye enlarged rapidly from hatching until 15 mm SL was achieved, and subsequently slowly reduced in all species and types. The eye size of fluvial *T. hakonensis* was the largest.

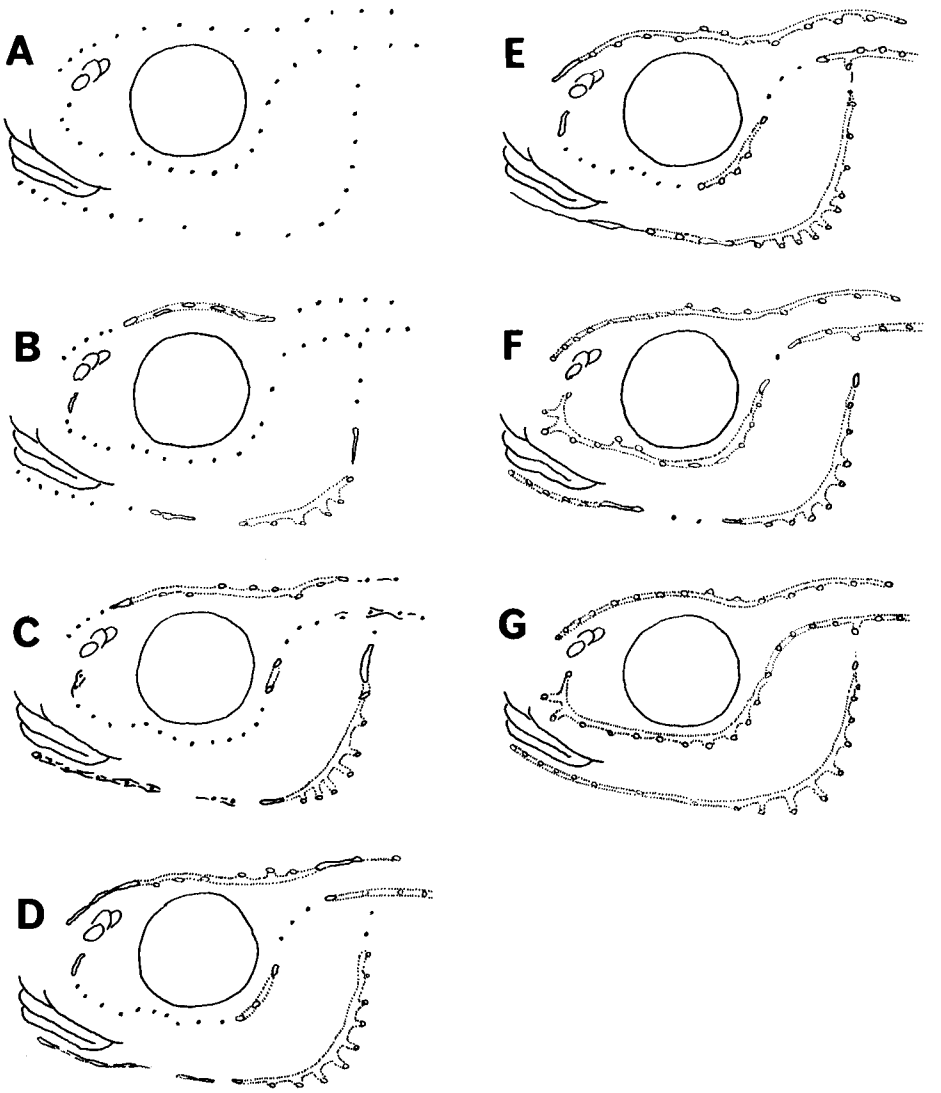

Fig. 13. Development of cephalic lateral-line system in *Tribolodon brandti* from the Mu River, Hokkaido. A, 17.0 mm SL; B, 22.3 mm; C, 23.3 mm; D, 27.6 mm; E, 34.1 mm; F, 47.3 mm; G, 59.9 mm.

Suborbital length (Fig. 19): The suborbital space gradually grew wider and reached approximately 3.5% of SL in *T. hakonensis* and about 4.5% of SL in *T. brandti* and *T. ezoe*.

Preanus length (Fig. 20): No peculiar growth pattern was obvious.

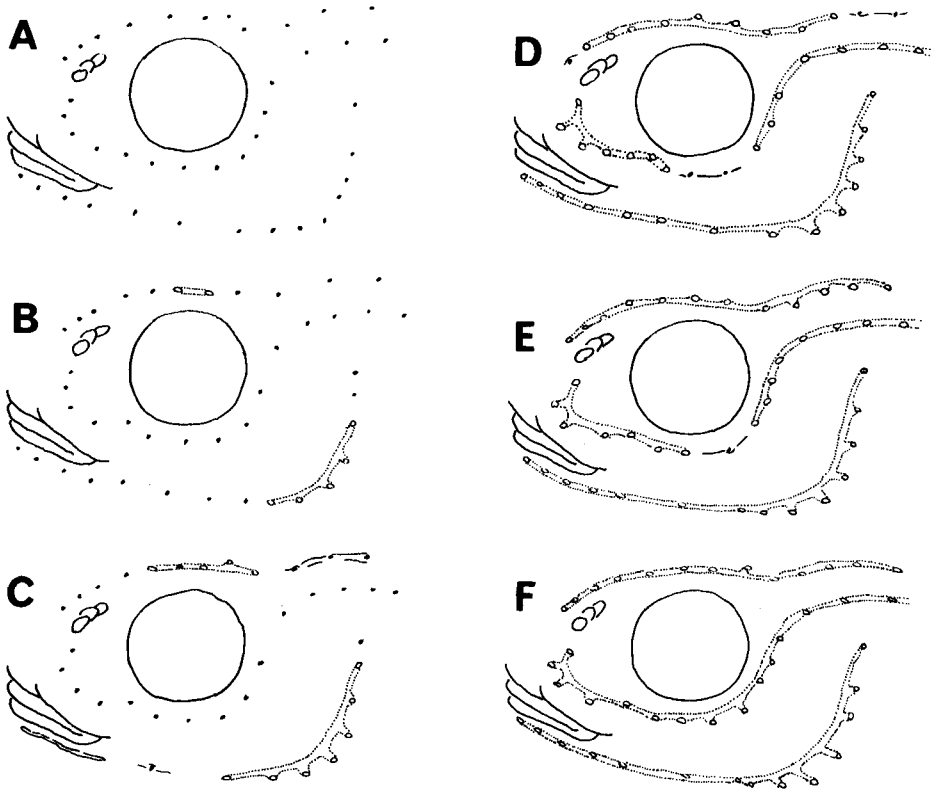

Fig. 14. Development of cephalic lateral-line system in *Tribolodon ezoe* from the Mu River, Hokkaido. A, 18.3 mm SL; B, 20.6 mm; C, 28.8 mm; D, 35.0 mm; E, 42.9 mm; F, 55.3 mm.

Discussion

Spawning of *Tribolodon* occurs en masse on rapids with gravel bottoms, with a preponderance of males (Okada, 1935; Kawajiri, 1956; Tabeta and Tsukahara, 1964; Nakamura, 1969; Ito, 1975; Dai et al., 1982; Gavrenkov, 1982; Gritsenko, 1982; Sakai, 1987, 1990). The eggs are attached to the under surfaces of the gravel. The larvae of three species of *Tribolodon* seemed to develop similar series of developmental intervals. Namely, hatched larvae migrate down into the interstices of gravel (Interval I), and then actively hide themselves deep in the gravel (Interval II) (Okada, 1935; Nakamura, 1963). The larvae emerge from the gravel at Interval III when they are able to eat large food such as water fleas. As the developmental intervals progress, the larvae acquire new feeding habits enabled by increased swimming ability. Finally the larvae metamorphose into juveniles during Interval VI, extending their habitat from the solw-flowing shallows to the riffles and ponds where many sessile organisms and/or drifting prey are available (Sakai, 1990).

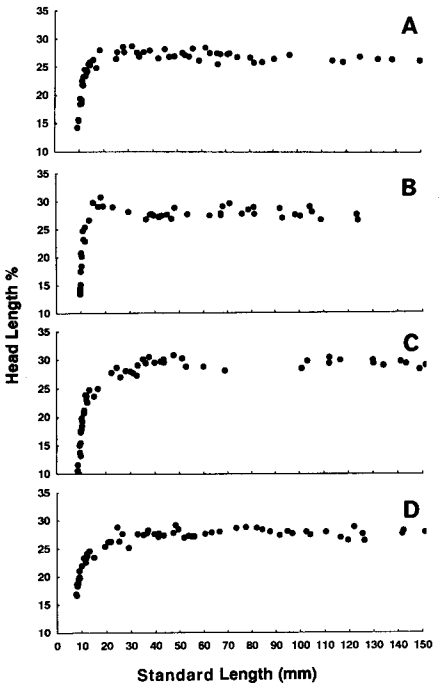

Fig. 15. Relative growth of head length in % of SL in *Tribolodon*. A, anadromous *T. hakonensis*; B, fluvial *T. hakonensis*; C. *T. brandti*; D, *T. ezoe*.

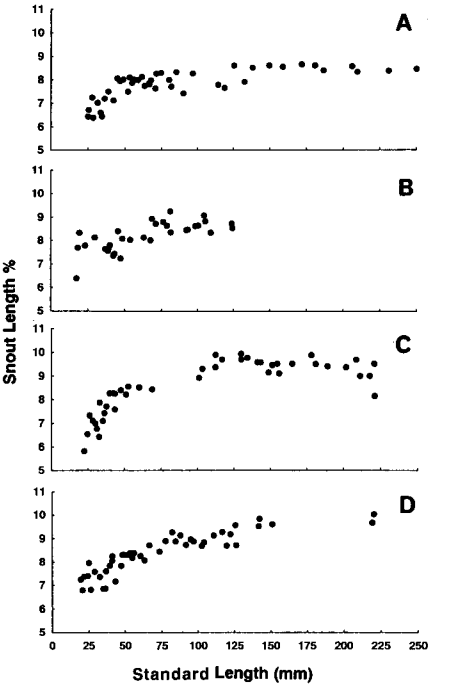

Fig. 16. Relative growth of snout length in % of SL in *Tribolodon*. A, anadromous *T. hakonensis*; B, fluvial *T. hakonensis*; C. *T. brandti*; D, *T. ezoe*.

These developmental intervals are thought to be characterized by a rich supply of yolk persisting beyond the start of notochord flexion (Sakai, 1990). This is probably closely related to the reproductive style of the species. *Tribolodon* is a lithophilous open substratum spawner (Balon, 1975a) and its larvae show strong photophobia which assists them in hiding under stones. The rich supply of yolk enables them to grow temporarily in the interstices of gravels without food. When they emerge from the gravel, they are large enough to eat large food.

Some subtle interval differences among species are the accelerated gas bladder development and the retarded fin development observed in interval II of *T. brandti* and *T. ezoe* as compared with that of *T. hakonensis*. These may be related to their smaller volume of yolk storage (smaller egg size, as described in the later chapter) than *T. hakonensis*. Cyprinid yolk volume often affects a series of larval developmental intervals, especially during the interval where feeding initiates (Sakai, 1990). However, differences described previously in *Tribolodon* are so subtle they never affect the series of developmental intervals, because the eggs of *T. brandti* and *T. ezoe* are larger than many other cyprinids (see Nakamura, 1969).

Cephalic lateral line canalization initiated in interval VI (juvenile) and completed at approximately 60 mm SL in all species of *Tribolodon*, except for the

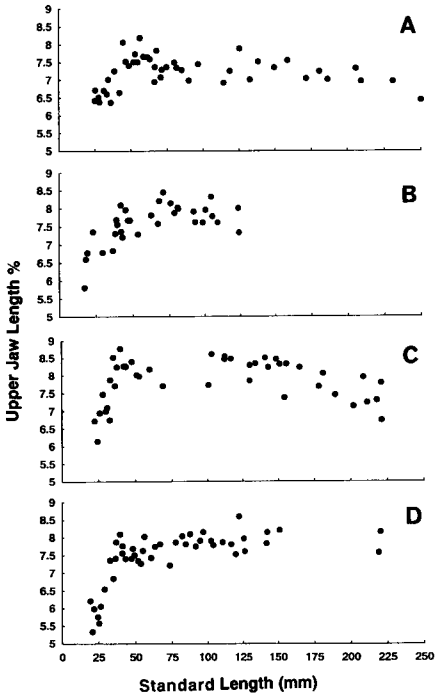

Fig. 17. Relative growth of upper jaw length in % of SL in *Tribolodon*. A, anadromous *T. hakonensis*; B, fluvial *T. hakonensis*; C. *T. brandti*; D, *T. ezoe*.

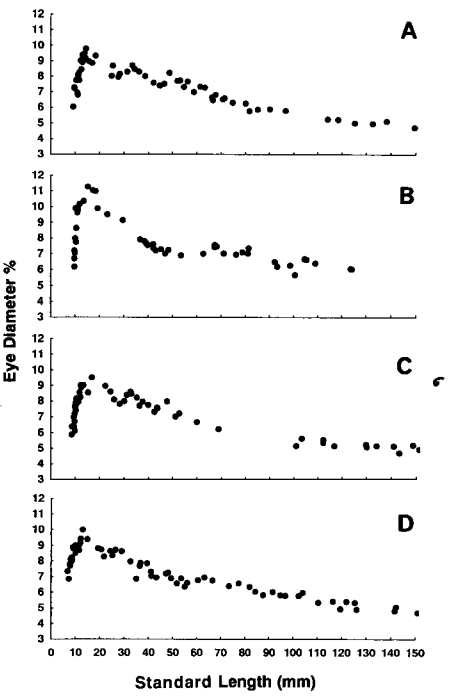

Fig. 18. Relative growth of eye diameter in % of SL in *Tribolodon*. A, anadromous *T. hakonensis*; B, fluvial *T. hakonensis*; C. *T. brandti*; D, *T. ezoe*.

connection between POC and POM in *T. brandti*, a characteristic distinguishing this species. These canals, POC and POM, must connect hypermorphically (Gould, 1977) in *T. brandti* as compared with the other species. The cephalic lateral line canalization is usually completed in 80 mm SL young (Kurawaka, 1977), and the interval IV is thought to continue in the 60-80 mm SL young fish. Achieving 60-80 mm SL the young may migrate to the sea in anadromous species (discussed in the next chapter).

In the relative growth patterns, the head length fixed and the eye diameter changed its relative size just before interval VI. The upper jaw length fixed at 50-70 mm SL, during the final stage of interval VI, in all species. The snout length fixed at approximately 70 mm SL, the end of interval VI, in *T. hakonensis*. The snout of *T. brandti* and *T. ezoe* continued to grow longer until 130 mm SL was realized. They have longer snouts than *T. hakonensis* (Nakamura, 1969). The snout development may be prolonged beyond interval VI in *T. brandti* and *T. ezoe* as compared with *T. hakonensis*.

The laval development of *Tribolodon* spp. is considered as a series of developmental intervals I to V, the the juvenile interval VI is thought to continue until 60-

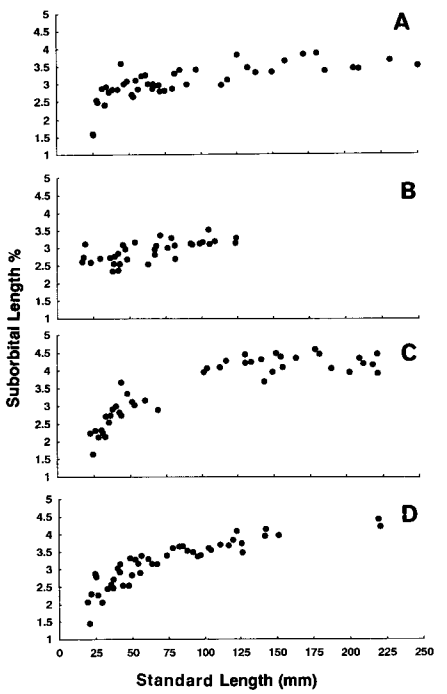

Fig. 19. Relative growth of suborbital length in % of SL in *Tribolodon*. A, anadromous *T. hakonensis*; B, fluvial *T. hakonensis*; C. *T. brandti*; D, *T. ezoe*.

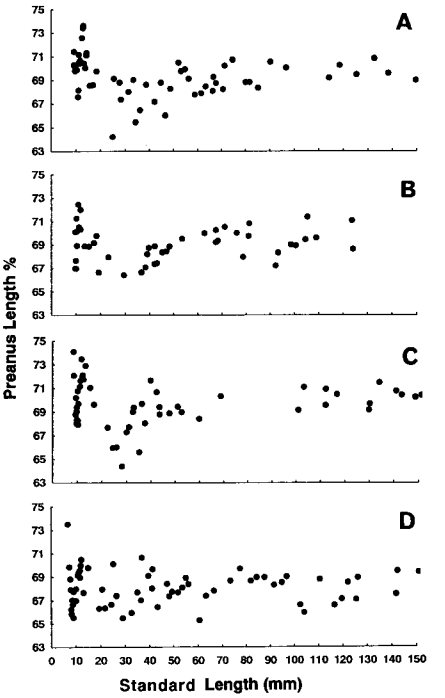

Fig. 20. Relative growth of preanus length in % of SL in *Tribolodon*. A, anadromous *T. hakonensis*; B, fluvial *T. hakonensis*; C. *T. brandti*; D, *T. ezoe*.

80 mm SL is achieved in all species. The differences among species during the intervals are slight.

IV. Developmental intervals in latter half of ontogeny

The previous chapter divided larval and juvenile development of *Tribolodon* into six intervals (interval I to VI), and all members in this study are thought to experience similar interval events. Subsequently, fish ontogenetic stages also exhibit developmental intervals (Balon, 1979, 1985) including *Tribolodon*.

Anadromous species migrate to the sea, descending rivers when young and ascending back during spawning periods (Nakamura, 1969). Additionally, the wintering migration is known in anadromous *Tribolodon* from Hokkaido (Kanoh, 1949). Therefore, the latter developmental intervals of *Tribolodon* involves their migration and maturation. The major differences observed in the life history among *Tribolodon* are exhibited during the latter half of ontogeny. Salinity tolerance, space preference, migration pattern, age and growth, and spawning age are utilized to describe developmental intervals during the latter half of ontogeny. The

Table 2. Materials and conditions for the experiments of salinity tolerance in juvenile *Tribolodon*.

Period	Range (mean) of WT°C	Salinity (‰) of 100% sea water	Range (mean) of SL mm					
			T. hakonensis		*T. brandti*	*T. ezoe*	*Carassius auratus*	*Pseudorasbora parva*
			anadromous	fluvial				
1982 (preliminary)								
7/18- 8/ 9	16.7-27.6	29.24-31.06	11.7-14.4 (13.3)		11.5-14.0 (12.4)	11.2-13.9 (12.3)		
9/21-10/21	15.4-24.5	32.68-37.61	18.6-29.5 (22.4)		16.6-35.0 (26.0)	16.6-27.6 (20.5)		
12/ 8- 1/10	19.1-20.0	31.91-36.42	23.6-32.6 (27.9)		19.5-37.9 (25.7)	28.6-39.0 (31.9)	29.3-44.9 (37.2)	37.5-52.2 (42.9)
1983								
8/ 6- 8/23	22.8-32.0 (28.1)	29.60-30.04	31.0-48.0 (37.5)*		41.9-53.0 (46.5)*			
9/30-10/10	13.2-19.4 (16.4)	29.92-32.47	22.9-39.1 (27.4)	21.0-40.9 (27.9)	22.1-35.2 (25.6)	20.3-38.1 (26.3)		
			47.1-64.2 (54.4)*		59.2-65.3 (63.2)*			
11/ 4-11/16	8.3-15.4 (11.5)	29.74-32.41	26.4-35.3 (29.9)	22.3-28.9 (26.2)	22.6-31.1 (25.7)	22.4-33.2 (27.2)		
			42.2-65.0 (51.7)*		38.2-69.2 (58.7)*			
1984								
2/14- 2/24	0.3-7.8 (4.0)	29.38-31.04	19.3-29.2 (25.3)	18.9-29.6 (23.9)	21.7-29.9 (24.2)	17.3-32.3 (23.7)		
			45.3-57.0 (51.7)*		53.0-66.9 (60.7)*			
4/23- 5/ 3	8.2-14.8 (11.4)	30.78-31.52	19.5-37.0 (26.1)	21.3-33.2 (28.2)	20.6-29.8 (25.5)	21.0-29.3 (23.4)		
5/22- 6/ 1	13.2-20.0 (17.3)	29.89-30.54	24.0-38.6 (31.7)	28.3-38.9 (33.5)	24.8-34.5 (27.7)	21.1-32.8 (27.4)		
			57.0-70.1 (64.4)*		66.6-75.8 (70.8)*			
8/ 5- 8/15	21.4-30.5 (27.8)	29.75-30.21	34.1-47.7 (42.9)	36.0-50.7 (42.8)	31.9-44.8 (38.8)	30.1-45.0 (37.6)		
9/21-10/ 1	14.5-25.5 (19.8)	29.38-30.95	41.4-57.9 (52.1)	41.7-56.9 (49.2)	42.0-56.7 (47.3)	38.0-56.7 (48.2)		

*: 1+ young.

histogram method utilized in dividing the larval developmental intervals was not adopted because the developmental events were not always conspicuous during these periods of development.

Materials and methods

1. Development of salinity tolerance

Experimental conditions and fish size utilized are shown in Table 2. Photoperiod and water temperature were not controlled except for one case in which the fish were acclimated to 20°C water temperature for one month prior to salinity tolerance testing under 20°C during the winter, 1982 experiment.

In 1982, preliminary one month experiments were conducted in summer, autumn and winter. Experimental fish were siblings of the fish observed the larval developments. Wild fish of *Carassius auratus langsdorfi* and *Pseudorasbora parva* (both cyprinids) caught from the Onuma Lake were used in the winter experiment for comparison. Four comcentrations of sea water (100% sea water≒30‰ salnity) were prepared, 80, 60, 40, and 20% sea water in summer, 100, 80, 60, and 40% sea water in autumn and winter. Survival rates of 30 individuals were determined in a 30 *l* closed circulating aquarium for one month at each experimental section under feeding condition if they had appetite.

In 1983 to 1984, 10 days experiments were replicated throughout the year in 1+ (to 2+) fish and in 0+ (to 1+) fish 5 and 7 times, respectively. The 1+fish were siblings of the fish observed the larval developments. Collections of parental fish for the 0+fish included ; one matured female, 283.0 mm SL, and one male, 248.0 mm SL of anadromous *T. hakonensis* from the Mu River on 19 June, 1983, one matured female, 152.5 mm SL, and one male, 110.0 mm SL of fluvial *T. hakonensis* from the Hime River on 1 July, 1983, one matured female, 375.0 mm SL, and one male, 332. 5 mm SL of *T. brandti* from the Mu River on 19 June, 1983, and one matured female, 207.0 mm SL, and one male, 145.0 mm SL of *T. ezoe* from the Atsuma River on 21 June, 1983. Approximately 1,000 eggs from each female were fertilized by the dry method and scattered on gravel containing 100 *l* closed circulating system for incubating and rearing.

100% sea water was prepared for 1+ fish. Three concentrations of sea water were prepared for 0+ fish, 100, 80, and 60% sea for *T. hakonensis* and *T. brandti*, 60, 40, and 20% sea water for *T. ezoe* in 1983, and 80, 60, and 40% sea water for *T. ezoe* in 1984. Ten individuals were introduced into a 3 *l* plastic jar and their survival rate was recorded from each experimental regime under non-feeding conditions.

2. Space preference of juveniles

The fish utilized in this section of the research were siblings of those used in the salinity tolerance experiments of 0+ fish in 1983. The hatched larvae of each species and type were reared for 1 year old. Ten individuals, 35.0-50.0 mm SL, from each population were selected and acclimated to each aquarium (30 *l*) for approximately one month, fed with commercially prepared crumbled pelleted food designed for carp. Feeding activity was recorded by video camera under two situations, feeding and non-feeding. Only the front face of the aquarium was

exposed to the camera set back 1.5 m. Feeding and video operations were remotely controlled. During the feeding situation, the introduced crumbles were all intially floating but gradually sank due to fish feeding attacks from the bottom and/or the aeration of water. During non-feeding situations, the fish usually set themselves near the bottom but were often attacking small air bubbles. In both experimental conditions, three 15 minute periods were video recorded and the number of feeding attacks during every third minute was counted. The feeding attacks were categorized as surface or middle layer events. Benthic feeding events were not scored due to the camera's resolution ability.

3. Migration

Kanoh (1949) described fish that wintered in Utonai Lake descended to the sea in spring and immediately accended the Mu River, 20 km east of Utonai Lake for spawning. Therefore, the anadromous *Tribolodon* from the Mu River is thought to hold three types of migration, sea-going, spawning, and wintering migrations. In this chapter, outlines of these migration patterns are described.

The distribution pattern of 0+ to 1+ fish in the Mu River was the first surveyed. Four to six sampling sites were selected from the river mouth to Kasuga Weir (Fig. 21). A preliminary survey was conducted in Oct., 1982 for 6 sampling sites. Distribution of 0+ (to 1+) fish born in 1983 was surveyed 4 times, Oct., 1983, Apr., Jun., and Jul., 1984, at 4 sampling sites; river mouth, Mukawabashi, Toyoshiro and Kasugabashi. In July, 1984, no fish were collected from Mukawabashi and Kasugabashi.

Young fish species of *Tribolodon* could not be easily morphologically identified. Therefore, electrophoretic species determinations were conducted following Sakai and Hamada (1985). The details of methods are described in chapters VI and VII.

Annual transitions of length distributions of young anadromous *T. hakonensis* and *T. brandti* from the Mu River were surveyed using a series of 12 samples

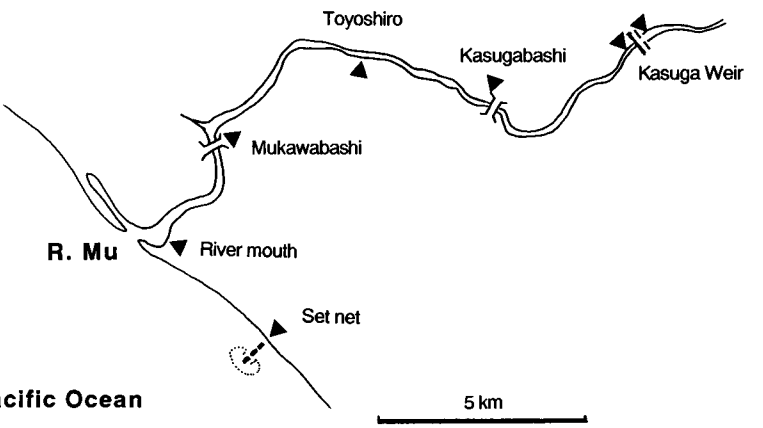

Fig. 21. Map showing sampling localities of *Tribolodon* in the Mu River and the set net in the Pacific coast.

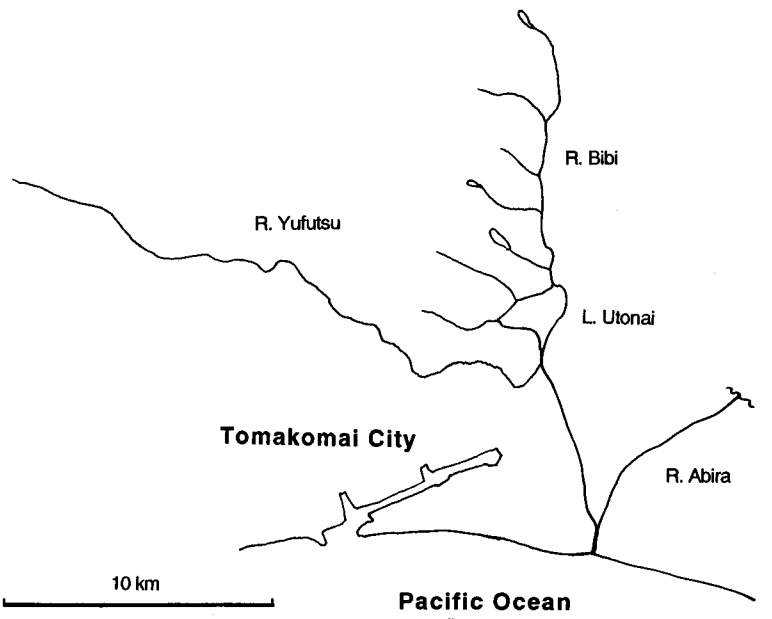

Fig. 22. Map showing the Yufutsu River system where many *Tribolodon* had wintered.

collected from Oct., 1980 to Oct., 1981 to clarify sea migration patterns. A series of 8 samples of fluvial *T. hakonensis* from Nov., 1982 to Sep., 1983 from the Hime River and a series of 10 samples of *T. ezoe* from Oct., 1980 to Oct., 1981 from the Atsuma River were also used for comparison. The sampling data are listed in Table 1.

For Consideration of wintering and spawning migrations, samples were occasionally collected from the wintering sites, Utonai Lake and the Yufutsu River shown in Fig. 22, from a set net in the sea shown in Fig. 21, and from the spawning site at the Kasuga Weir of the Mu River (Fig. 21). The sample data are listed in Table 1.

Growth patterns of wintering fish from Utonai Lake were extrapolated from reading scales. Potential migration patterns from the sea are discussed briefly. The scale reading method is described in the next section.

4. Age and growth

Age determination was accomplished by counting annual rings. The scales examined were selected from 5 to 10 scales from the dorso-lateral portion of the body between the dorsal fin and lateral line. A scale of *T. hakonensis* (4 years old) is shown in Fig. 23. Annual rings can appear as a slow growth zone in *Tribolodon* (Tanaka and Miyazaki, 1976). The zone appeared from autumn through June. Initiation of a rapid growth zone was observed in late June. The month of June is the most active spawning period for *Tribolodon* in Hokkaido (Sakai, 1987). Therefore, the age determined by counting the annual rings indicates the full age. The same situation has been reported in other cyprinids such as *Carassius* (Suzuki and

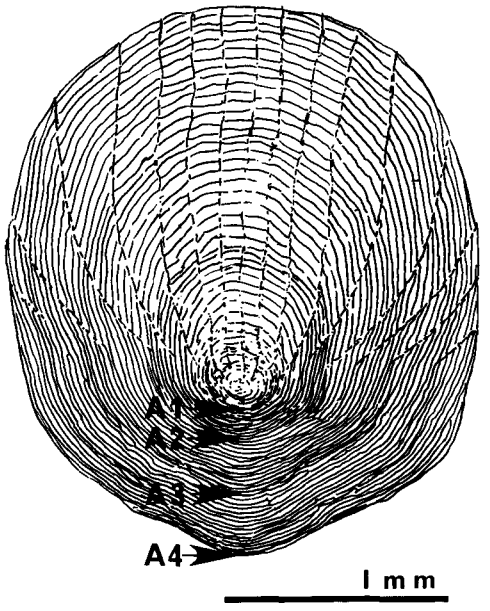

Fig. 23. A scale of anadromous *Tribolodon hakonensis*, 4 years old, 172.5 mm SL. Annual rings can be read as slow growth zones.

Kimura, 1977). Using specimens collected in March to June (Table 1), actual growth patterns were investigated for each species and type.

As the *Tribolodon* scales grow in proportion to the standard length, standard length extrapolations of previous ages are possible (Tanaka and Miyazaki, 1976). Extrapolation of previous age length was calculated by examining the relationship between the distance from the focus to an annual ring and the length from the focus to the edge of the hidden part of the scale. Calculated growth were determined utilizing specimens from all seasons as listed in Table 1.

5. Spawning age

Age of spawning adults of anadromous *T. hakonensis*, *T. brandti* and *T. ezoe* from the Mu River, and fluvial *T. hakonensis* from the Hime River was determined by reading scales as listed in Table 1.

Results

1. Development of salinity tolerance

larval stage fish exhibit a considerable tolerance to sea water (Fig. 24) in the summer experiment. Some *T. brandti* could tolerate even 80% sea water. Several fish of *T. hakonensis* and *T. ezoe* survived 60% and 40% sea water, respectively. In the autumn (Fig. 25), juveniles exhibited increased salinity tolerance. Limited numbers of *T. hakonensis* and *T. brandti* survived exposure to 100% sea water. A large portion of the observed *T. ozoe* was alive in 40% sea water. In the winter warm water experiment (about 20°C) (Fig. 26), all fish of *T. ezoe* survived in 40% sea water. No *T. hakonensis* and *T. brandti* tolerated 100% sea water. The least

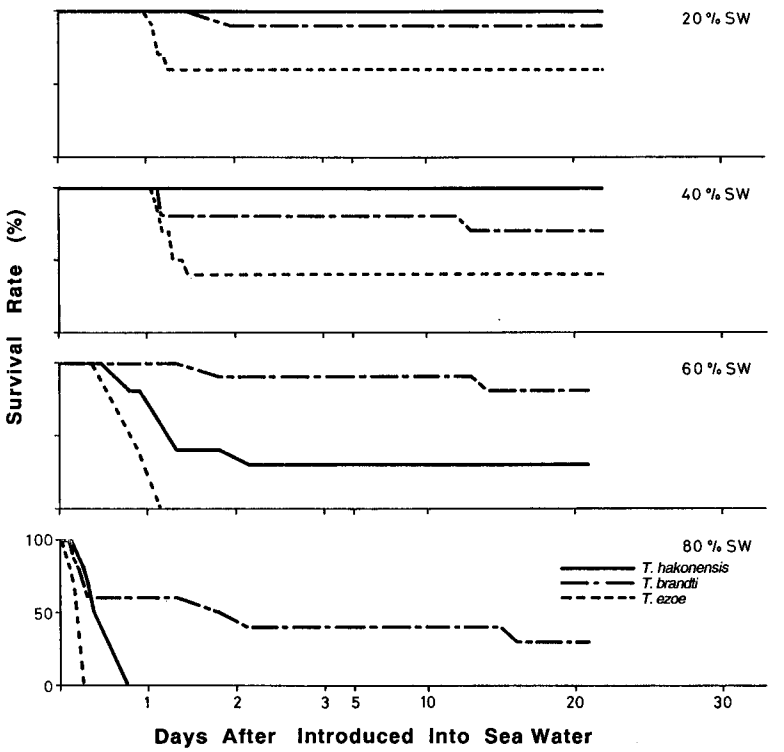

Fig. 24. Changes in survival rate of 0+ fish of *Tribolodon* from the Mu River reared in differently diluted sea water (100% sea water≒salinity 30‰), from July to August, 1982.

tolerant, *T. ezoe* , exhibited greater salinity tolerance than other cyprinids such as *C. auratus* and *P. parva*.

0+ fish of anadromous *T. hakonensis* and *T. brandti* survived exposure to 100% sea water in Oct. 1983, Apr., May, and Sep. 1984 (Fig. 27). A large percentage of 0+ fluvial *T. hakonensis* was tolerant to 100% sea water on Oct. 1983 and Sep. 1984. In April and May 1984, fluvial *T. hakonensis* showed slightly higher survival rates when compared with those in winter and summer. Similar results were recorded for *T. ezoe*, namely the survival rate decreased in winter and summer, and all fish survived 60% sea water exposure in May and Sep. 1984.

Similar tendencies were exhibited by 1+ to two years fish of *T. hakonensis* and *T. brandti* (Fig. 28). All fish vigorously survived exposure to 100% sea water on Oct. 1983 and May 1984.

2. Space preference of juveniles

Mean feeding attack frequencies of every third minutes are shown in Table 3. During non-feeding situation, both types of *T. hakonensis* attacked more on the surface and middle layers than *T. brandti* and *T. ezoe*. During the feeding situa-

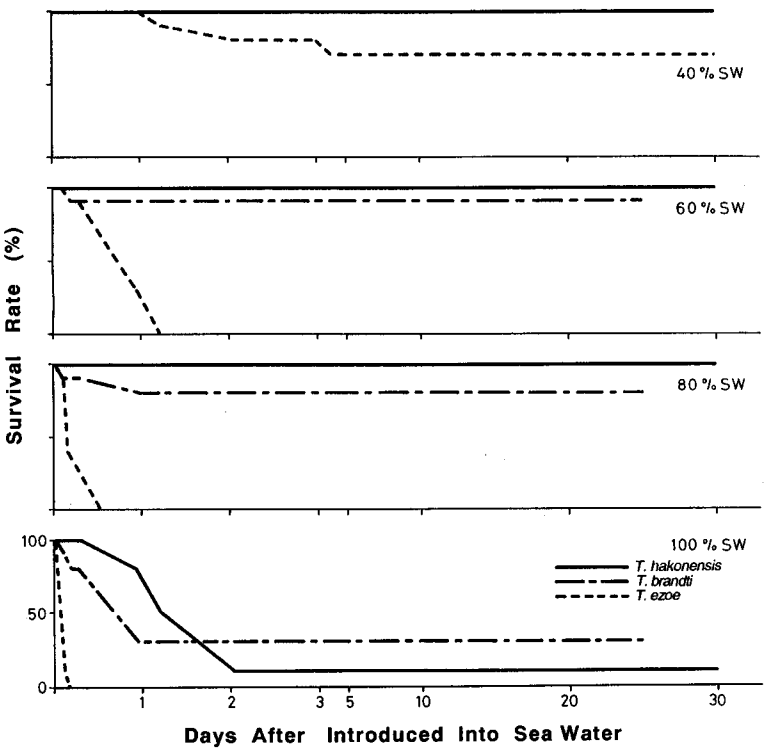

Fig. 25. Changes in survival rate of 0+ fish of *Tribolodon* from the Mu River reared in differently diluted sea water (100% sea water≒salinity 30‰), from September to October, 1982.

tions, both types of *T. hakonensis* fed much more on the surface and middle layers at the first minute than subsequent minutes. The fish attacked the surface floating crumbles at the onset then attacked the descending food. *T. brandti* fed more in the middle layer during the initial minute than subsequent minutes, but they rarely attacked the surface layer. Conversely, *T. ezoe* never attacked the surface but eagerly fed on bottom sunken foods due to aeration. Initial attacking frequencies were not high.

3. Migration

Sea-going migration of juveniles. Results of preliminary surveys are schematized in Fig. 29. The most conspicuous feature was *T. brandti* of which distribution restricted to the river mouth. Conversely, only *T. ezoe* was collected from the upper part of the Kasuga Weir which prevented anadromous spawners from ascending further. In the river course, *T. hakonensis* was distributed more to the lower reaches, but *T. ezoe* was less frequent.

Similar tendencies were recognized for juveniles born in 1983 (Fig. 30). From autumn to spring and summer of the next year, most juvenile fish of *T. brandti* were

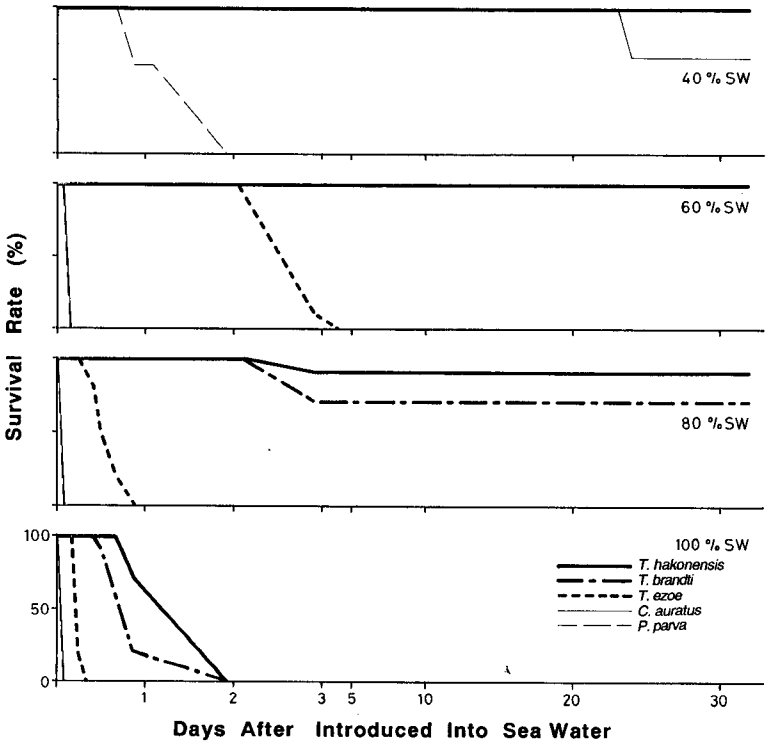

Fig. 26. Changes in survival rate of 0+ fish of *Tribolodon* from the Mu River, and *Carassius auratus langsdorfi* and *Pseudorasbora parva* from the Onuma Lake reared in differently diluted sea water (100% sea water≒salinity 30‰), from December, 1982 to January, 1983.

collected from the lower reaches. Most juveniles of *T. brandti* seem to descend to the river mouth by the middle of autumn. *T. ezoe* was collected more from the upper reaches, while *T. hakonensis* distribution varied from the upper to the lower reaches.

Anadromous *T. hakonensis* length composition transition from the Mu River is illustrated in Fig. 31 in which spawning runners are omitted. The largest composition change occurred in June, namely 0+ fish appeared and older fish than 2 years disappeared in June 20, 1981. A minor change was seen in November, namely larger than 100 mm SL, older than 2+ fish, appeared. They were thought to come from the sea for wintering.

A similar situation was also recognized in *T. brandti* length composition transition from the Mu River (Fig. 33). Older fish greater than 2+ appeared in November and disappeared in June.

Such phenomena were not obvious in the transition of length composition of the fluvial *T. hakonensis* from the Hime River or *T. ezoe* from the Atsuma River (Figs. 32 and 34, respectively). Comparatively wide range of length was collected

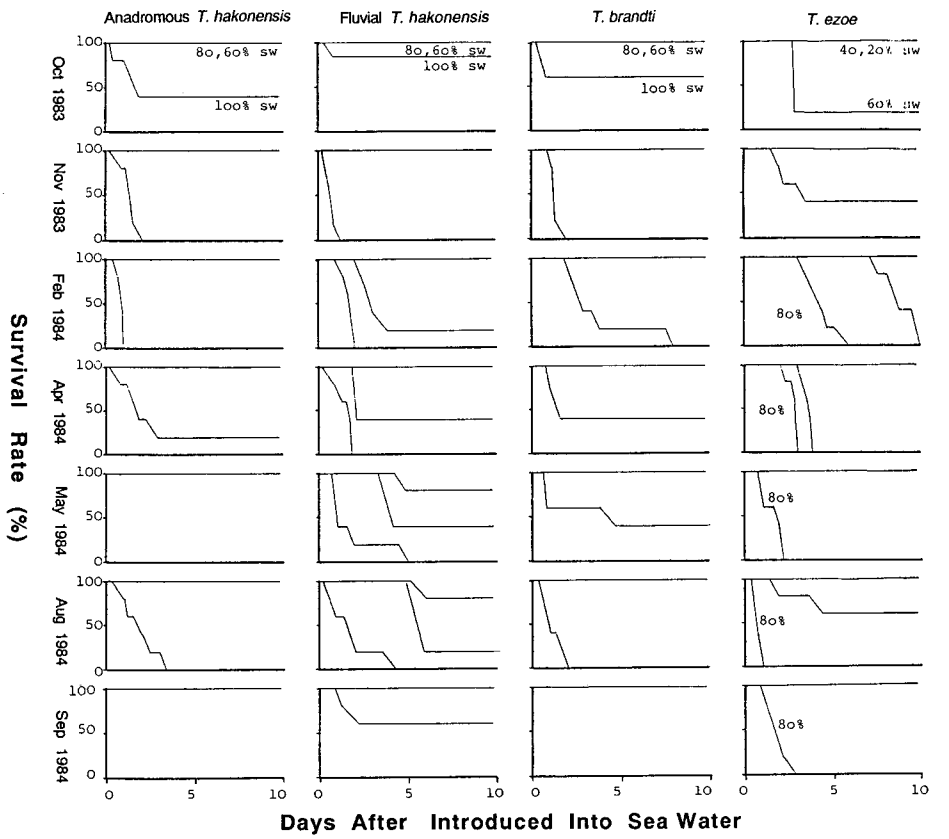

Fig. 27. Changes in survival rate of 0+ to 1+fish of *Tribolodon* from the Mu River and from the Hime River (fluvial *T. hakonensis*) reared in differently diluted sea water (100% sea water≒salinity 30‰), from Octocer, 1983 to September, 1984.

throughout the year.

Wintering migration. The Abira River system is illustrated in Fig. 22 where large stocks of *Tribolodon* winter at Utonai Lake, the Yufutsu and Bibi Rivers. According to fisherman living near Utonai Lake, wintering fish stay in the river system from October to April. Many anglers were fishing for them at the Yufutsu and Bibi rivers and many professional fishermen were catching them by gill nets at Utonai Lake in October, November, December, March and April. Specimens of such wintering populations collected from the Yafutsu and Bibi rivers and Utonai Lake are listed in Table 1. Wintering stocks also ascended the Mu River.

Extrapolated growth curves of wintering fish collected from Utonai Lake are shown in Fig. 35. Anadromous *T. hakonensis* and *T. brandti* developed logistic curves and *T. ezoe* resulted in a straight line similar to the results of the next section. Increased growth rate in *T. hakonensis* and *T. brandti* at age 3 to 5 clearly indicates the sea-going life of them which will be discussed in subsequent section.

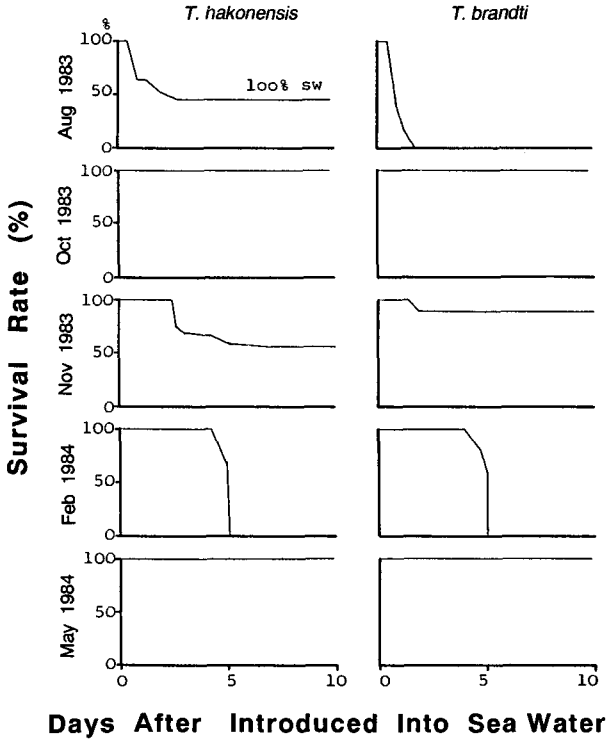

Fig. 28. Changes in survival rate of 1+ to 2 years fish of *Tribolodon hakonensis* and *T. brandti* from the Mu River in 100% sea water, from August, 1983 to May, 1984.

Spawning migration. Spawning runs in the Mu River were collected from May to July (Table 1). On May 2, 1981, a sample of adult fish was collected by a set net at Shiomi, 5 km south-east from the mouth of the Mu River. Spawning runners wintered in Utonai Lake or the Mu River from October to April may descend to the sea with the thawing of snow in April and may ascend the Mu River again for spawning from May. Details of the spawning season are described in the next chapter.

4. Age and growth

In both actual and extrapolated growth patterns, male and female were very similar with a tendency that males were slightly smaller than females (Figs. 36-38) except actual growth of *T. ezoe* (Fig. 39). The difference seen in the actual growth patterns of *T. ezoe* (Fig. 39) might be attributed to small sample size of the study organisms. Actual and extrapolated growth patterns (mean lengths) of both sexes were similar to each other except for *T. ezoe*.

Comparisons of four species growth patterns (mean length) are presented in Fig. 40. In all cases, extrapolated patterns traced smoother lines than actual ones. The anadromous two drew logarithmic growth curves and grew larger than the fluvial

Table 3. Attacking frequencies (mean of 3 trials) per 10 juveniles of *Tribolodon* to the surface and middle layers in every third consecutive minutes.

Lapsed minute after starting	Anadromous *T. hakonensis*		Fluvial *T. hakonensis*		*T. brandti*		*T. ezoe*	
	Surface	Middle	Surface	Middle	Surface	Middle	Surface	Middle
Feeding condition								
0- 1	32.7	18.3	78.7	42.3	1.0	36.3	0.0	12.3
3- 4	2.3	3.0	2.7	30.0	0.0	8.0	0.0	5.3
6- 7	0.0	2.7	0.7	13.7	0.0	7.7	0.0	3.7
9-10	0.7	1.3	5.3	18.3	0.0	5.7	0.0	0.3
12-13	2.7	1.7	3.3	8.7	0.3	2.3	0.0	2.7
Non-feeding condition								
0- 1	18.3	65.3	7.7	16.7	0.0	1.7	0.0	3.0
3- 4	15.0	39.3	0.7	23.3	0.0	3.0	0.0	7.0
6- 7	16.0	59.7	1.7	14.0	0.0	5.3	0.0	8.3
9-10	21.7	80.0	5.0	16.7	0.0	4.0	0.0	7.3
12-13	23.7	70.7	6.3	18.3	0.0	3.3	0.0	2.7

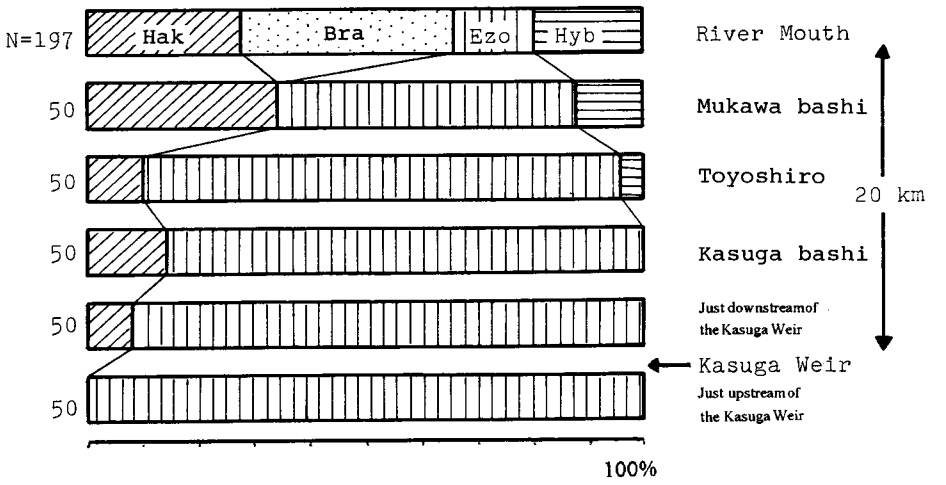

Fig. 29. Distribution pattern of 0+ fish of *Tribolodon hakonensis* (Hak), *T. brandti* (Bra), *T. ezoe* (Ezo), and their hybrid (Hyb) in October, 1982, in the river course of the Mu River. The sampling sites are shown in Fig. 21.

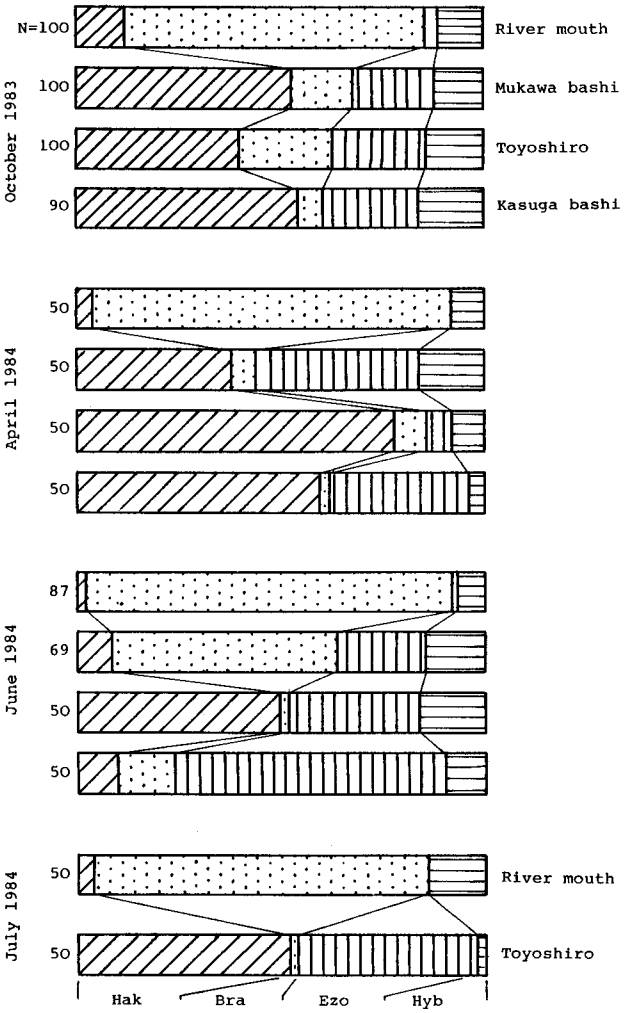

Fig. 30. Distribution pattern of 0+ to 1+ fish of *Tribolodon hakonensis* (hak), *T. brandti* (Bra), *T. ezoe* (Ezo), and their hybrid (Hyb) from October, 1983 to July, 1984, in the river course of the Mu River. The sampling sites are shown in Fig. 21.

two which drew nearly straight growth lines. The standard length (L_t) and age (t) relationships were represented in the following equations:

for anadromous *T. hakonensis*, $Lt=306.034/(1+e^{-0.7352832(t-3.60487)})$, ss (sum of squares)$=114.213$

for fluvial *T. hakonensis*, $Lt=12.5964+21.4369t$, r (corelation coefficient)$=0.9984$

for *T. brandti*, $Lt=347.756/(1+e^{-0.735271(t-3.86975)})$, $ss=48.8136$

for *T. ezoe*, $Lt=2.44279+22.9572t$, $r=0.9979$

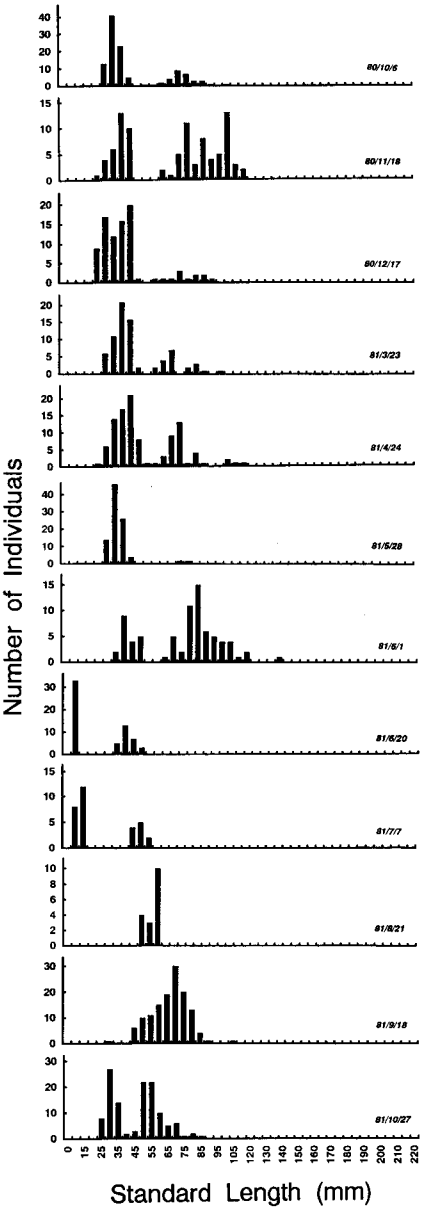

Fig. 31. Change in length composition of anadromous *Tribolodon hakonensis* from the Mu River from October, 1980 to October, 1981.

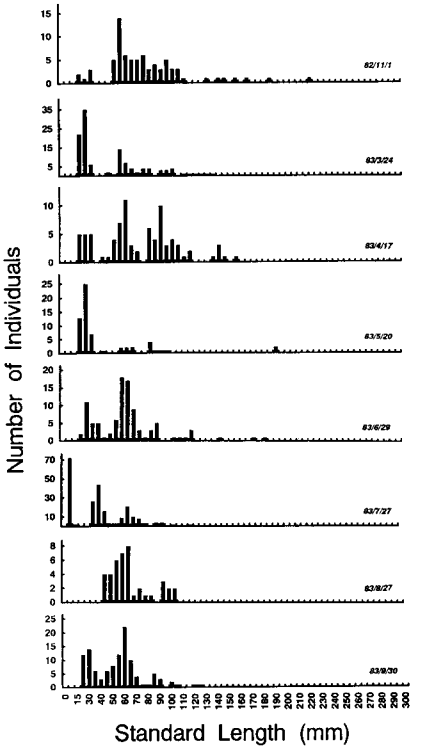

Fig. 32. Change in length composition of fluvial *Tribolodon hakonensis* from the Hime River from November, 1982 to September, 1983.

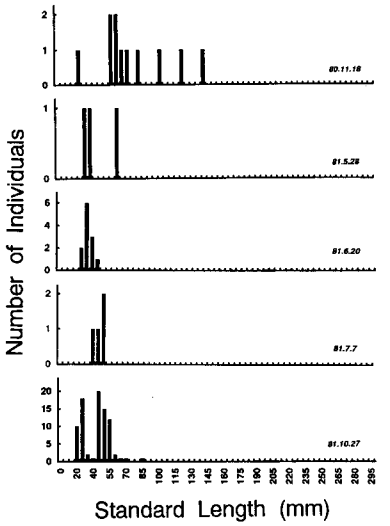

Fig. 33. Change in length composition of *Tribolodon brandti* from the Mu River from November, 1980 to October, 1981.

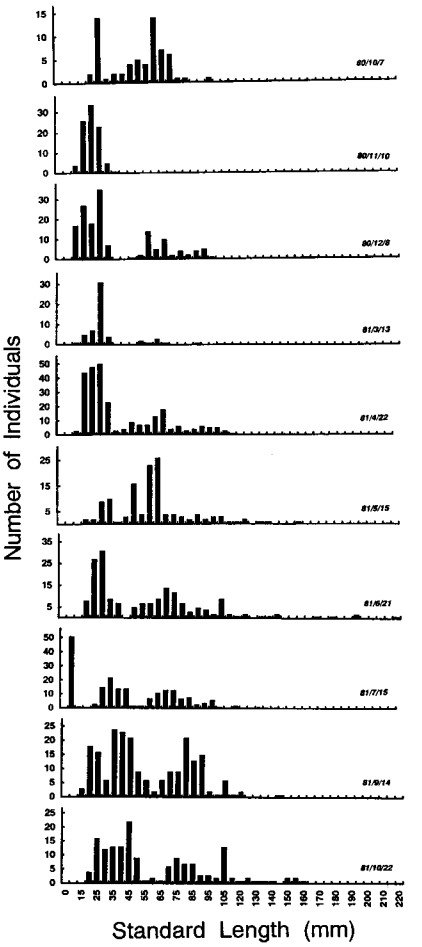

Fig. 34. Change in length composition of *Tribolodon ezoe* from the Atsuma River from October, 1980 to October, 1981.

The rapid growth rate from age 2 in anadromous species and type must be related to their sea-going life. Tanaka and Miyazaki (1976) also drew geometric growth lines in *Tribolodon* species from the Zinzu River and discussed the lack of old fish in the study sample might cause such results.

5. Spawning age

The youngest spawners were 3 year old fish of fluvial *T. hakonensis* (Table 4). Five year old *T. brandti* were the oldest among the three species in the initial spawning age. Anadromous *T. hakonensis* and *T. ezoe* spawners appeared at age 4.

Composition differences existed between anadromous and fluvial types and/or

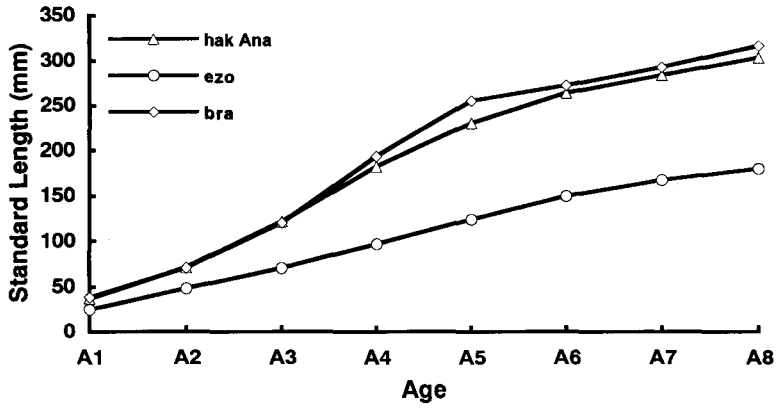

Fig. 35. Extrapolated growth curves of wintering fish in mean length collected from Utonai Lake listed in Table 1. The length was extrapolated from the result of scale reading.

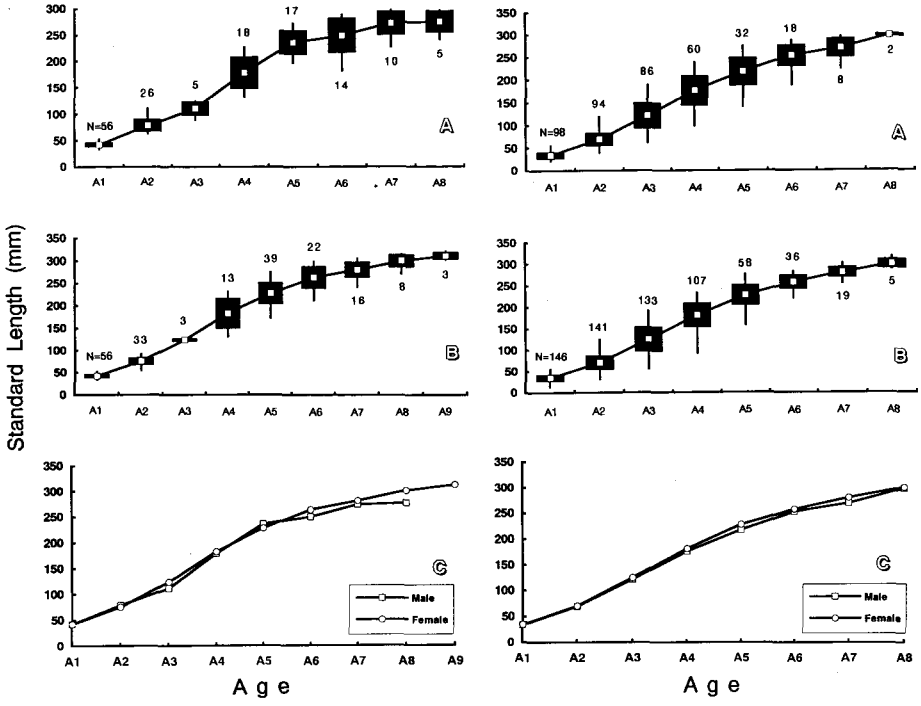

Fig. 36. Growth (left) and extrapolated growth curves (right) of anadromous *Tribolodon hakonensis* from the Mu and Yufutsu River system, in male (A), female (B), and mean length of both sexes (C). Bar, black square and white square indicate range, ±SD and mean length, respectively.

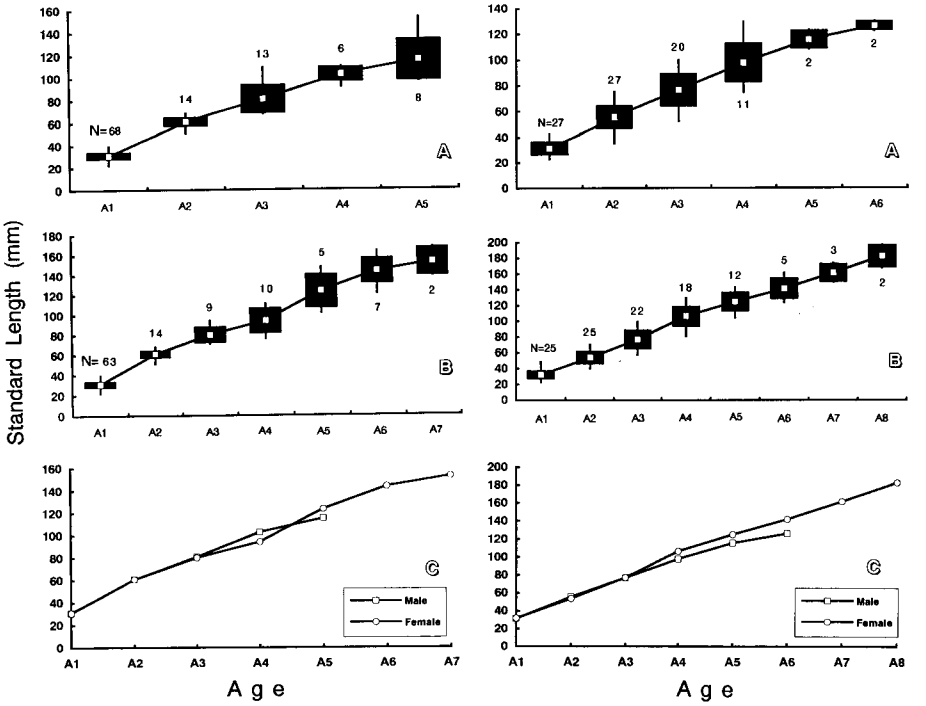

Fig. 37. Growth (left) and extrapolated growth curves (right) of fluvial *Tribolodon hakonensis* from the Hime River, in male (A), female (B), and mean length of both sexes (C). Bar, black square and white square indicate range, ±SD and mean length, respectively.

species. In anadromous *T. hakonensis* and *T. brandti*, mean age composition of females and males were very similar and males were slightly older than females. Conversely, those of fluvial *T. hakonensis* and *T. ezoe* were quite different, males were much younger than females and the oldest female was older than females of anadromous type and/or species.

Discussion

The results of salinity tolerance tests clearly indicate that the anadromous *Tribolodon* species exhibited strong ability to enter sea water in autumn and spring, especially in September, October and May. Equivalent salinity tolerances were seen in the fluvial species of *Tribolodon*, though *T. ezoe* never migrated to the sea at least in Japan. Even *T. ezoe* exhibited greater salinity tolerance than other cyprinids such as *Carassius* and *Pseudorasbora*.

The distribution of juveniles and young fish in the river indicated that both anadromous *T. hakonensis* and *T. brandti* migrated to the sea in June when they were 2 years old. According to the results of salinity tolerance tests, 0+ to 1+ fish

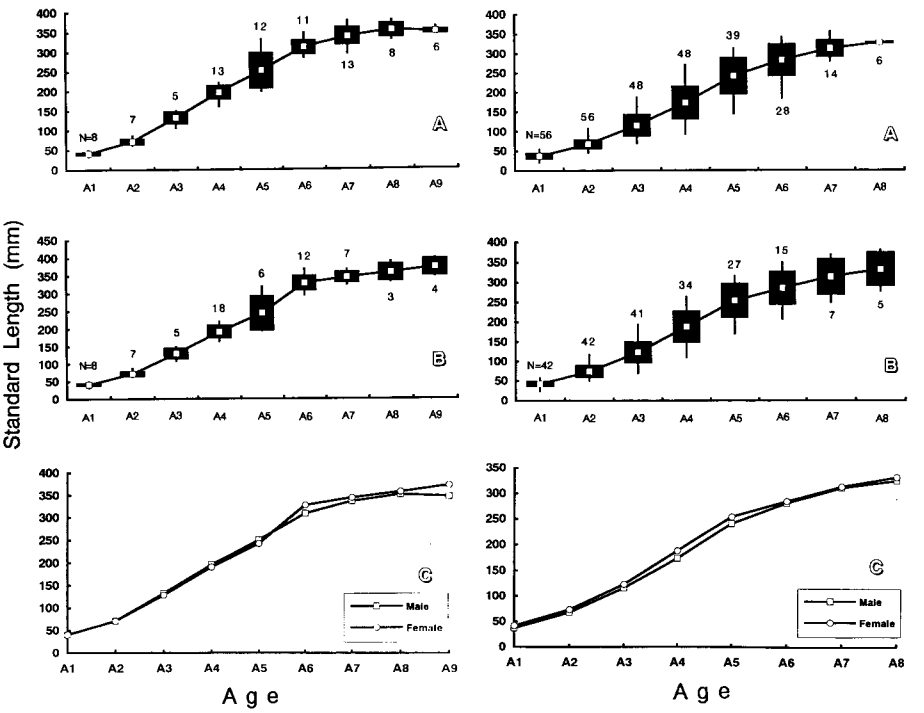

Fig. 38. Growth (left) and extrapolated growth curves (right) of *Tribolodon brandti* from the Mu and Yufutsu River system, in male (A), female (B), and mean length of both sexes (C). Bar, black square and white square indicate range, ±SD and mean length, respectively.

of *T. hakonensis* and *T. brandti* maintained viability in sea water during autumn and spring. In natural conditions, they must go to the sea in spring to early summer when they are 2 years old. The increase in growth rates occurred from age 2 in the anadromous species, relating to their sea-going life.

The mean lengths of two year anadromous *T. hakonensis* and *T. brandti* were 75.4 (8.9 SD) mm and 71.3 (9.1 SD) mm, respectively (Figs. 36 and 38). This size coincides well with the length when the developmental interval VI ends as discussed in the previous chapter. Meanwhile, in fluvial *T. hakonensis* and *T. ezoe*, this size was not attained until the fish reached 3 years. It is concluded that the devolopmental interval VI (juvenile) extends to the approximate length of 70 mm SL at 2 years old in anadromous and at 3 years in fluvial *Tribolodon* in Hokkaido. Subsequently, young fish will go to the sea in anadromous *Tribolodon*.

The results of space preference experiments indicate *Tribolodon* juveniles are different in utilizing feeding space from species to species. Juveniles of *T. hakonensis* use all layers of the water, those of *T. brandti* use middle and bottom layers, and those of *T. ezoe* scarcely use surface and middle layers to feed. Such differences were realized in the interval VI and not clearly evident in the early larval

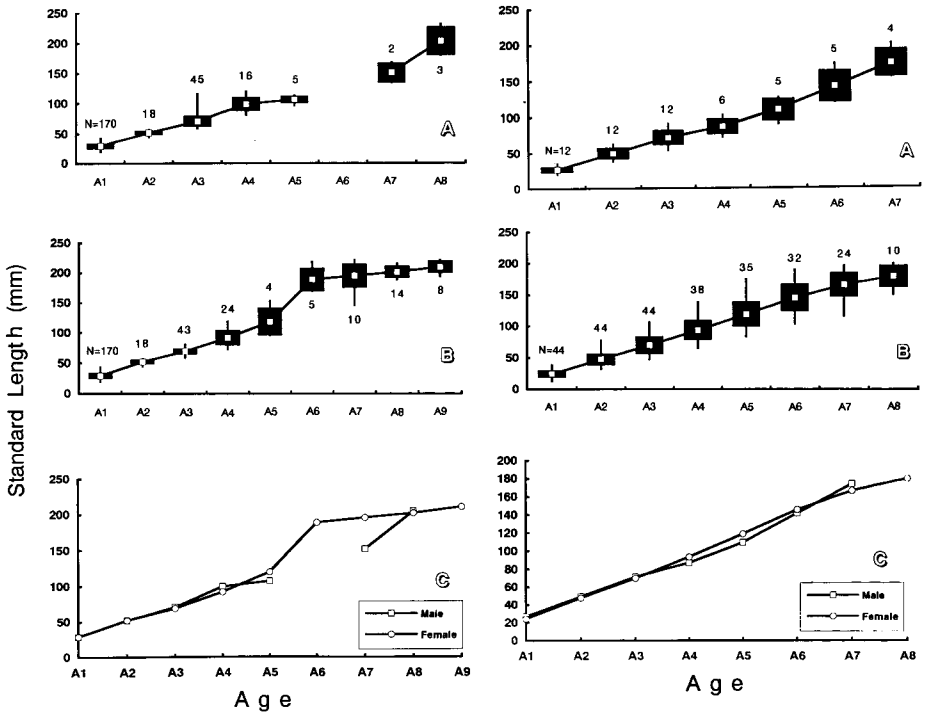

Fig. 39. Growth (left) and extrapolated growth curves (right) of *Tribolodon ezoe* from the Mu and Yufutsu River systems, in male (A), female (B), and mean length of both sexes (C). Bar, black square and white square indicate range, ±SD and mean length, respectively.

intervals.

In freshwater fish communities, resource partitioning is thought to be one of the major mechanisms permitting similar species coexistence (Ross, 1986). Two coexisted species often undergo microhabitat niche shifts and/or food partitioning through interaction (Ishigaki, 1969, 1984; Nakano et al. 1992). If there are innate differences in habitat or feeding space preference, the niche shifts will soon occur effectively through the interaction (Ishizaki, 1969, 1984).

This must be true for *Tribolodon* species. Space and vertical segregation between surface and bottom among species can be reinforced through their interactions. In the coexisting condition, fluvial *T. hakonensis* will use upper layer more than *T. ezoe* predominately in the river, and *T. brandti* will feed more in lower layer than anadromous *T. hakonensis* at the sea. Such feeding tendencies have already been suggested by Nakamura (1969) and Sakai (1989). *T. brandti* and *T. ezoe* seldom coexist except for short periods during their spawning season and juvenile stages. Their mouth morphology may support these situations. *T. ezoe* has the lowest positioned mouth and *T. brandti* exhibits a lower positioned mouth than a large majority of *T. hakonensis* (Nakamura, 1969, also see Fig. 1 of this study).

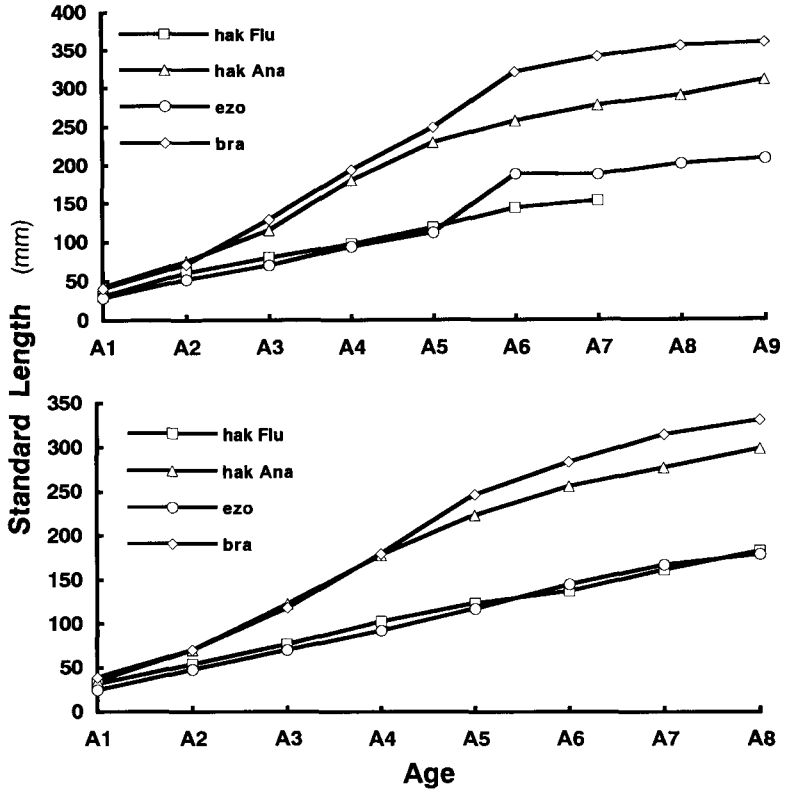

Fig. 40. Comparisons of observed growth (upper) and extrapolated growth (lower) among anadromous *Tribolodon hakonensis* (hak Ana), fluvial *T. hakonensis* (hak Flu), *T. brandti* (bra), and *T. ezoe* (ezo) in mean length including both sexes.

However, if there are no or different interactions between species, the niche preference can be modified according to the situations. *T. ezoe* of the upper reaches of the Atsuma River was observed actively catching the surface floating foods in the mono-specific situation (personal observation), while fluvial *T. hakonensis* from the upper reaches of rivers on middle Honshu has a low positioned mouth and utilizes the bottom dwelling insects most under co-existence with many other cyprinids (Nakamura, 1969).

Horizontal difference of space utilization (sea and river) occur during the next interval, VII. In the surface feeding group, anadromous *T. hakonensis* goes to the sea while fluvial *T. hakonensis* remains. In the bottom feeding group, *T. brandti* goes to the sea while *T. ezoe* remains. Previous to interval VII, *T. brandti* juveniles descended to the river mouth. Interval VII may extend to the time of maturation. Therefore, fluvial *T. hakonensis* interval VII is very short due to maturation initiating at 3 years when interval VI ends, in portions of the population.

Adult interval VIII can be defined as the spawning interval. Initiation is

Table 4. Age composition of spawners in *Tribolodon*. Anadromous type of *T. hakonensis*, *T. brandti*, and *T. ezoe* are collected from the Mu River, and fluvial type of *T. hakonensis* is collected from the Hime River.

		Age									
		3	4	5	6	7	8	9	10	11	12
T. hakonensis anadromous	Female		7	17	15	10	7	2			
	Male		8	15	15	12	7	1	2		
fluvial	F	7	9	5	7	3		2	1	1	
	M	9	16	5							
T. brandti	F				4	3	3				
	M			2	4	10	8	5	1		
T. ezoe	F		3	9	4	8	6	6			1
	M		6	11	8	4		1			

quicker and duration is longer in fluvial as compared to anadromous species and types. Although the length of this interval by an individual is unknown, it ranges more than one year in anadromous species (Gavrenkov, 1982; Gritsenko, 1982). In fluvial species, especially in female *T. ezoe*, the individual interval length must be extended because they tend to ovulate only a portion of their ovarian eggs (discussed in the next chapter).

Consequently, the latter half of *Tribolodon* ontogeny can be divided into three intervals, VI, VII, and VIII. In the interval VI, all body characteristics are completed, and the vertical space preferences are exhibited which results in space segragation through interactions, surface feeding for *T. hakonensis* and bottom feeding for *T. brandti* and *T. ezoe*. The horizontal space partitioning occurs from the interval VII, sea running for anadromous *T. hakonensis* and *T. brandti* and river dwelling for fluvial *T. hakonensis* and *T. ezoe*. At last they spawn in the interval VIII which continues to their death.

V. Reproductive characteristics

Many reports have been published on the breeding ecology of *Tribolodon*. However, till recently, the classification of *Tribolodon* species was confused and it is difficult to say which species of the genus relates to published observations. The few exceptions are fragmentary. Tabeta and Tsukahara (1964) described the spawning habits of anadromous *T. hakonensis* in the northern Kyushu. Kawajiri (1956) and Mizuno et al. (1958) reported those of fluvial *T. hakonensis* from Honshu Island. Dai et al. (1982) documented some biological aspects of *T. brandti* from the Tumen River, China, Gavrenkov and Ivankov (1979) and Gavrenkov (1982) compared some reproductive characteristics of anadromous *T. hakonensis* and *T. brandti* from the

southern Maritime Territory. Knowledge of reproductive habits and characteristics of *T. ezoe* are the most scarce. Only brief documents have been published on such matters of *T. ezoe* by Ito (1975) and Sakai (1987) from the Mu River, Hokkaido and by Gritsenko (1982) from Sakhalin Island, in comparisons among three species, *T. hakonensis*, *T. brandti*, and *T. ezoe*. Descriptions by Nakamura (1969) on the biology of *Tribolodon* species are the most comprehensive, but the knowledge on *T. ezoe* is scarce as compared with those on *T. hakonensis* or *T. brandti*. In this chapter, additional detailed descriptions of the reproductive characteristics of *Tribolodon* such as spawning season, spawning site, gonad-somato index, and fecundity are presented.

Materials and methods

1. Spawning season

Documenting the spawning period of *Tribolodon* is very difficult. In the Mu River, spawning runs of *Tribolodon* are prevented from further upstream migration by the Kasuga Weir (Fig. 21) and spawn there in high concentrations (Kanoh, 1949; Ito, 1975; Sakai, 1987; Sakai and Hamada, 1985). Therefore, every day to every third day observations of spawning occurrence were conducted at the Kasuga Weir from May through July of 1981 and 1982. During spawning observations, some fish were caught and their species determined. Identical methods were utilized for fluvial *T. hakonensis* at the Hime River in 1983.

2. Spawning site

Spawning site measurements included surface water velocity and depth. Data were collected from the Kasuga Weir of the Mu River in 1982 and a spawning site of fluvial *T. hakonensis* in the Hime River in 1983. One to three days after massive spawning, a grid of 1-3 meter squares was put on the spawninsg grounds (rapids).

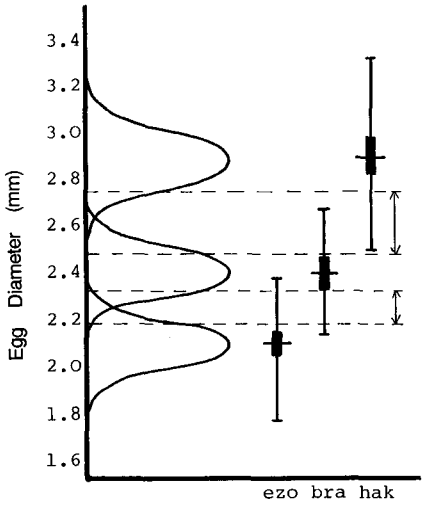

Fig. 41. Range (bar) and ±SD (black square) of diameter of fertilized eggs in anadromous *Tribolodon hakonesis* (hak), *T. brandti* (bra), and *T. ezoe* (ezo) from the Mu River. Omitting the eggs sized between dashed lines, species discrimination of eggs is performed effectively by their size.

Surface water velocity and water depth at the intersections of the grid were measured utilizing a current meter and a plastic scale, respectively. Approximately 2 *l* gravel cores at each intersection were taken with a 13 cm (diameter) plastic pipe. All eggs attached to the collected gravel were picked up and fixed with 5% formalin solution.

The collected eggs were classified to species by size. Size differences of eggs among species were investigated beforehand by measuring artificially fertilized eggs. Seventeen females of anadromous *T. hakonensis*, 231.0-324.9 mm SL, 8 females of *T. brandti* 350.5-387.5 mm SL, and 20 females of *T. ezoe*, 108.4-203.5 mm SL, all collected in June, 1982, had their eggs squeezed out, and the eggs were fertilized. After one to two days, approximately 30 eggs from each batch were fixed with 5% formalin solution and the diameters measured. The data was analyzed and normal distribution curves were developed for each species using probability paper (Fig. 41). Three females of fluvial *T. hakonensis* from the Hime River, 124.4-150.5 mm SL, collected in June, 1983, were also squeezed and their egg size determined.

3. Gonad-somato index

Gonad-somato indices (GSI = 100 × gonad weight/body weight) were determined in more than 4 year old anadromous *T. hakonensis*, more than 3 year old fluvial *T. hakonensis*, more than 5 year old *T. brandti*, and more than 4 year *T. ezoe* listed in Table 1. Anadromous *T. hakonensis* and *T. brandti* were from the Mu River and Yufutsu River system and from the sea. Fluvial *T. hakonensis* was from the Hime River, and *T. ezoe* was from the Mu River, Atsuma River and the Yufutsu River system.

4. Fecundity

Fecundity of pre-spawning (pre-ovulated) females collected from November to July (listed in Table 1) were calculated by the method reported by Imai et al. (1986). The ovary of *T. ezoe* consisted of several different size groupings of eggs and only the group with the largest diameter was utilized in calculations. Distribution of ovarian egg size was also examined in other specimens from each species and/or type, measuring the largest diameter of approximately 150 eggs per ovary using an ocular micrometer attached to a dissecting microscope.

Results

1. Spawning season

Spawning of *Tribolodon* in the Mu River occurred in the same manner in 1981 and 1982 (Fig. 42). A number of spawning concentrations were observed several days after rainfall from May through July. The spawning period of *T. hakonensis* was the longest, starting first in May and ending in July. The next was that of *T. brandti*, occurring intermittently from June through July. The final and shortest was that of *T. ezoe*. The spawning concentration of *T. ezoe* was observed only twice in 1981 and once in 1982 from June through July at the Kasuga Weir. However, additional spawning continued to the end of July at the upper reaches of the Mu River.

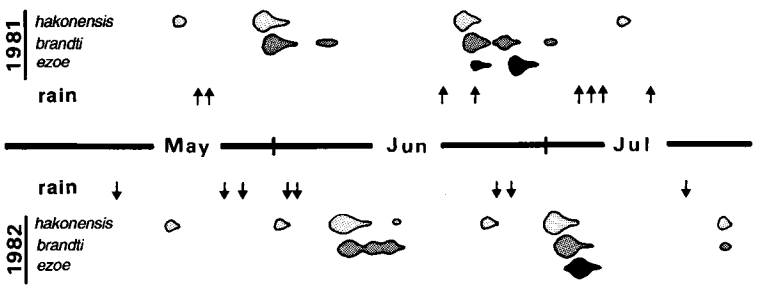

Fig. 42. Symbolized presentation of spawning concentrations of *Tribolodon* at the Kasuga Weir of the Mu River in 1981 and 1982 from May to July. The length and width of a symbol reflect the duration and degree of a spawning concentration, respectively. The arrow indicates rainy day.

Two spawning concentrations in June of very large multi-specific runs were observed. During spawning event, *T. hakonensis* came first, *T. brandti* joined next, and *T. ezoe* spawned last and they rarely mingled (described in chapter VII).

In the Hime River, five spawning concentrations were observed, May 16, 28, June 23, 28 and July 1, 1983. All occurred several days after rainfall, as was the case in anadromous *T. hakonensis* of the Mu River.

2. Spawning site

The preliminary investigation on the size differences of eggs among species clarified totals of 545, 266 and 664 eggs ranging from 2.50–3.12 (mean 2.89, SD 0.126), 2.14–2.67 (mean 2.40, SD 0.076), and 1.77–2.38 (mean 2.10, SD 0.092) mm diameter

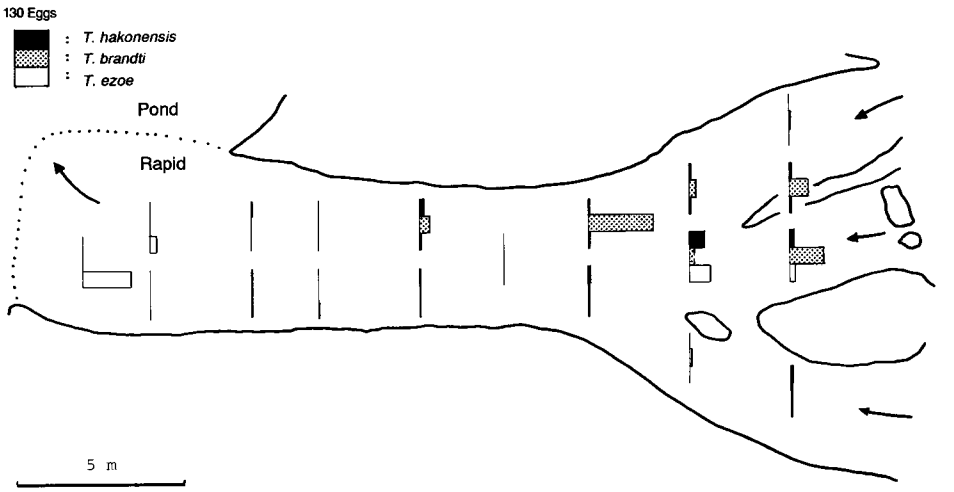

Fig. 43. An example of egg distribution pattern in a rapid at the Kasuga Weir of the Mu River on July 2, 1982. Arrows indicate the direction of water flow.

for anadromous *T. hakonensis*, *T. brandti* and *T. ezoe*, respectively (Fig. 41). If the eggs measuring 2.18-2.33 mm and 2.47-2.75 mm, overlapping zones between species, are excluded from the analysis, it is possible to determine 75% of *T. hakonensis* eggs, 50% of *T. brandti* eggs, and 75% of *T. ezoe* eggs with an error margin of approximately 1.0% of total eggs. This criterion was applied to the field samples. A total of 95 eggs of fluvial *T. hakonensis* were measured ranging from 2.39 to 2.85 (mean 2.61, SD 0.073) mm diameter.

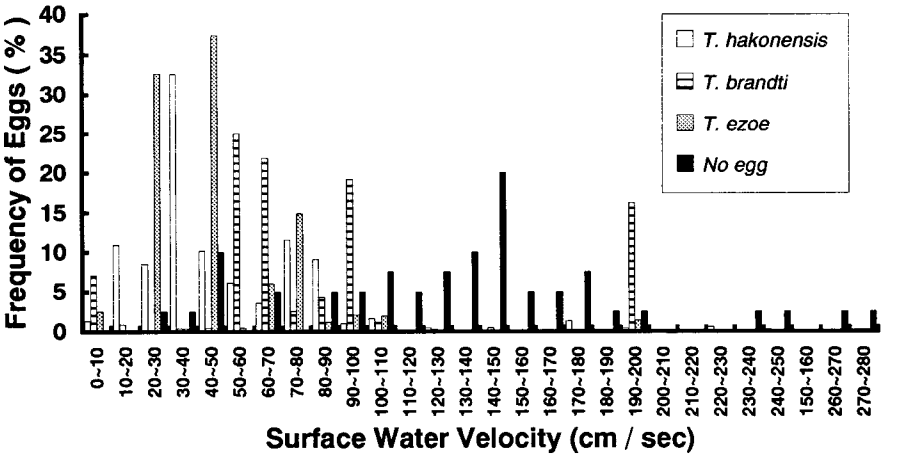

Fig. 44. Histograms of the frequency distribution of spawned eggs of *Tribolodon* at each surface water velocity in the just down stream area from the Kasuga Weir of the Mu River.

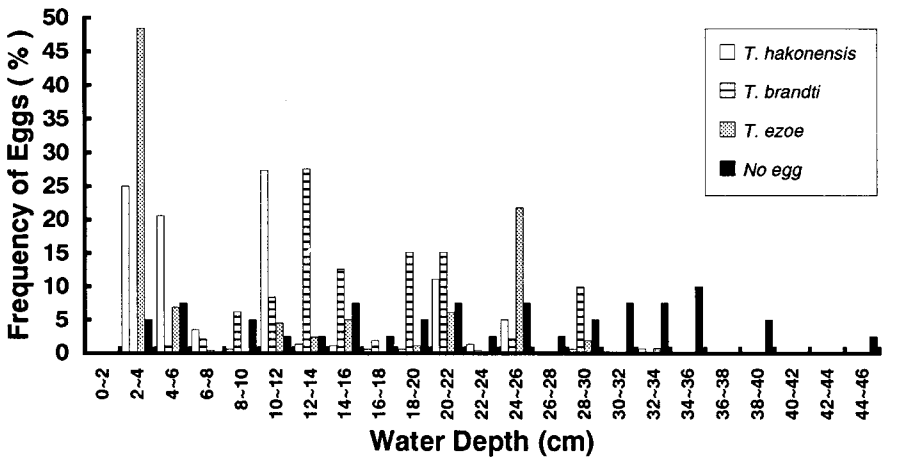

Fig. 45. Histograms of the frequency distribution of spawned eggs of *Tribolodon* at each water depth in the just down stream area from the Kasuga Weir of the Mu River.

Egg distribution from a rapid at the Kasuga Weir investigated on July 2, 1982, is shown in Fig. 43. Large concentrated spawning by all three species were performed on the previous day. A rough tendency was recognized. Eggs of *T. hakonensis* and *T. brandti* were distributed in the upper and those of *T. ezoe* in the lower portion of the rapid, but there were many sites with eggs of three species.

A total of 77 sites (intersections) at the Kasuga Weir of the Mu River and a total of 17 sites (intersections) at the Hime River were established to investigate egg distributions, water velocities and depth. A total of 1,394 eggs from 49 sites of anadromous *T. hakonensis*, 123 eggs from 4 sites of fluvial *T. hakonensis*, 1,043 eggs from 28 sites of *T. brandti*, and 560 eggs from 20 sites of *T. ezoe* were collected, and 30 sites yielded zero eggs.

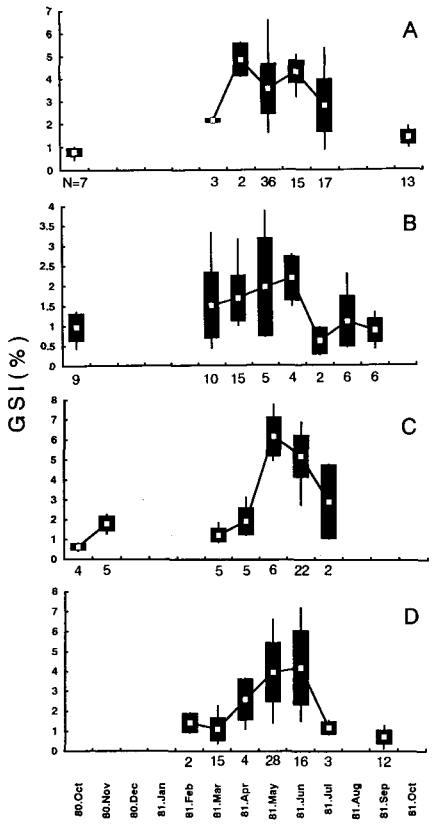

Fig. 46. Change of male GSI in anadromous *Tribolodon hakonensis* (A), fluvial *T. hakonensis* (B), *T. brandti* (C), and *T. ezoe* (D) from October, 1980 to October, 1981. Bar, black square and white square indicate range, ±SD and mean length, respectively.

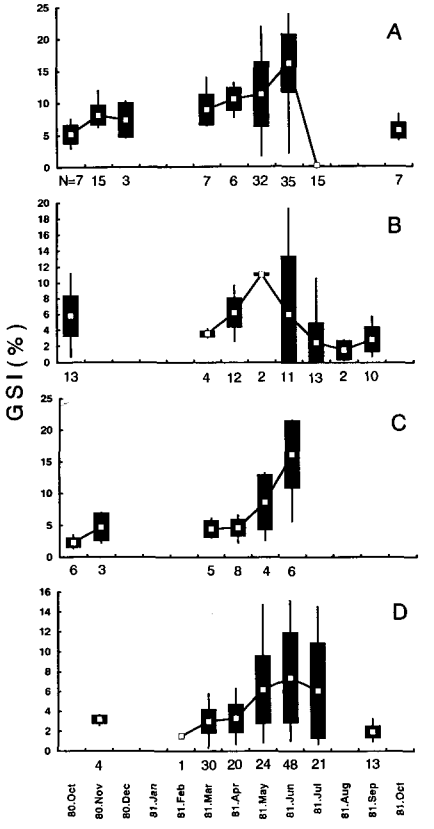

Fig. 47. Change of female GSI in anadromous *Tribolodon hakonensis* (A), fluvial *T. hakonensis* (B), *T. brandti* (C), and *T. ezoe* (D) from October, 1980 to October, 1981. Bar, black square and white square indicate range, ±SD and mean length, respectively.

Distributions of egg frequencies were illustrated in Figs. 44 and 45 for surface water velocity (SWV), and water depth (WD) respectively, for the 77 sites of the Mu River. Means per egg were 52.0 cm/s SWV and 9.6 cm WD in anadromous *T. hakonensis*, 88.7 cm/s and 15.9 cm in *T. brandti*, and 47.1 cm/s and 11.0 cm in *T. ezoe*. Means per site where eggs were not collected were 138.8 cm/s SWV and 22.3 cm WD. *T. brandti* spawned at more rapid and deeper sites than the other two species. The differences of mean SWV and WD were not significant between *T. hakonensis* and *T. ezoe*, while those were significant between *T. brandti* and the other two species and between the sites with zero eggs and all the three species (ANOVA, $P < 10^{-7}$).

In the case of fluvial *T. hakonensis* from the Hime River, the mean SWV and WD per egg were 32.8 cm/s and 11.9 cm, respectively. Those per site with zero eggs were 46.0 cm/s and 15.2 cm.

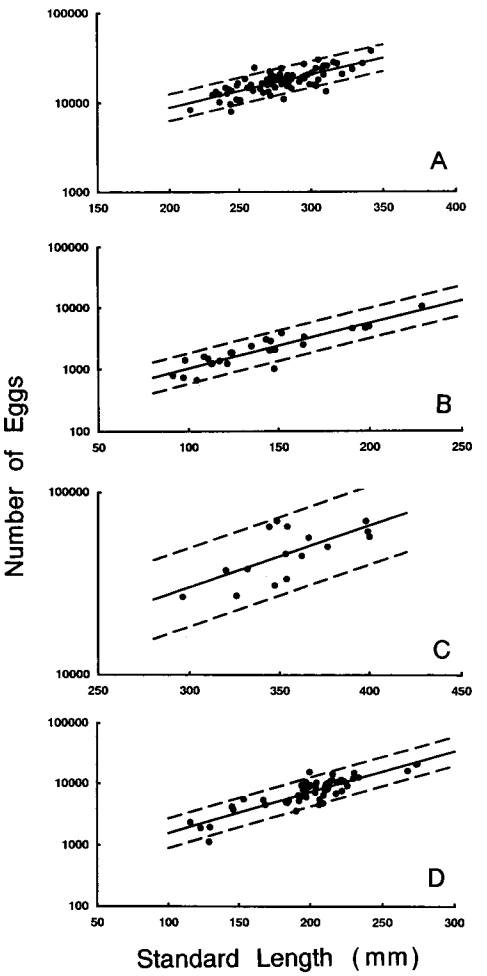

Fig. 48. Relationships of number of ovarian ripe eggs and standard length of anadromous *Tribolodon hakonensis* (A), fluvial *T. hakonensis* (B), *T. brandti* (C), and *T. ezoe* (D) from the Mu and Yufutsu River systems and from the Hime River (fluvial *T. hakonensis*). Dashed lines around the regression line indicate 95% confidence limits.

3. Gonad-somato index (GSI)

In male, anadromous and fluvial *T. hakonensis* and *T. brandti* recorded the highest GSI in may, 6.5, 3.9 and 7.8%, respectively, while *T. ezoe* did in June, 7.2% (Fig. 46). In all cases, GSI of male were the highest just prior to the most active spawning periods (described later). In female, anadromous and fluvial *T. hakonensis*, *T. brandti*, and *T. ezoe* recorded the highest indices in June, 23.9, 19.4, 19.7, and 15.2%, respectively (Fig. 47). In all cases, GSI of females were the highest in the most active spawning periods.

4. Fecundity

The maximum and minimum number of eggs recorded were 37,663 (341.9 mm SL) and 8,075 (244.4 mm SL) in anadromous *T. hakonensis*, 10,434 (228.0 mm SL) and 666 (104.6 mm SL) in fluvial *T. hakonensis*, 70,078 (398.0 mm SL) and 26,834 (296.0 mm SL) in *T. brandti*, and 21,086 (273.2 mm SL) and 1,127 (128.9 mm SL) in *T. ezoe*.

Relationships between number of ovarian eggs (y) and standard length (x) (Fig. 48) were represented as the following equations:

for anadromous *T. hakonensis*, $\log y = 0.003637x + 3.222$, ($r = 0.755$)
for fluvial *T. hakonensis*, $\log y = 0.007345x + 2.277$, ($r = 0.904$)
for *T. brandti*, $\log y = 0.003401x + 3.461$, ($r = 0.648$)
for *T. ezoe*, $\log y = 0.006738x + 2.513$, ($r = 0.876$)

The two anadromous and the two fluvial groups exhibited different slopes (Fig. 49).

Frequency distributions of ovarian egg diameters of anadromous *T. hakonensis* (Fig. 50) and *T. brandti* (Fig. 52) were bi-modal, and the larger eggs were from

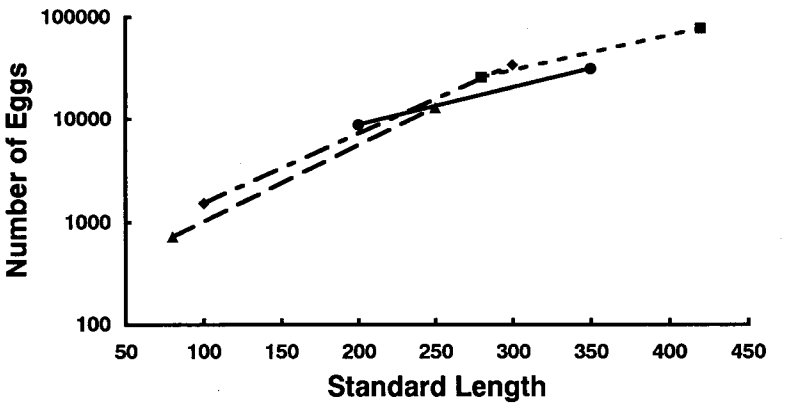

Fig. 49. Comparison of regression lines of relationships between number of ovarian ripe eggs and standard length of anadromous *Tribonodon hakonensis* (closed circle and solid line), fluvial *T. hakonensis* (triangle and long dashed line), *T. brandti* (square and short dashed line), and *T. ezoe* (diamond and skipped line) from the Mu and Yufutsu River systems and from the Hime River (fluvial *T. hakonensis*).

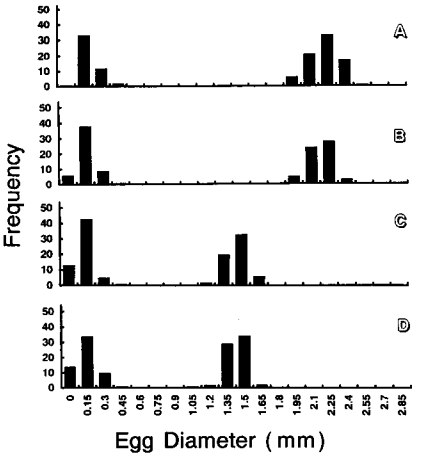

Fig. 50. Histograms of size distribution of ovarian eggs of anadromous *Tribolodon hakonensis* collected from the Mu River on June, 1981. A, 278.5 mm SL, 8 years old ; B, 253.0 mm, 7 years ; C, 237.5 mm, 5 years ; D, 222.0 mm, 6 years.

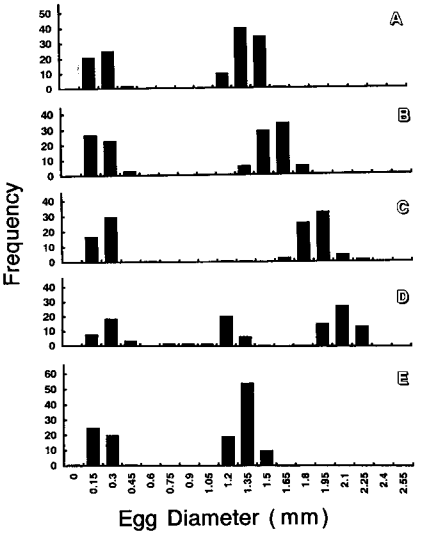

Fig. 51. Histograms of size distribution of ovarian eggs of fluvial *Tribolodon hakonensis* collected from the Hime River on April, 1983. A, 145.0 mm SL, 6 years old ; B, 148.0 mm, 5 years ; C, 164.0 mm, 6 years ; D, 147.5 mm, 6 years ; E, 143.0 mm, 7 years.

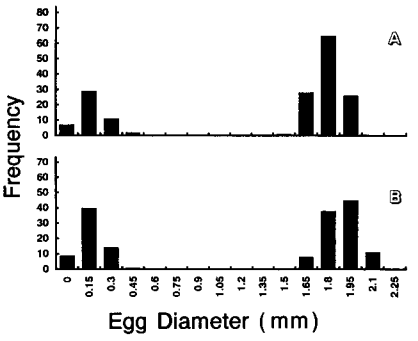

Fig. 52. Histograms of size distribution of ovarian eggs of *Tribolodon brandti* collected from the Mu River on April, 1981. A, 296.0 mm SL, 7 years old ; B, 353.0 mm, 10 years.

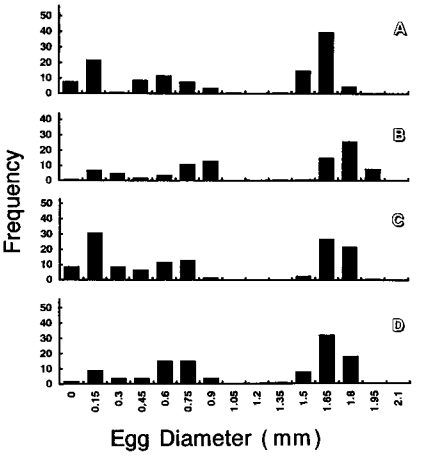

Fig. 53. Histograms of size distribution of ovarian eggs of *Tribolodon ezoe* collected from the Mu River on June, 1981. A, 210.1 mm SL, 8 years old ; B, 216.5 mm, 7 years ; C, 223.5 mm, 8 years ; D, 191.5 mm 8 years.

maturing ones. All four individuals of *T. ezoe* exhibited tri-modal distribution (Fig. 53). Conversely, fluvial *T. hakonensis*, one individual had tri-modal and the other four individuals exhibited bi-modal ovaries (Fig. 51).

Discussion

Tribolodon spawning starts earlier in more southern regions (early to mid spring) and later in the more northern region (early to mid summer) (Nakamura, 1969). In the Mu River, *T. hakonensis* spawned first, from late May through July, *T. brandti* next, from June through July, and last *T. ezoe*, from late June through July. These results agree with those of Ito (1975) from the Mu River, and Gritsenko (1982) from Sakhalin. These three species spawned in high concentrations several days after episodes of rain, several times a spawning season. These results also agree with the results reported by other workers (e.g. Kawajiri, 1956; Mizuno et al., 1958; Tabeta and Tsukahara, 1964; Nakamura, 1969; Ito, 1975; Gavrenkov and Ivankov, 1979; Gavrenkov, 1982; Gritsenko, 1982).

In a spawning ground, the three species occasionally spawned at the same time as reported by Ito (1975), but *T. brandti* spawned in the swiftest and deepest part of the spawning rapid as documented by Gavrenkov (1982) and Gritsenko (1982).

The fecundity of anadromous *T. hakonensis* from the Mu Miver in this study, 8,075-37,663, was slightly larger than fish from other regions. Gavrenkov (1982) and Imai et al. (1986) reported the fecundity of anadromous *T. hakonensis* as 5,854-34,536 from the southern Maritime Territory and as 2,200-16,400 from Iwate Prefecture, Tohoku District, respectively. Also in *T. brandti*, the fecundity in this study, 26,834-70,078, was larger than the results by other workers. Nakamura (1969) described the fecundity of *T. brandti* from the Tama River, Kanto District, as 30,000-50,000, Gavrenkov (1982) reported the fecundity from the southern Maritime Territory as 15,772-54,989, and Dai et al. (1982) reported that from the Suifun River, China, as 17,760-57,030. However, it is unclear whether or not these differences of fecundity in anadromous *T. hakonensis* and *T. brandti* are significant, because they did not calculate the relationships between number of eggs and standard length.

The fecundity of fluvial *T. hakonensis* from the Hime River in this study, 666 (104.6 mm SL)-10,434 (228.0 mm SL), was much smaller than that from the Tama River (Nakamura, 1969), 723 (84.7 mm SL)-5,981 (134.8 mm SL). The size of eggs are not appreciably different (2.5-2.6 mm diameter by Nakamura, 1969), therefore differences in the fecundity is thought to be significant.

Nakamura (1969) described that a pair of ovaries of *T. hakonensis* consisted of eggs of the same maturation stage, and suggested that it would spawn once in a spawning season. On the contrary, twenty percent of fluvial *T. hakonensis* is this study had ovaries with tri-modal ovarian egg size distribution similar to *T. ezoe* is this study.

There has been no record of *T. ezoe* fecundity until the results of this study, 1,127 (128.9 mm SL)-21,086 (273.2 mm SL). The regression line of *T. ezoe* was closely similar to that of fluvial *T. hakonensis* from the Hime River. If *T. ezoe* and fluvial *T. hakonensis* spawn twice or more in one spawning season, their fecundity

per season may become greater, and the difference in the fecundity of fluvial *T. hakonensis* between two localities mentioned above as well as the difference in slope of the regression lines between anadromous and fluvial species may become smaller.

According to the definition by Yamamoto and Yamazaki (1961), the oocytes of anadromous *T. hakonensis* and *T. brandti* might exhibit the development of group-synchronous type, those of *T. ezoe* might be asynchronous type, and fluvial *T. hakonensis* might have both types of development within a population. Fish of asynchronous oocyte development type such as *T. ezoe* and a part of fluvial *T. hakonensis* populations have a possibility to ovulate twice or more in one spawning season (Takano, 1989).

In fact, a freshwater fish Ayu, *Plecoglossus altivelis altivelis*, have asynchronous type of oocyte development, spawn twice in the spawning season, and compensate for insufficient number of eggs per batch under unfavorable environmental conditions, while spawn once under good condititons (Matsuyama and Matsuura, 1982, 1983, 1984a, 1984b, 1985; Sakai et al., 1991a). Like Ayu, a part of fluvial *T. hakonensis* and *T. ezoe* in this study may do so under the severe conditions to live a fluvial life in Hokkaido.

VI. Genetic relationships

Several methodologies have been developed to analyze the phylogeny of certain groups, namely cladistic analysis, numerical analysis, etc. (e.g. Arai, 1988). Cladistic analysis by Hennig (1966) has been successful in analyzing many fish taxa (e.g. American Society of Ichthyologists and Herpetologists, 1984). However, it has been adopted mainly for higher taxa than genus level because it requires the characteristics to be so distinct as to be able to judge the polarity of the character state series. Analyzing lower taxa than genus level such as the case with *Tribolodon* species where their osteological differences are very subtle (Naito, 1992), the numerical method using genetic characteristics such as allozyme genes, DNA sequences, etc. has achieved good results (Nei, 1990; Nozawa, 1994).

Also in *Tribolodon*, their genetic relationships have already been reported at the inter-specific level (Hanzawa and Taniguchi, 1982a, 1982b) as well as the intra-specific level (Hanzawa et al., 1987, 1988). However, their description and phylogenetic analyses are so brief that it is impossible to say the reports clarified *Tribolodon* systematics completely.

The goal of this chapter is to infer the phylogenetic interrelationship of *Tribolodon* using electrophoreticaly distinguished allozymes. More detailed descriptions of genetic controls of allozymes and analysis of *Tribolodon* systematics are presented than those available heretofore.

Materials and methods

1. Allozyme electrophoresis and genetic variability

Five samples of anadromous and 2 of fluvial *T. hakonensis* from 4 rivers and a harbor near the river mouth of the Hime River, 3 samples of *T. brandti* from 2 rivers, 5 samples of *T. ezoe* from 3 rivers, and one sample of *T.* sp. from the Mogami River,

Table 5. Sixteen populations surveyed allelic frequencies.

Population number	Locality	Date	No. specimens
T. hakonensis			
1	Mu River	1991 Autumn	33
2	Mu River	1984 Autumn	35
3 (fluvial type)	Kikonai River	1991 Autumn	9
4	Assabu River	1991 Autumn	8
5	Assabu River	1984 Autumn	43
6 (fluvial type)	Hime River	1984 Autumn	70
7	Otobe River	1984 Autumn	80
T. brandti			
8	Mu River	1991 Autumn	34
9	Mu River	1984 Autumn	34
10	Assabu River	1991 Autumn	12
T. ezoe			
11	Mu River	1991 Autumn	35
12	Mu River	1984 Autumn	35
13	Kikonai River	1991 Autumn	4
14	Assabu River	1991 Autumn	5
15	Assabu River	1984 Autumn	40
T. sp.			
16	Mogami River	1991 Autumn	2
T. hakonensis for *SOD** only			
	Hime River		
	middle reach	1984 Autumn	40
	river mouth	1984 Autumn	40

Yamagata Pref., were collected in the autumns of 1984 and 1991 (Table 5).

The fish were immediately frozen after collection and stored at −70°C until processed for horizontal starch-gel electrophoresis (12% gel) and zymogram method (e.g. Shaw and Prasad, 1970; Harris and Hopkinson, 1976; Murphy et al., 1990). The 12 enzymes and/or protein analyzed, 21 loci recognized, tissues and buffer systems utilized, and other conditions analyzed are described in Table 6. Locus and gene nomenclature follows Shaklee et al. (1990). The most common allele at a locus of *T. hakonensis* was designated as ***100***. Proportion of polymorphic loci (*pp*, most common allele does not exceed 0.95), observed average heterozygosity (*Ho*) and expected average heterozygosity (*He*, Nei's (1978) biased estimate) were calculated for each sample.

2. Gene uniformity test between anadromous and fluvial *T. hakonensis*

For the determination of whether the anadromous and the fluvial populations

Table 6. Enzymes, enzyme numbers, loci, tissues, and buffer systems used.

Enzyme	Enzyme number	Locus	Tissue	Buffer
Aspartate aminotransferase	2.6.1.1	*AAT-1**	Muscle	AC
		*AAT-2**	M	AC
Alcohol dehydrogenase	1.1.1.1	*ADH**	Liver	AC
Glycero-3-phosphate dehydrogenase	1.1.1.8	*G3PDH**	M	AC
Glucose-6-phosphate isomerase	5.3.1.9	*GPI-1**	M	RW
		*GPI-2**	M	RW
Isocitrate dehydrogenase	1.1.1.42	*IDH-1**	L	AC
		*IDH-2**	L	AC
		*IDH-3**	M	AC
L-Lactate dehydrogenase	1.1.1.27	*LDH-1**	M	RW
		*LDH-2**	M	RW
		*LDH-3**	L	AE
Malate dehydrogenase	1.1.1.37	*MDH-1**	M	AC
		*MDH-2**	L, M	AC
		*MDH-3**	L, M	AC
Phosphogluconate dehydrogenase	1.1.1.44	*PGDH**	L	AE
Phosphoglucomutase	5.4.2.2	*PGM**	M	RW
Superoxide dismutase	1.15.1.1	*SOD**	L	RW
Sarcoplasmic protein		*SP-2**	M	RW
		*SP-3**	M	RW
Xanthine dehydrogenase	1.1.1.204	*XDH**	L	RW

AC : Amine (N-(3-Aminopropyl)-morpholine) citrate buffer (pH 6.1) by Clayton and Tretiak (1972), 4 mA/cm^2 for 3 hours.
RW : Tris-citric acid (gel pH 8.5), lithium hydroxide-boric acid (tray pH 8.5) buffer system by Ridgway et al. (1970), 4 mA/cm^2 for 2 hours.
AE : Amine (N-(3-aminopropyl)-diethanolamine) citrate buffer (pH 7.0) by Clayton and Tretiak (1972), 4 mA/cm^2 for 3 hours.

of *T. hakonensis* possess common gene pool, additional samples from the middle reach and the river mouth of the Hime River were collected. Gene frequencies on the locus *SOD**, where the greatest gene differentiation was found between populations from the upper reach of the Hime River and the Otobe Harbor, were determined. The fish collected from the Otobe Harbor were possibly born in the Hime River as it is located nearly 500 m from the Hime River mouth. Chi-square tests for *SOD** gene uniformity were performed between pairs of 4 samples from the upper reach, middle reach and river mouth of the Hime River, and the Otobe Harbor.

3. Cluster analysis

Nei's (1978) unbiased genetic distance (ND for short) and Cavalli-Sforza and

Edwards' (1967) chord distance (CD for short) were calculated between pairs of 16 populations. A dendrogram of ND by the UPGMA (Sneath and Sokal, 1973), a Wagner's tree (Farris, 1972; Swofford, 1981) of D, and two unrooted trees of the two distances (mean by species and types) by neighbor-joining method (Saitou and Nei, 1987; Nei, 1990) were calculated, compared and discussed. The first is an expected distance tree and the other three are realized distance trees (Nei, 1990).

Results

1. Genetic control of isozymes

The allele frequencies for 21 loci are given in Table 7. Allelic displacements between species were seen in 9 loci, *AAT-2**, *GPI-1**, *IDH-1**, *IDH-2**, *LDH-2**, *LDH-3**, *PGM**, *SP-2**, and *SP-3** (Fig. 54).

AAT (dimer): The muscle-dominant *AAT-1** in the anodal zone and *AAT-2** in the cathodal zone were identified. The allelic displacement was seen in *AAT-2** between *T. hakonensis* (**-100*) and the other species (**-90*).

ADH (dimer): The liver-specific *ADH** was monomorphic in all species.

G3PDH (dimer): Three alleles were seen in muscle-dominant *G3PDH**, but **-100* predominated in all species. Rare alleles **-40* and **-130* were seen in several populations of *T. hakonensis*.

GPI (dimer): *GPI-1** in the anodal zone, *GPI-2** in the cathodal zone, and their heterodimer were exhibited in muscle. The allelic displacement was recognized in *GPI-2** between *T. hakonensis* (**100*) and the other species (**86*). Four alleles for *GPI-2** were identified, and **-100* in *T. hakonensis* and *T. brandti* and **-67* in *T. ezoe* and *T.* sp. were dominant. The allele **-30* was secondary dominant in *T. ezoe*, and the rare allele **-120* was only seen in *T. brandti*.

IDH (dimer): The liver-dominant *IDH-1** and *IDH-2**, and muscle-specific *IDH-3** were identified. The heterodimer between *IDH-1** and *IDH-2** was also exhibited. The allelic displacements were recognized between *T.* sp., **74* in *IDH-1** and **68* in *IDH-2**, and the other species, **100* in *IDH-1** and **100* or **53* in *IDH-2**. *IDH-3** was monomorphic in all species.

LDH (tetramer): Three loci coded for LDH, the muscle-dominant *LDH-1** and *LDH-2** in the anodal zone, and the liver-specific *LDH-3** in the cathodal zone were identified. Three bands of heterotetramer were also exhibited between *LDH-1** and *LDH-2**. In *LDH-1**, a rare allele **86* was seen in one population of *T. hakonensis*, but all the other populations and species were monomorphic in allele **100*. The allelic displacements were recognized in *LDH-2** between *T. ezoe* (**280*) and the other species (**100*), and in *LDH-3** among *T. hakonensis* and *T.* sp. (**-100*), *T. brandti* (**-142*) and *T. ezoe* (**-139*).

MDH (dimer): Three loci coded for MDH, the muscle-specific *MDH-1** in the anodal zone, the liver dominant *MDH-2** in the anodal zone, and the liver-dominant *MDH-3** in the cathodal zone were identified. The latter two were also present in muscle. The heterodimer between *MDH-1** and *MDH-2** was observed. All species were monomorphic in *MDH-1** and *MDH-3**. In *MDH-2**, the allele **9* was dominant in *T.* sp., but the other species were monomorphic in **100*.

PGDH (dimer): Three alleles were recognized in *PGDH** in the liver. The

Table 7. Number of individuals surveyed and allele frequencies at 21 loci in 16 populations of *Tribolodon*.

Locus	*T. hakonensis*							*T. brandti*			*T. ezoe*					*T.* sp.
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
(N)	33	35	9	8	43	70	80	34	34	12	35	35	4	5	40	2
*AAT-1**																
**140*	.015	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
**100*	.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
*AAT-2**																
**-90*	.000	.000	.000	.000	.000	.000	.000	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	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
*ADH**																
**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
*G3PDH**																
**-40*	.030	.000	.000	.000	.000	.000	.000	.000	1.000	.000	.000	.000	.000	.000	.000	.000
**-100*	.970	1.000	1.000	1.000	1.000	.986	.981	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
**-130*	.000	.000	.000	1.000	.000	.014	.019	.000	1.000	.000	.000	.000	.000	.000	.000	.000
*GPI-1**																
**100*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
**86*	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
*GPI-2**																
**-30*	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.200	.257	.250	.300	.338	.000
**-67*	.288	.299	.167	.125	.245	.143	.188	.015	.029	.042	.657	.557	.625	.400	.425	1.000
**-100*	.712	.771	.833	.875	.755	.857	.812	.882	.897	.958	.143	.186	.125	.300	.237	.000
**-120*	.000	.000	.000	.000	.000	.000	.000	.103	.074	.000	.000	.000	.000	.000	.000	.000
*IDH-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	.000
**74*	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000
*IDH-2**																
**100*	.985	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.914	1.000	1.000	1.000	.000
**68*	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000
**53*	.015	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.086	.000	.000	.000	.000
*IDH-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**																
**100*	.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
**86*	.015	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000

*LDH-2**																
**280*	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	1.000	1.000	1.000	.000
**100*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	1.000
*LDH-3**																
**-100*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
**-139*	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	1.000	1.000	1.000
**-142*	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	.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	1.000	1.000	1.000	1.000	1.000	.250
**9*	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.750
*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
*PGDH**																
**170*	.000	.000	.000	.000	.012	.000	.044	.000	.000	.000	.000	.000	.000	.000	.000	.000
**100*	1.000	1.000	1.000	1.000	.988	1.000	.956	.926	.838	1.000	1.000	1.000	1.000	1.000	1.000	1.000
**53*	.000	.000	.000	.000	.000	.000	.000	.074	.162	.000	.000	.000	.000	.000	.000	.000
*PGM**																
**114*	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	1.000	1.000	.000	.000
**107*	.000	.000	.000	.000	.000	.000	.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	1.000
**100*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
*SOD**																
**100*	.955	.843	1.000	.812	.872	.757	.875	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
**-67*	.045	.157	.000	.188	.128	.243	.125	.000	.000	.000	.000	.000	.000	.000	.000	.000
*SP-2**																
**117*	.000	.000	.000	.000	.000	.000	.000	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	1.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
*SP-3**																
**325*	.000	.000	.000	.000	.000	.000	.000	.000	.000	.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	1.000	1.000	1.000	1.000	.000	.000	.000	.000	.000	.000
*XDH**																
**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

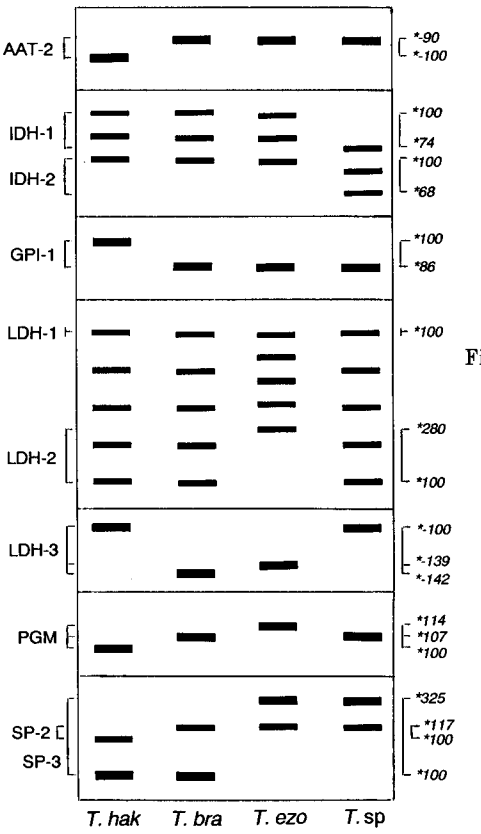

Fig. 54. Diagrammatic electrophoregrams of 9 marker loci diagnostic of *Tribolodon* spp., *T. hakonensis* (*T. hak*), *T. brandti* (*T. bra*), *T. ezoe* (*T. ezo*), and *T.* sp. Alleles are indicated on the right side.

allele **100* was predominant in all species. Rare alleles **170* and **53* were only seen in two populations of *T. hakonensis* and two populations of *T. brandti*, respectively.

PGM (monomer): The muscle-dominant *PGM** was exhibited in the anodal zone. The allelic displacement was recognized among species, **100* in *T. hakonensis*, **107* in *T. brandti* and *T.* sp., and **114* in *T. ezoe*.

SOD (dimer): The liver-specific *SOD** was recognized in the anodal and/or cathodal zones. The allele **100* dominated in all species. The second allele *-*67* was scored from *T. hakonensis* populations in various frequencies.

SP (monomer): Some stained zones were observed in muscle extract, and very clearly recognized *SP-2** and *SP-3** were scored. The allelic displacements were seen in *SP-2** between *T. hakonensis* (**100*) and the other species (**117*), and in *SP-3** between *T. hakonensis* and *T. brandti* (**100*) and *T. ezoe* and *T.* sp. (**325*).

XDH (ambiguous, according to Murphy et al., 1990): The liver-dominant *XDH** was seen in the anodal zone. It was monomorphic in all species.

2. Genetic variability

Of 21 loci scored, *GPI-2**, *PGDH**, and *SOD** were polymorphic (the most

common allele did not exceed 0.95) at least in some populations. Deviations of observed genotypic frequencies from the expected values of the Hardy-Weinberg equilibrium were not significant for these 3 loci of all populations examined with more than 30 individuals.

The genetic variability data are shown in Table 8. Proportions of polymorphic loci (*pp*) ranged from 0 to 0.095 (mean 0.072). Observed heterozygosities (*Ho*) ranged from 0.004 to 0.048 (mean 0.028) and those expected (*He*) ranged from 0.004 to 0.036 (mean 0.026). The average ratio of *Ho*/*He* was 1.07.

3. Gene uniformity test between anadromous and fluvial *T. hakonensis*

The allele frequencies in *SOD** of *T. hakonensis* from the upper reach, middle reach, Hime River mouth, and Otobe Harbor are shown in Table 9. Frequencies of

Table 8. Proportion of polymorphic loci and average heterozygosity in 16 populations of *Tribolodon* at 21 loci. Sample numbers correspond to those in Table 5.

Population	Polymorphic loci*	Average heterozygosity	
		observed	expected**
T. hakonensis			
1	.048	.035	.031
2	.095	.034	.029
3	.048	.016	.013
4	.095	.030	.025
5	.095	.028	.030
6	.095	.031	.031
7	.095	.031	.031
T. brandti			
8	.095	.018	.017
9	.095	.025	.023
10	.000	.004	.004
T. ezoe			
11	.048	.022	.024
12	.095	.038	.036
13	.048	.036	.025
14	.048	.048	.036
15	.048	.033	.035
T. sp			
16	.048	.024	.018
Mean	.072	.028	.026

* : a locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95.

** : biased estimate (Nei, 1978).

Table 9. Allele frequency at *SOD** in 4 samples of *Tribolodon hakonensis*, from upper reach, middle reach and river mouth of the Hime River, and from Otobe Harbor.

	N	**100*	**-67*
Hime River			
upper	70	.757	.243
middle	40	.812	.188
river mouth	40	.937	.063
Otobe Harbor	80	.875	.125

Table 10. Chi-square test for *SOD** gene uniformity between pairs of 4 samples of *Tribolodon hakonensis* from the Hime River and Otobe Harbor, chi-square (above diagonal) and probability (below diagonal).

	Hime River			Otobe Harbor
	upper	middle	river mouth	
Hime River				
upper		0.889	13.560	7.046
middle	$.3<p<.5$		5.715	1.701
river mouth	$p<.001$*	$.01<p<.02$**		2.195
Otobe Harbor	$p<.01$*	$.1<p<.2$	$.1<p<.2$	
Total of 4 samples		chi-square = 14.624		$p<.01$*

* : the difference is significant at 1% level.
** : significant at 2% level.

**100* increased and **-67* decreased from the upper to the lower localities.

The results of chi-square tests for *SOD** gene uniformity among four populations are expressed in Table 10. The frequency differences were fairly significant between upper reach vs. river mouth populations, upper reach vs. Otobe Harbor populatitons, and middle reach vs. Otobe Harbor populations. Significant differences exist among all four populations determined at the 1% level.

4. Cluster analysis

ND (Nei, 1978) and CD (Cavalli-Sforza and Edwards, 1967) matrix between pairs of populations and between pairs of species and types (mean distance) are presented in Tables 11 and 12, respectively. The intra-specific distance was very close in all species, even between pairs of anadromous and fluvial types of *T. hakonensis*. The largest distance was scored between *T. hakonensis* and *T.* sp. (0.521 ND and 0.565 CD), and the closest between *T. brandti* and *T. ezoe* (0.233 ND and 0.409 CD). Two situations are highlighted from the mean distance matrix (Table

Table 11. Nei's (1978) unbiased genetic distance (above diagonal) and Cavalli-Sforza and Edwards' (1967) chord distance (below diagonal) between pairs of 16 populations of *Tribolodon*. Sample numbers correspond to those in Table 5.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
T. hakonensis																
1	*****	.000	.000	.001	.000	.003	.001	.283	.285	.281	.438	.439	.438	.430	.435	.496
2	.048	*****	.001	.000	.000	.000	.000	.283	.285	.281	.444	.444	.443	.434	.439	.506
3	.053	.057	*****	.001	.000	.002	.000	.278	.280	.275	.443	.442	.442	.431	.436	.506
4	.058	.020	.062	*****	.000	.000	.000	.281	.283	.278	.452	.451	.451	.439.	.445	.519
5	.049	.022	.056	.031	*****	.001	.000	.283	.285	.280	.443	.442	.442	.433	.438	.504
6	.064	.027	.073	.019	.038	*****	.001	.284	.286	.282	.455	.454	.454	.442	.448	.521
7	.058	.036	.061	.039	.029	.037	*****	.282	.284	.280	.448	.447	.447	.436	.441	.511
T. brandti																
8	.450	.450	.445	.449	.449	.451	.449	*****	.000	.000	.247	.244	.246	.233	.239	.303
9	.451	.451	.446	.450	.450	.452	.450	.027	*****	.001	.249	.246	.247	.235	.240	.305
10	.445	.445	.440	.444	.445	.446	.445	.060	.071	*****	.247	.244	.246	.233	.239	.303
T. ezoe																
11	.531	.533	.532	.537	.531	.537	.535	.421	.421	.415	*****	.000	.000	.000	.002	.318
12	.531	.534	.532	.537	.532	.538	.535	.421	.421	.415	.044	*****	.000	.000	.001	.319
13	.532	.534	.533	.538	.532	.538	.536	.422	.422	.416	.009	.043	*****	.000	.000	.318
14	.529	.531	.529	.533	.528	.534	.532	.414	.415	.409	.037	.047	.035	*****	.000	.330
15	.531	.532	.530	.535	.530	.536	.534	.417	.417	.412	.033	.045	.029	.010	*****	.330
T. sp.																
16	.556	.559	.559	.564	.559	.565	.561	.457	.457	.452	.469	.471	.469	.476	.475	*****

Table 12. Nei's (1978) unbiased genetic distance (above diagonal) and Cavalli-Sforza and Edwards' (1967) chord distance (below diagonal) between pairs of 4 species 2 types of *Tribolodon* in mean.

	T. hakonensis		*T. brandti*	*T. ezoe*	*T.* sp.
	anadromous	fluvial			
T. hakonensis					
anadromous		.001	.282	.441	.507
fluvial	.043		.284	.451	.521
T. brandti	.447	.450		.245	.304
T. ezoe	.553	.537	.421		.325
T. sp.	.560	.565	.464	.473	

12). First, all species other than *T. brandti* were closely related to *T. brandti* at similar distances (0.245-0.304 ND and 0..421-0.464 CD), in other words, *T. brandti* was close to all the other species. Second, all species other than *T. hakonensis* were neatly related to each other (0.245-0.325 ND and 0.421-0.473 CD), in other words, *T. hakonensis* was distantly related to other two species except for *T. brandti* (0.441-0.521 ND and0.537-0.565 CD).

A dendrogram by UPGMA of ND among 16 populations of *Tribolodon* (Fig. 55) is an expected distance tree. Specific populations formed each cluster (ND=0.000). *T. brandti* and *T. ezoe* were linked in a cluster (ND=0.245), then *T.* sp. was linked to this (ND=0.317), and finally the cluster of *T. hakonensis* was linked together (ND=0.398). Two fluvial populations of *T. hakonensis*, from the Hime and Kikonai Rivers, as well as year-different populations from the same river, such as the Mu River populations and the Assabu River populations, did not form a pair with

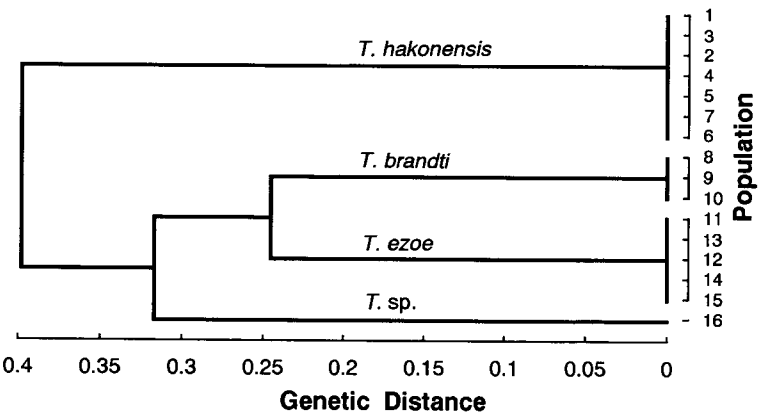

Fig. 55. Dendrogram (UPGMA) of Nei's unbiased genetic distance among 16 populations of *Tribolodon*. Population numbers correspond to those in Table 5.

each other in the cluster analysis.

An unrooted realized distance tree (a network) of mean ND by the neighbor-joining method (Fig. 56) reflects the actual distance data fairly well as compared with the dendrogram above. *T. brandti* was branched from approximately the center of the network and located at similar distances from the other three species. Contrary from the above dendrogram by UPGMA, *T. ezoe* and *T.* sp. formed a pair of neighbors with each other.

Wagner's tree of CD among 16 populations of *Tribolodon* presents a realized distance tree (Fig. 57). First, *T. hakonensis* and the other species were branched, next, *T. brandti* and a cluster of *T. ezoe* and *T.* sp. were branched at the branch length 0.004 from the root, and finally *T. ezoe* and *T.* sp. were branched at the

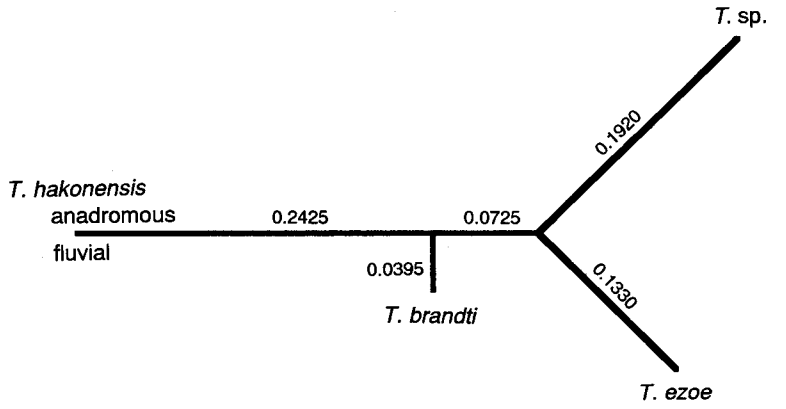

Fig. 56. Unrooted neighbor-joining tree of Nei's unbiased distance among 4 species of *Tribolodon*.

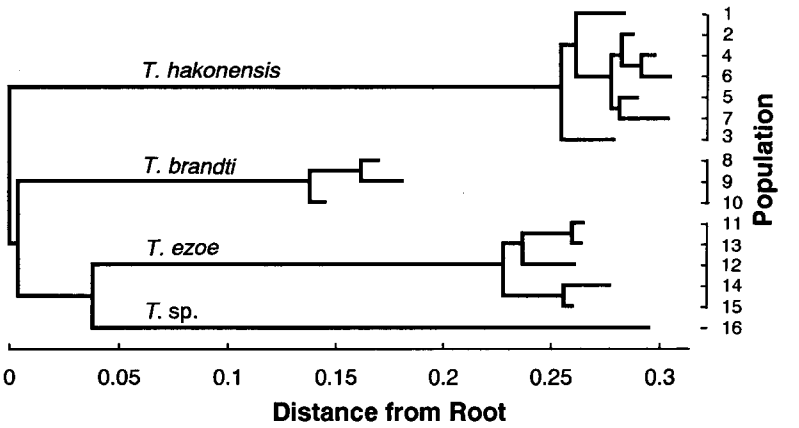

Fig. 57. Wagner's tree of Cavalli-Sforza and Edwards' chord distance among 16 populations of *Tribolodon*. Population numbers correspond to those in Table 5.

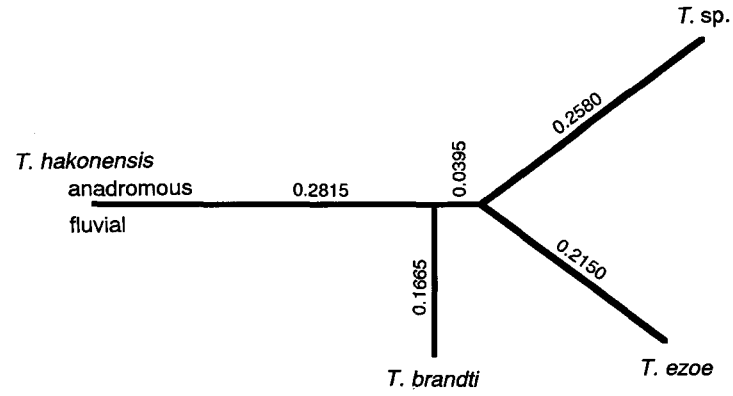

Fig. 58. Unrooted neighbor-joining tree of Cavalli-Sforza and Edwards' chord distance among 4 species of *Tribolodon*.

branch length 0.034 (0.004+0.034=0.038 from the root). The lengths of species branches were 0.255, 0.134, 0.190 and 0.257 from each branching point, and 0.257, 0.138, 0.228 and 0.295 from the root, for *T. hakonensis*, *T. brandti*, *T. ezoe* and *T.* sp., respectively. Populations within species formed a well-assembled cluster. Two fluvial populations of *T. hakonensis*, as well as year-different populations, did not form a pair in the species cluster, as in the case of the dendrogram of ND.

Another unrooted neighbor-joining tree based on the mean SD reflects the actual distance data fairly well (Fig. 58). Its branching pattern was the same as the first unrooted tree of ND (Fig. 56) except for the branch lengths.

Discussion

Genetic variability data ($pp=0.072$, $Ho=0.028$) of this study are similar to the results of the same four species of *Tribolodon* from Fukushima Prefecture reported by Hanzawa and Taniguchi (1982a) ($pp=0.099$, $Ho=0.023$) but lower than those from 21 populations of *T. hakonensis* reported by Hanzawa et al. (1988) ($pp=0.22$, $Ho=0.041$). The values reported by Hanzawa et al. (1988) are within the data range from studies reported by Ostariophysi by Nevo (1978) ($pp=0.04$-0.25, mean $pp=0.155$, $Ho=0.0006$-0.112, mean $Ho=0.045$).

The genetic variation of diadromous species is larger than that of related freshwater species (Gyllensten, 1985; Goto and Andoh, 1990; Taniguchi et al., 1990). This phenomenon, however, was not evident in *Tribolodon*. The genetic variability values of fluvial *T. hakonensis* from the Hime River ($pp=0.095$, $Ho=0.031$) were quite similar to those of *T. hakonensis* from the sea (Otobe Harbor). The mean values of *T. ezoe* (fluvial species) ($pp=0.057$, $Ho=0.035$) were at similar or larger levels in *Ho* than those of *T. brandti* (anadromous species) ($pp=0.063$ $Ho=0.016$). These results may indicate the effective size of fluvial populations is not as small as compared with that of anadromous *Tribolodon* populations.

The chi-square test for *SOD** gene uniformity indicated that the fluvial and anadromous populations of *T. hakonensis* from the Hime River, had different gene

pools as compared to each other. There are some weirs along the Hime River and the fluvial population in this study was collected from the site above the upper most weir. Therefore, gene flow must be restricted between the upper fluvial and the lower anadromous populations. The same fact was also documented in *T. hakonensis* from the Shimanto River, Kochi Prefecture (Hanzawa et al., 1988). Contrarily, two fluvial populations of *T. hakonensis* did not form a sister-pair in the species cluster (Figs. 55 and 57). Hanzawa et al. (1988) reported that the genetic differentiation exhibited between anadromous and fluvial populations from the same river was rather smaller than that between geographical populations (the distance was even subspecies level between northern and southern groups). Accordingly, slight difference in allelic constitution observed between anadromous and fluvial populations appears to be occasional.

The genetic distances (ND) between species of *Tribolodon* ranged from 0.282 to 0.521, which corresponded fairly to the interspecific level applied to marine and freshwater fish species by other workers (Shaklee et al., 1982; Buth, 1984). These measurements coincided well with the results of the same 4 species from Fukushima Prefecture (0.229–0.438, Hanzawa and Taniguchi, 1982b). Genetic relationships, distinct or close pairing, were equal to results reported by Hanzawa and Taniguchi (1982b). The coincidence is best expressed in the dendrogram presented in Fig. 55 and that reported by Hanzawa and Taniguchi (1982a). *T. brandti* and *T. ezoe* linked first, *T.* sp. formed a cluster with them next, and at last *T. hakonensis* was connected.

The dendrograms above do not reflect that all species other than *T. brandti* were closely related to *T. brandti* at similar distances, because a dendrogram by UPGMA is an expected distance tree (Nei, 1990). Meanwhile, the unrooted realized distance tree by neighbor-joining method (Fig. 56) expressed the genetic relationships perfectly, in which *T. ezoe* and *T.* sp. formed a neighboring pair and *T. brandti* was branched from near the center of the tree approximately the same distances from the other three species. The genetic inter-relationships among *Tribolodon* spp. expres-

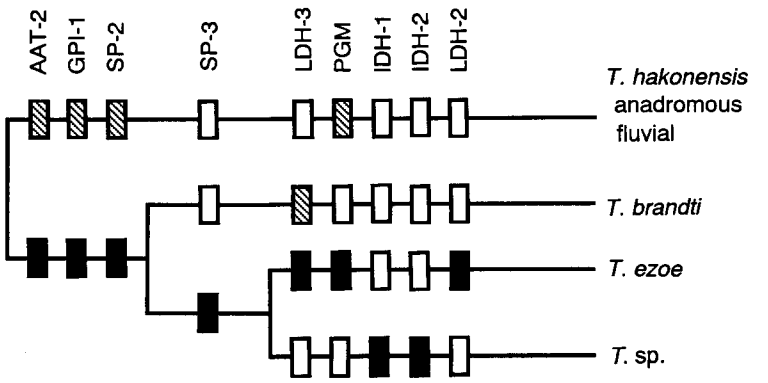

Fig. 59. Distribution of alleles on 9 diagnostic loci among 4 species of *Tribolodon*. Closed and striped squares are alleles diagnostic of monophyletic cluster. Open squares are those characterizing paraphyletic lines.

sed in the unrooted tree strongly suggests a certain difference in evolutionary rates among species. If true, the performance of the dendrogram by UPGMA would be inferior to trees by all other methods (Saitou and Nei, 1987; Nei, 1990).

The Wagner's tree of CD (Fig. 57) expressed the genetic relationships of *Tribolodon* species very well (in this method, non-metric distance data such as Nei's distance are omitted (Farris, 1972)), in which *T. hakonensis* branched first, *T. brandti* branched next, *T. ezoe* and *T.* sp. separated last, and *T. brandti* was located near the root. The unrooted tree of CD by neighbor-joining method (Fig. 58) coincided with the Wagner's tree as wall as the first unrooted tree of ND (Fig. 56) in the relationships. If the phylogenetic relationships of *Tribolodon* species were as expressed in the Wagner's tree, the minimum number of evolutionary steps in allele displacement (11 steps) are performed at the same time in the tree (Fig. 59). In fact, there is no allele which characterizes *T. brandti* and *T. ezoe* as a monophyletic pair (Fig. 59), although they were the closest pair in genetic distance as expressed in the dendrogram of ND (Fig. 55).

The facts support the phylogenetic relationships indicated in Figs. 56-59 as reasonable. If it is assumed that the evolutionary rate of *T. brandti* was lower and then has less derived allele than the other lines, the genetic relationships of *Tribolodon* species came from the allozyme data are fully explainable without inconsistency. Accordingly, the phylogenetic relationships of *Tribolodon* is presumed to be as follows; *T. hakonensis* line branched first from the ancestral *Tribolodon* stock, *T. ezoe-T.* sp. line separated next, *T. ezoe* and *T.* sp. were divided at last, and *T. brandti* was derived directly from the ancestral stock with less allozymic changes than the other species.

VII. Hybridization and reproductive isolation

Species of *Tribolodon* are morphologically very similar, but each of them exhibits a wide range in meristic variation (Nakamura, 1969). Much overlapping in meristics are also seen among these species (Onodera and Honma, 1976). Therefore, identification of species within the genus is difficult. This is particularly ture of juveniles in which the key characters such as cephalic sensory canals, etc. are incompletely developed. Moreover, classification is further complicated when hybrids are considered (Sakai and Hamada, 1985).

Authors have suggested the many advantages in adopting electrophoretically distinct isozymes as species markers to distinguish morphologically similar species including hybrids (e.g. Asspinwall and Tsuyuki, 1968; Nyman, 1970; Brassington and Ferguson, 1976; Fujio, 1977; Avise and Van Den Avyle, 1984; Ohkubo and Kudo, 1986; Campton, 1987). In these methods, one reliable allelic displacement among species can make a distinct identification of hybrids. Examination of plural loci bearing allelic displacements makes it possible to distinguish F_1 hybrids and later filial generations (Brassington and Ferguson, 1976; Fujio, 1977; Avise and Van Den Avyle, 1984; Campton, 1987).

The availability of allelic displacement in some allozyme and protein loci has been suggested as a method to identify particular *Tribolodon* species (Hanzawa and Taniguchi, 1982a, 1982b; Bushuyev et al., 1980; Gavrenkov et al., 1984) as well as

their hybrids (Hanzawa et al., 1984; Sakai and Hamada, 1985).

In the course of this research (previous chapter), nine allelic displacements among species were recognized. Many hybrids were found during the investigation of 0+ fish river distribution (chapter IV). Initial results were previously reported by Sakai and Hamada (1985), and Sakai (1987) discussed the population genetics of hybridization in *Tribolodon* briefly. However, their discussion was no more than superficial because the isozyme markers utilized could determine parental species of hybrids but not the hybrid mother species.

Such information is obtainable from the analysis of mtDNA polymorphism since it exhibits strict maternal inheritance (e.g. Campton, 1987; Dowling et al., 1990; Nei, 1990). Therefore, restriction fragment length polymorphisms (RFLPs) were examined on certain portions of the samples, and careful consideration of hybridization population genetics and its direction is made in this study as compared to previous reports (Sakai and Hamada, 1985; Sakai, 1978).

According to Mayr (1963, 1970), distinct species, utilizing his concept of biological species, are isolated reproductively by premating and/or postmating isolating mechanisms. Contrarily, less differentiated taxon, such as semispecies, may hybridize and introgress, and in extreme cases the barrier between the two breaks down completely so that the parental species are replaced by a hybrid swarm forming a continuous bridge between the two species under environmentally disturbed conditions by artificial means (Hubbs, 1955, 1961; Mayr, 1963, 1970). Sakai (1987, 1994) suggested environmental disturbances were involved in *Tribolodon* interbreeding (Sakai, 1987, 1994). The ecological and environmental conditions producing many hybrids and the isolation mechanims presumably functioning among *Tribolodon* species are discussed in the population genetic consideration.

Materials and methods

1. Occurrence pattern of hybrids

Intense sampling was accomplished on the Mu River because it contained large stocks of the three *Tribolodon* species. Mu River samplings were as follows; 0+ fish in autumn 1982, spring and autumn 1983, spring 1984, and autumn 1991, spawning adults in spring 1983, and parental fish for artificial hybrids in spring 1982.

Electrophoresis methodology was described in the previous chapter. For the technical convenience, however, only five of nine loci displaced among species, *GPI-1**, *LDH-2**, *PGM**, *SP-2** and *SP-3** expressed in the muscle extracts (Fig. 60), utilizing the discontinuous buffer system (Ridgway et al., 1970) were analyzed for the hybrids determinatiton study.

During the first electrophoretic inspection, the allelic displacements were confirmed by examination of three species from the Mu 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 confirm the allelic displacements because only one species was isolated in these rivers. Secondly, the artificial hybrids (all crosses exhibited greater than 90% ratio in hatching as well as in fertilization) were examined to check for heterozygotic isozyme patterns. Then the other fish were electrophoreticaly analyzed and determined to which species or

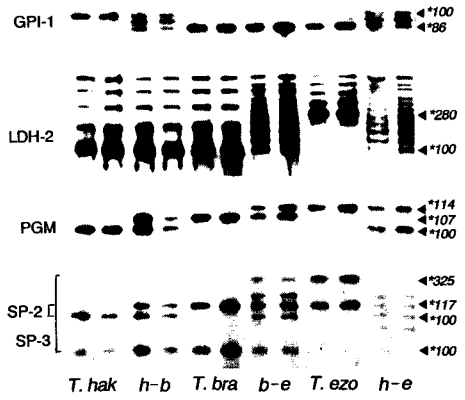

Fig. 60. Electrophoregrams of glucose phosphate isomerase (GPI), lactate dehydrogenase (LDH), phosphoglucomutase (PGM), and sarcoplasmic protein (SP) of muscle extract in *Triborodon hakonensis* (*T. hak*), *T. brandti* (*T. bra*), *T. ezoe* (*T. ezo*), and their hybrids (*h-b*, *b-e*, and *h-e*). The hybrids were artificially made.

hybrid they belonged.

Individuals were identified by species-specific bands. Those demonstrated heterozygotic patterns in all marker loci were identified as F_1 hybrids. Fish demonstrating heterozygosity in certain parts of the marker loci were judged as backcrosses of F_1 hybrids or later filial generations. In this method, approximately 3% of the backcross are mistakenly included in the F_1 hybrids or in a good species.

2. RFLPs analysis of mtDNA for mother species determination

The 0+ fish samples collected from the Mu River in 1991 were frozen again after the allozyme electrophoresis, and used for RFLPs analysis of mtDNA by eight restriction enzymes (6 base pairs recognized), *Bam* HI, *Eco* RI, *Hin* dIII, *Kpn* I, *Pst* I, *Pvu* II, *Xba* I, and *Xho* I. Each of 25 individuals of *T. hakonensis*, *T. brandti* and *T. ezoe*, judged as separate species by allozyme patterns, were analyzed for their mtDNA restriction patterns. As a result, three restriction enzymes, *Bam* HI, *Hin* dIII and *Pvu* II, were revealed to exhibit different restriction patterns among three species with no variation (Figs. 61-63, respectively). These results were reinforced by examining additional samples from other localities; *T. hakonensis*, 3 from the Assabu River, 5 from the Hine River, and 3 from the Kikonai River; *T. brandti*, 3 from the Assabu River; *T. ezoe*, 3 from the Assabu River, and 2 from the Kikonai River. Samples judged as hybrids by isozyme patterns were examined for their mtDNA restriction patterns by these three restriction enzymes. Mother species of 56 out of 77 hybrids could be determined (see the results).

Extraction of total DNA from muscle: Frozen muscle (0.2-1.0 g) was homogenized in 4.5 *ml* homogenizing buffer (50 *mM* Tris-HCl, 150 *mM* NaCl, 10*mM* EDTA, pH 8.0) using Potter-Elvehjem homogenizer and 0.5 *ml* of 10% SDS was added and incubated for 1 hr at 37°C. The homogenate was centrifuged at 12,000 rpm for 10 min, then the upper layer was transferred to a new polypropylene tube and phenol extracted (1 : 1) twice. This was followed by an extraction (8,000 rpm, 10 min) with phenol-chloroform-isoamyl alcohol (25 : 24 : 1), twice removals (8,000 rpm, 10 min) of the phenol by ether (1 : 1), and a precipitation of total DNA by adding 1/10 volumes of isopropanol and 1/10 volumes of 3*M* potassium acetate. The sample

was kept at freezing for more than 8 hr. Afterward the sample was centrifuged at 12,000 rpm for 15 min, the solution was decanted, washed by 70% ethanol once, and the tube was dried until the white pellet turned clear. The DNA pellet was resuspended in sterile distilled deionized water.

mtDNA purification for probe: mtDNA was purified by the method of Kijima et al. (1990) with slight modification. Ovary tissues of *T. hakonensis* from the Assabu River (1.0 g) were homogenized in 5 m*l* homogenizing buffer (200 *mM* sucrose, 50 *mM* Tris, 10 *mM* EDTA, 1.5% KCl, pH 7.5) using Potter-Elvehjem homogenizer. Nuclei and debris were removed by centrifuging at 3,000 rpm for 15 min, and then mitochondria were collected by centrifuging the supernatant at 12,000 rpm for 30 min. The pellet was resuspended in 2 m*l* of 1% SDS for 15 min, allowed to stand 10 min over ice with adding 0.4 *N* NaOH and adjusting pH 12.0-12.5. After 1 *M* Tris-HCl was added to the sample to adjust pH 8.0-8.5, 3 *M* potassium acetate was also added to make the final density 1 *M* potassium acetate, and allowed to stand 1 hr over ice. This was followed by removals of proteins, nucleus DNA and open circular mtDNA (8,000 rpm, 15 min), and the supernatant was introduced into the same extraction processing (phenol, phenol-chloroform-isoamyl alcohol, ether, and isopropanol extraction) as in the total DNA extraction. The mtDNA pellet was resuspended in the TE buffer (10 *mM* Tris, 1 *mM* EDTA, pH 8.0). The purified mtDNA were digested with *Hin* dlll and used as the probe templates.

Restriction enzyme digestion and electrophoresis: Each restriction enzymes of 5-10 units digested 10 μl total DNA solution of each sample at 37°C for 10-14 hr. λDNA was also digested with *Eco* T14 and used as size markers. Agarose slab gel electrophoresis (1% agarose, 4 *mM* Tris-acetate, 1 *mM* EDTA, pH 8.0) was performed.

Southern transfer and fragment detection: Nylon membranes (Zeta-probe, Bio-Rad Co. Ltd.) were used for 8-12 hr southern transfer with 0.4*N* NaOH as the transfer solution. The membrane was washed with 2×SSC solution, and baked at 80°C for more than 2 hr. The mtDNA probe and the λDNA probe were labeled using Dig DNA labeling kit (Boehringer Mannheim). DNA fragments were detected using Dig nucleic acid detection kit (Boehringer Mannheim). All procedures were carried out according to the methods recommended by the supplier.

3. Observation of spawning schools

When *Tribolodon* experienced high spawning concentrations, frequently more than two species were spawning side by side, at the same rapid, and at the same time. To ascertain whether the contamination of another species occurred or not, a direct observation of spawning schools was done by the skin diving into the spawning ground on June 22, 1981. A stake was driven near the spawning concentrations and spawning schools were watched for approximately five hours utilizing a water glass and clinging to the stake. On June 22 and 23, 1981, species concentrations and/or contaminations were confirmed by catching fish with a casting net. Several casts were made for the spawning concentration of each species and fish were counted and identified by their spawning color.

4. Model simulation of hybridization

To simulate the successive hybridization process, a simplified model of introgressive hybridization between two equally situated species under certain selective coefficients was schemed (Box 1) and calculated in several cases.

Box 1

It is assumed that species X and Y hybridize to a certain degree (p) for both directions and quite the same things occur in both species. Then the frequency change proceeding in the population X ($=Y$) is calculated here. In t generations after the first hybridization, frequencies in the population in 0+ fish are

$$x_t+h_t+l_t=1$$

where x_t, h_t and l_t are frequencies of good species, F_1 hybrid and offspring of F_1, respectively, judged by some genetic markers (n loci).

If these hybrids only contribute to the next generation in selection coefficients s_1 and s_2 for F_1 and offspring of F_1, respectively, the equation in the adult phase become

$$x_t+h_t(1-s_1)+l_t(1-s_2)=\mathrm{m}$$

In their gametes, the gamete frequency which exhibits markers of species X and Y in all loci (n) are

$$(1-p)x_t/\mathrm{m}+(1-s_1)h_t/2^n\mathrm{m}+(1-s_2)/2^n\mathrm{m}=X_t \qquad \text{and}$$
$$py_t/\mathrm{m}+(1-s_1)h_t/2^n\mathrm{m}+(1-s_2)l_t/2^n\mathrm{m}=Y_t \qquad \text{respectively.}$$

The gamete frequency which exhibits markers of both species at the same time are

$$1-X_t-Y_t=Z_t$$

These gametes make pairs randomly. The frequencies in 0+fish of $t+1$ generation are

$$(X_t+Y_t+Z_t)^2=X_t^2+Y_t^2+Z_t^2+2X_tY_t+2X_tZ_t+2Y_tZ_t=1$$

where X_t, Y_t and $2X_tY_t$ are frequencies of those judged as species X, Y and their F_1 hybrid, respectively. Fish of the frequency Z_t^2 should include some $\{1/(2^n-2)\}$ which exhibit heterozygotes in all loci examined. The frequency of fish judged as Y should be equal to that fish judged as X from the population of species Y. Therefore, the frequencies of good species, F_1 hybrid and offspring of F_1 in the next generation are predicted to be

$$x_{t+1}=X_t^2+Y_t^2,$$
$$h_{t+1}=2X_tY_t+Z_t^2/(2^n-2),$$
$$l_{t+1}=1-x_{t+1}-h_{t+1},$$

respectively.

Results

1. Occurrence pattern of hybrids

Table 13 denotes the change of the occurrence pattern by year and/or between 0+fish and adults in the Mu River. The results from 1991 determinations are shown in Table 14.

Of a total of 2,332 individuals examined, 103 individuals and 174 individuals were judged as F_1 hybrids and hybrids of later filial generations, respectively. The amount of F_1 hybrids was too much if the introgression among species was assumed to be proceeding, especially in the hybridization between *T. brandti* and *T. ezoe*

Table 13. Change in occurrence pattern of three species of *Tribolodon* and their hybrids in the Mu River. In the hybrids, for example, *h-b* shows those of *T. hakonensis* × *T. brandti* and *h-b-b* denotes their backcross hybrids.

	0+ young					Spawning adult
	'82 autumn	'83 spring	'83 autumn	'84 spring	total	'83 spring
hakonensis	53	83	154	155	445	53
brandti	80	76	118	218	492	22
ezoe	25	141	67	131	364	107
h-b	6	14	12	8	40	5
b-e	17	9	3	5	34	2
h-e				3	3	
h-b-h			9	1	10	
h-b-b	5	17	19	27	68	2
b-e-b	2	4	3	1	10	
b-e-e	8	3	2	2	15	
h-e-e				1	1	
the others	1	2	3	4	10	
Total	197	349	390	556	1492	191
Hybrid %	19.8	14.0	13.1	9.4	12.8	4.7
% of *hak*+*bra*						
F_1 (*h-b*)	4.2	7.4	3.8	2.0	3.8	6.1
offspring of F_1	3.5	8.9	9.0	6.8	7.4	2.4
% of *bra*+*ezo*						
F_1 (*b-e*)	12.9	3.9	1.6	1.4	3.7	1.5
offspring of F_1	7.6	3.0	2.6	0.8	2.7	
% of *hak*+*ezo*						
F_1 (*h-e*)				1.0	0.4	
offspring of F_1				0.3	0.1	

(Table 13) in which the rate of F_1 was even higher than that of the later generations.

In the Mu River, the rate was always high in 0+fish (9.4–19.8%, mean 12.8% in 1982–1984, 11.9% in 1991), but significantly lower in spawning adult (4.8%) (X^2 test, $p<0.01$). In 0+juveniles, the rate decreased significantly in spring after the fish experienced winter (X^2 test, autumn in 1982 and 1983 vs. spring in 1982 and 1983, $p<0.02$).

In the Mu River, only 7 hybrids between *T. hakonensis* and *T. ezoe* were found from a total of 277 hybrids out of 2,332 individuals examined. All other hybrids were between *T. brandti* and the other two species.

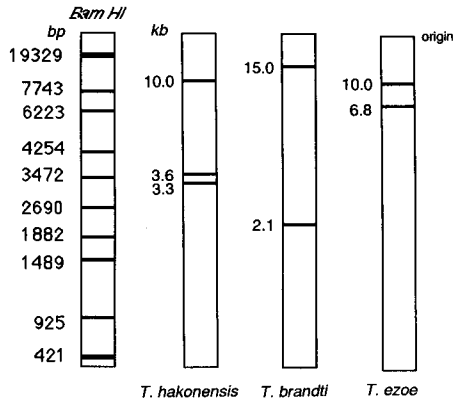

Fig. 61. Diagrammatic representation of *Bam* HI digestion profiles of mtDNA in three species of *Tribolodon*. The left most lane is molecular size markers (λDNA digested by *Eco* T14).

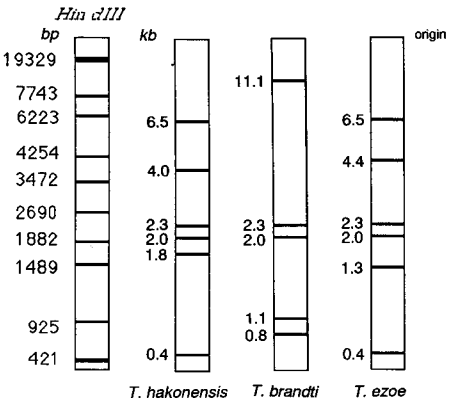

Fig. 62. Diagrammatic representation of *Hin* dIII digestion profiles of mtDNA in three species of *Tribolodon*. The left most lane is molecular size markers (λDNA digested by *Eco* T14).

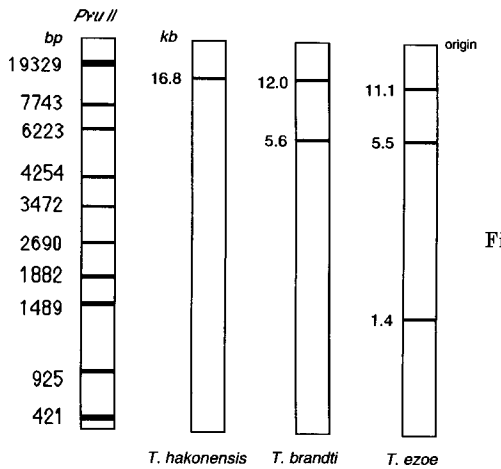

Fig. 63. Diagrammatic representation of *Pvu* II digestion profiles of mtDNA in three species of *Tribolodon*. The left most lane is molecular size markers (λDNA digested by *Eco* T14).

2. Mother species of hybrids

Restriction morphs and size of mtDNA: Restriction patterns of three species of *Tribolodon* with *Bam* HI, *Hin* dIII and *Pvu* II were monomorphic in surveyed individuals from each species. In *Bam* HI restriction, *T. hakonensis*, *T. brandti* and *T. ezoe* exhibited 3 (10.0, 3.6 and 3.3 kilobase pairs (kbp)), 2 (15.0 and 2.1 kbp), and 2(10.0 and 6.8 kbp) fragments, respectively (Fig. 61). Two bands of *T. hakonensis* (3.6 and 3.3 kbp) might be two subdivisions of the 6.8 kbp fragment of *T.ezoe*. In *Hin* dIII restriction, the three species exhibited 6 (6.5, 4.0, 2.3, 2.0, 1.8 and 0.4 kbp), 5 (11.1, 2.3, 2.0, 1.1 and 0.8 kbp), and 6 (6.5, 4.4, 2.3, 2.0, 1.3 and 0.4 kbp) fragments respectively (Fig. 62). The two fragments, 2.3 and 2.0 kbp, were common in the three species. In *Pvu* II restriction, the three species exhibited 1 (16.8 kbp), 2 (12.0 and 5.6 kbp), and 3 (11.1, 5.5 and 1.4 kbp) fragments, respectively (Fig. 63). Two bands of *T. brandti* (12.0 and 5.6 kbp) might be two subdivisions of the 16.8 kbp

Table 14. Occurrence pattern of hybrids of three species of *Tribolodon* (0+ young) from the Mu River in 1991. For abbreviations of hybrids, see legend of Table 13.

	0+ young
hakonensis	75
brandti	415
ezoe	82
h-b	7
b-e	11
h-e	1
h-b-h	1
h-b-b	36
b-e-b	8
b-e-e	4
h-e-h	1
h-e-e	1
the others	7
Total	649
Hybrid %	11.9
% of *hak*+*bra*	
F_1 (*h-b*)	1.3
offspring of F_1	6.9
% of *bra*+*ezo*	
F_1 (*b-e*)	2.1
offspring of F_1	2.3
% of *hak*+*ezo*	
F_1 (*h-e*)	0.6
offspring of F_1	1.3

Table 15. Mother species of 56 hybrids of *Tribolodon* judged from mtDNA haplotype. For abbreviations of hybrids, see legend of Table 13.

		Mother species		
		hakonensis	*brandti*	*ezoe*
h-b	5	1	4	
b-e	11		7	4
h-e	1			1
h-b-b	29	1	28	
b-e-b	5		5	
b-e-e	1			1
h-e-h	1	1		
h-b-e-b	3		3	
Total		3	47	6

band, and two of three bands in *T.ezoe* (11.1 and 1.4 kbp) might be two subdivisions of the 12.0 kbp fragment of *T. brandti*. At any rate, the three species of *Tribolodon* can be discriminated clearly with these fragment patterns, and the identification of mother species of their hybrids can be done as well. The sizes of mtDNA were 16.8–17.0 kbp in *T. hakonensis*, 17.1–17.6 kbp in *T. brandti*, and 16.8–18.0 kbp in *T. ezoe*. These measurements coincide well with the available data of *T. hakonensis* (Hanzawa et al., 1987) as well as those of other fish species (Kijima, 1991).

Mother species of hybrids: Out of 649 0+fish surveyed in 1991 from the Mu River, 77 individuals (11.9%) were judged as hybrids by their allozyme patterns (Table 14). Of them, mother species of 56 hybrids were determined with the RFLPs analysis of mtDNA (Table 15). One of the F_1 between *T. hakonensis* and *T. brandti* (*h-b*), one of later hybrid between *T. hakonensis* and *T. brandti* (*h-b-b*), and one of later hybrid between *T. brandti* and *T. ezoe* (*h-e-h*) exhibited the *T. hakonensis* pattern of mtDNA RFLPs in all three restriction enzymes. Four of the F_1 between *T. brandti* and *T. ezoe* (*b-e*), one of F_1 between *T. hakonensis* and *T. ezoe* (*h-e*), and one of the later hybrid between *T. brandti* and *T. ezoe* (*b-e-e*) showed the *T. ezoe* pattern. All the remaining 47 hybrid individuals (83.9%) exhibited the mtDNA haplotype of *T. brandti*.

3. Contamination of spawning schools

Table 16 indicates the number of individuals collected with a casting net per cast for the specific spawning concentrations in the same spawning ground, on the same day at just under the Kasuga Weir, Mu River. Apparently no breeding contamination occurred in populations of *T. hakonensis*. A little contamination by other species was exhibited in populations of *T. ezoe*. Meanwhile, many matured males and premature fish of *T. ezoe* and some matured males of *T. hakonensis* were captured with the spawners of *T. brandti*.

This phenomenon was observed by skin diving into the spawning ground.

Table 16. Number of individuals collected by a casting net (about 8 m²) per cast for the spawning concentration of each species of *Tribolodon* in June, 1981, at teh Kasuga Weir, the Mu River.

For concentration of		Number of individuals		
		hakonensis	*brandti*	*ezoe*
T. hakonensis (6 casts)				
	cast 1	15	0	0
	2	18	0	0
	3	24	0	0
	4	21	0	0
	5	12	0	0
	6	27	0	0
	mean	19.5	0	0
T. brandti (4 casts)				
	cast 1	3	11	18
	2	1	8	25
	3	0	9	32
	4	1	9	15
	mean	1.3	9.3	22.5
T. ezoe (5 casts)				
	cast 1	0	1	35
	2	0	0	17
	3	1	0	19
	4	2	0	28
	5	0	0	15
	mean	0.6	0.2	22.8

Some males of *T. hakonensis* were observed following the trailing end of the spawning school of *T. brandti* with some smaller males of *T. brandti*, which were followed next by many *T. ezoe* picking up the spawned eggs. Similar situations were never observed in the spawning schools of *T. hakonensis* and in *T. ezoe*.

4. Simulation of hybridization

When the number of genetic marker loci (n) is set as infinite, only a few contamination (p) with only a few survival ($1-s$) leads to a successive gradual increase in hybrid offsprings. In the case of *Tribolodon* hybrid in this study, the number of genetic markers are three in both combinations between *T. brandti* and *T. hakonensis* or *T. ezoe*. The results of 10 generations of hybridization with three loci and settled p value ($=0.02$) are shown in Fig. 64 for various situations of selection coefficients where some results which are very close to the actual data of hybrids can be calculated. When s_1 and s_2 are set as 0 and 1, respectively, the

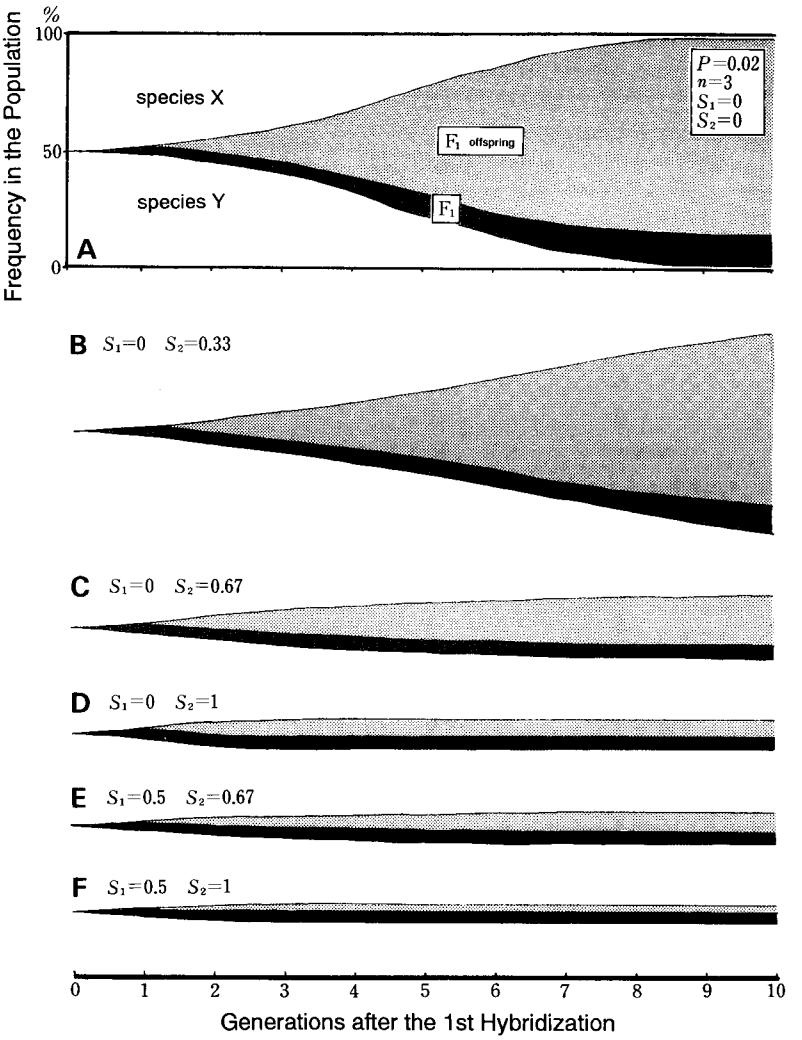

Fig. 64. Simulated hybridizations under various selection coefficients. Proportion of species contamination (p=0.02) and number of genetic markers between species (n=3) are settled, and selection coefficients for F_1 hybrid (s_1) and offspring of F_1 (s_2) are varied.

frequencies of F_1 and offsprings of F_1 converge to 4.8% and 7.6%, respectively (Fig. 64D), which are very close to the natural data of hybrids between *T. brandti* and *T. hakonensis*, 3.8 and 7.4% (Table 13). If s_1 is reset as 0.5, the frequencies become 4.3% and 3.4%, respectively (Fig. 64F). These results are similar to the natural data detected in the hybrids between *T. brandti* and *T. ezoe* (Table 13). The relative low number of offsprings of F_1 hybrid can only be derived from the situation that a considerable part of F_1 hybrids and any of offsprings of F_1 can not produce

the next generation.

Discussion

Tribolodon species are similar and difficult to distinguish morphologically from each other, and various opinions have been presented concerning their classification (e.g. Jordan and Fowler, 1903; Tanaka, 1931; Ikeda, 1936, 1938; Okada and Ikeda, 1937; Onodera and Honma, 1976). The classification must be more perplexing when they hybridze. Such wild hybrids have further complicated the classification of the genus.

The intense research on the Mu River in this study revealed that more than 10% of 0+fish were hybrids. However, only a few hybrids between *T. hakonensis* and *T. ezoe* were collected in the Mu River. In contrast to the fluvial type, the anadromous type of *T. hakonensis* may not easily hybridize with *T. ezoe* which lives an entirely fluvial life.

In the Mu River, the three species of *Tribolodon* often simultaneously spawned on the same rapids, in high concentrations, where the Kasuga Weir prevented the spawning runs from continuing further upstream migration. Such forced crowding of fish on a limited spawning ground must be one of the causes resulting in many natural hybrids (Hubbs, 1955). Nevertheless, it remains unclear why *T. hakonensis* and *T. ezoe* did not easily hybridize.

Some males of *T. hakonensis* and many fish of *T. ezoe* were observed following the trailing end of the spawing school of *T. brandti*. The similar phenomenon was never observed in the spawning schools of *T. hakonensis* and in *T. ezoe*. All these observations quite agree with the occurrence pattern of hybrids in the Mu River. Males of *T. hakonensis* and *T. ezoe* are supposed to have run and crossed with females of *T. brandti*. This supposition was supported strongly in this study by the fact that approximately 84% of the hybrids involved the *T. brandti* female.

Tribolodon is a lithophilous open substratum spawner (Balon, 1975a), spawning en masse in stream rapids with its sex ratio skewed to males (Okada, 1935; Kawajiri, 1956; Tabeta and Tsukahara, 1964; Nakamura, 1969; Ito, 1975). When *T. brandti* came to the spawning ground, the peak of *T. hakonensis* spawning concentration had already passed (chapter V). The males of *T. hakonensis* following *T. brandti* might be those which missed their own spawning opportunities.

Hubbs (1995), in his review of fish hybridization, predicted that the natural hybridization between species would show a clinal gradation from north temperate to tropical zones, as well as from freshwater to the sea, and stated that frequency of hybridization was inversely correlated with the number of species in given areas, especially where the environment was unstable due to natural or artificial disturbance. In reality, there exist only a few primary freshwater fishes in Hokkaido (Goto et al., 1978; Goto, 1982, 1991; Maekawa and Goto, 1982; Goto and Nakano, 1993), and conversely the population size of a species is large in Hokkaido, and this is especially true of anadromous *Tribolodon* which comes back to the limited river spawning ground in a big run from the sea (Sakai and Hamada, 1985; Sakai, 1987). If the spawning runs of two species were disrupted by a weir across the river under such situation, the gene of one species might be apt to "overflow" into the other, and

the direction of "gene overflow" would be affected by the habitual difference between the participating species. In this manner, gene of *T. hakonensis* which missed its own spawning concentration might overflow into *T. brandti* female. In the more southern area, in Honshu, where the population size of each species are thought to be smaller than in Hokkaido, hybridization has not been detected (Hanzawa et al., 1984).

Another mechanism may act as well on the hybridization between *T. brandti* and *T. ezoe*. Spawning school of *T. ezoe* (as well as that of fluvial *T. hakonensis*) consists of various sized fish, and the smaller males follow the trailing end of the spawning school. They were observed rushing or streaking into the spawning-ejaculating center or devouring the scattered eggs (personal observation). The many males of *T. ezoe* following the spawing school of *T. brandti* were also eating the eggs scattered and/or already attached to stones. Although the act has not been observed, some of them might streak into the just spawning-ejaculating point of *T. brandti*, and then hybridize with *T. brandti* as the mother species. The actual data, however, included hybrids between *T. brandti* and *T. ezoe* which had the mtDNA haplotype of *T. ezoe*. Some males of *T. brandti* must occasionally hybridize with relatively larger females of *T. ezoe*.

Why didn't the males of *T. ezoe* follow the spawning school of *T. hakonensis*? Why didn't they easily hybridize? Only the answer available now may be their genetic relationships. *T. hakonensis* and *T. ezoe* are genetically very distant from each other, although both are closely related to *T. brandti* at the same time. These genetic interrelationships agree with the occurrence pattern of hybrids. The closer two species may hybridize, while the less closely related two may not. In the discussion of the genetic relationships of *Tribolodon* (chapter VI), *T. brandti* was presumed to be genetically most similar to the ancestral species. Namely, *T. hakonensis* as well as *T. ezoe* were presumed to have derived from the ancestor which was similar to *T. brandti*. This presumption may also explain the direction of observed hybridization. Males of derived species may be apt to hybridize mistakenly with females of mother (ancestral) species (Watanabe and Kawanishi, 1979, 1981, 1983). All hybrid occurrence patterns are not contradictory to the genetic interrelationships of *Tribolodon*.

If successive generations of hybridization had occurred, this would have lead to continuous intergradation of phenotypes and finaly fusion of the participating species. In reality, the scale number was overlapped and somewhat integrated among the parental species and their hybrids (Sakai and Hamada, 1985). 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 equilibrium that no statistical analysis was necessary. The relative frequency of F_1 hybrids was so high as compared with that of offsprings of F_1 that the introgressive hybridization between species has not been proceeding. The results calculated by the hybridization model of this study strongly suggested that F_1 hybrids of *Tribolodon* could not produce their grandchildren. In reality, the number of hybrid individuals in 0+fish was significantly decreased after wintering. In adult fish, the ratio of hybrids decreased more (Table 13). These data suggest the reduced adaptability of hybrid individuals than good species.

Such a phenomenon is known as the hybrid breakdown which serves as an inhibitor of introgression between species (e.g. Mayr, 1963, 1976; Dobzhansky, 1970).

Three species of *Tribolodon* sometimes spawn at the same ground at the same time. But the majority of them spawn with their own species in a spawning school. One of the important cues in discriminating conspecifics (the premating reproductive isolating mechanism) may be their olfactory information (Sakai and Yoshii, 1990). When hybridized, most of the hybridization occurs on the female of *T. brandti*, which is thought to be genetically closest to the ancestral *Tribolodon*. However, the introgression between species does not proceed because of the hybrid breakdown (the postmating reproductive isolating mechanism).

VIII. Distribution

Nishimura (1974) emphasized that the genus *Tribolodon* is one of the typical groups which exhibit the circum-Japan Sea distribution pattern, and discussed that it had originated from the Sea of Japan. However, not all members of the genus are distributed to the circum-Japan Sea area (Kurawaka, 1977). The classification of species within this genus had been complicated previously, and there were only fractional reports concerning their distribution, especially abroad. This is also true of Hokkaido to a degree. There are large stocks of the three *Tribolodon* species, *T. hakonensis*, *T. brandti* and *T. ezoe*, but the distribution pattern in Hokkaido as well as the distribution in a river of three species is unclear. In this chapter, the geographical distribution pattern, distribution in Hokkaido, and the distribution pattern of three species of the genus in several rivers are reviewed and presented using actual specimens as well as reports from the literature.

Materials and methods

1. Geographical distribution

In order to clarify the geographical distribution patterns of four species of *Tribolodon*, evidences by actual fish specimens were not used except for the case of Hokkaido because it seemed quite impractical to check all specimens from all localities especially from abroad. Maps were drawn from reports which documented distributions based on the proper classification of *Tribolodon* species which is based on the qualitative characteristics such as the spawning color, the cephalic lateral line system, the gas-bladder morphology, etc.; Ikeda (1938), Onodera and Honma (1976), Kurawaka (1977), Takeuchi and Hashimoto (1990) and Honma (1991) for Japan, Gritsenko (1974) for Sakhalin, and Gavrenkov and Ivankov (1979), Dai et al. (1982) and Jeon and Sakai (1984) for from the Southern Maritime Territory to the Korean Peninsula.

2. Distribution in Hokkaido

The distribution maps in Hokkaido were made by plotting actual records of fish specimens collected by the Laboratory of Embryology and Genetics, Faculty of Fisheries, Hokkaido University, from 1978 to 1982. Thirteen rivers in Hokkaido, the Teshio, Tokachi, Tokoro, Saru, Shiribeshi-Toshibetsu, Charo, Rumoi, Onbetsu,

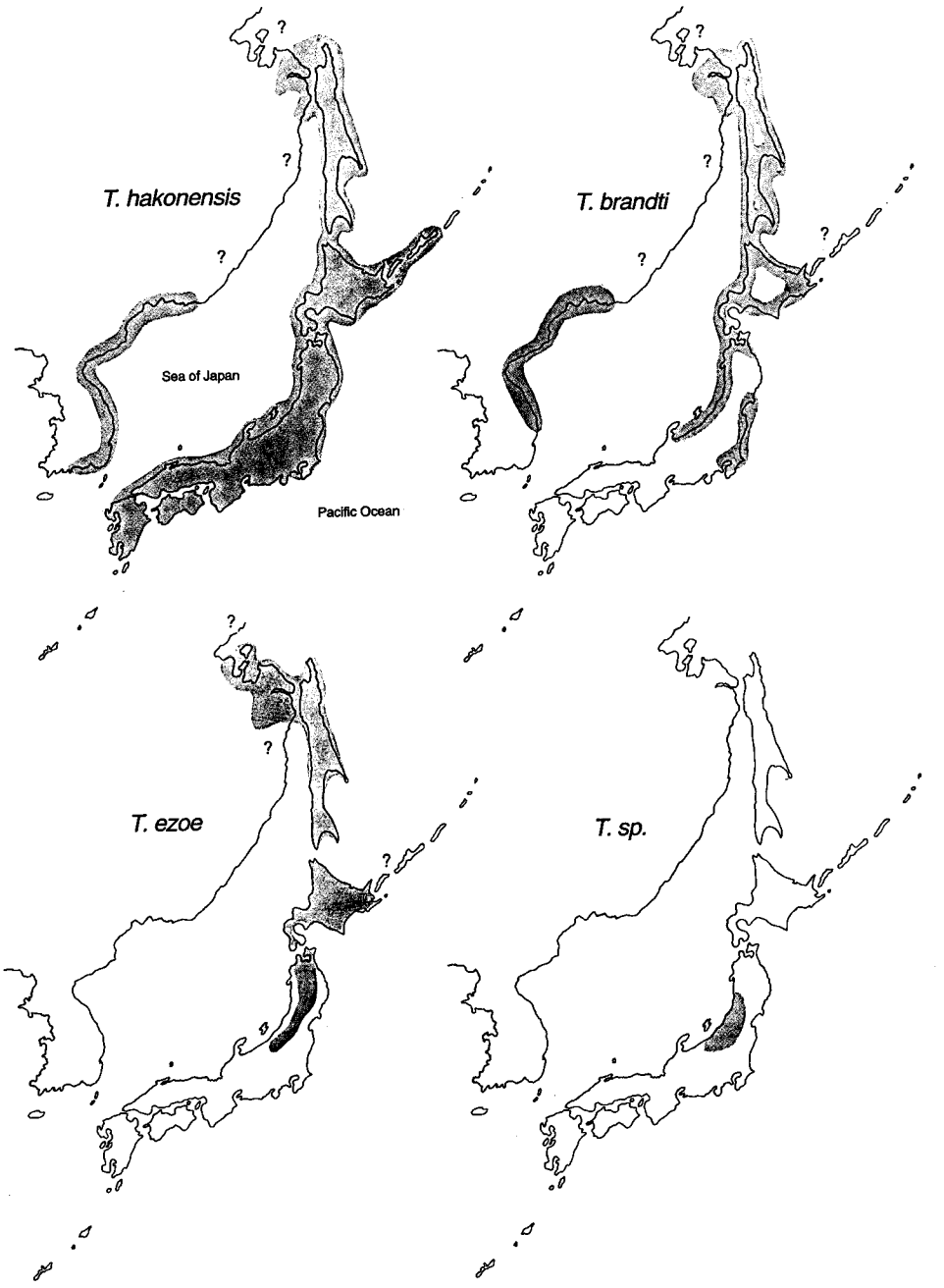

Fig. 65. Map showing distribution ranges of *Tribolodon* spp. The fluvial type of *T. hakonensis* is not scored from the continental side.

Amano, Futoro, Hime, Uttsu and Shosanbetsu Rivers, were sampled at several stations from upper to lower reaches in order to clarify the distribution pattern of species along the river courses. The specimens were classified using some diagnostic characters such as number of scales (Nakamura, 1969; Sakai and Hamada, 1985), the cephalic lateral line system (Nakamura, 1963, 1969; Kurawaka, 1977) and the morphology of gas-bladder (Kahata, 1981; Churikov and Sabitov, 1982).

Results

1. Geographical distribution

The geographical distribution area (Fig. 65) of *T. hakonensis* ranges from the Amur basin to Kyushu, including South Kuril, and from the Southern Maritime Territory to the east coast of Korea. In the Maritme Territory proper, *Tribolodon* is certainly distributed but the species name still remains unclear (Svetovidova, 1973). The northen limit is also unclear. The anadromous form is commonly seen north of Sendai Bay in the Pacific slope and north of Toyama Bay in the Japan Sea slope of Japan, and intermittently extends to Mikawa Bay in the Pacific slope (Kurawaka, 1977, 1992) and to Northern Kyushu in the Japan Sea slope (Tabeta and Tsukahara, 1964). The anadromous form is certainly distributed from the Southern Maritime Territory to the Korean Peninsula, but the fluvial form does not exist there

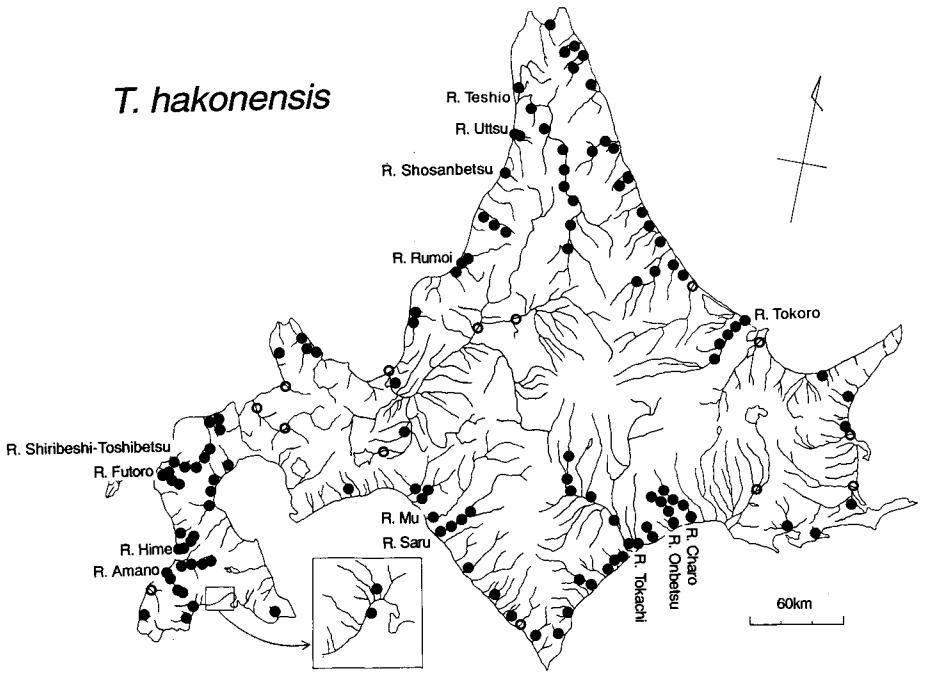

Fig. 66. Map of Hokkaido showing rivers *Tribolodon hakonensis* was recorded. Open marks are records in literatures.

(Uchida, 1939 ; Gavrenkov and Ivankov, 1979 ; Gavrenkov, 1982 ; Dai et al., 1982 ; Jeon and Sakai, 1984 ; Jeon, 1987).

The geographical distribution area of *T. brandti* extends from the Amur basin to Tokyo Bay in the Pacific slope and to Toyama Bay in the Japan Sea slope of Japan, and from the Southern Maritime Territory to the north-east coast of Korea, but the habitat is rather restricted to the locality where a large body of brackish water exists especially in the southern part of the range (Kurawaka, 1977, 1992). Both the northern and the eastern limits are unknown. It is also unclear whether it exists along the Maritime Territory proper or not.

The distribution range of *T. ezoe* extends from Shanter Island to Fukushima Prefecture, Japan. All populations in Japan live a fluvial mode of life, but in the northern part of the range, in Sakhalin for example, more or less some members of a population migrate to the sea (Gritsenko, 1974). The habitat is restricted to the upper reaches of comparatively large rivers in the southern part of the range, in Honshu Island (Kurawaka, 1977, 1992 ; Takeuchi and Hashimoto, 1990). The northern and the southern limits in the Maritime Territory are unknown.

T. sp. has been recorded from the Koyoshi, Mogami, Agano and Shinano Rivers on Akita, Yamagata, Niigata and Fukushima Prefectures (Sakai et al., 1991b ; Honma, 1991).

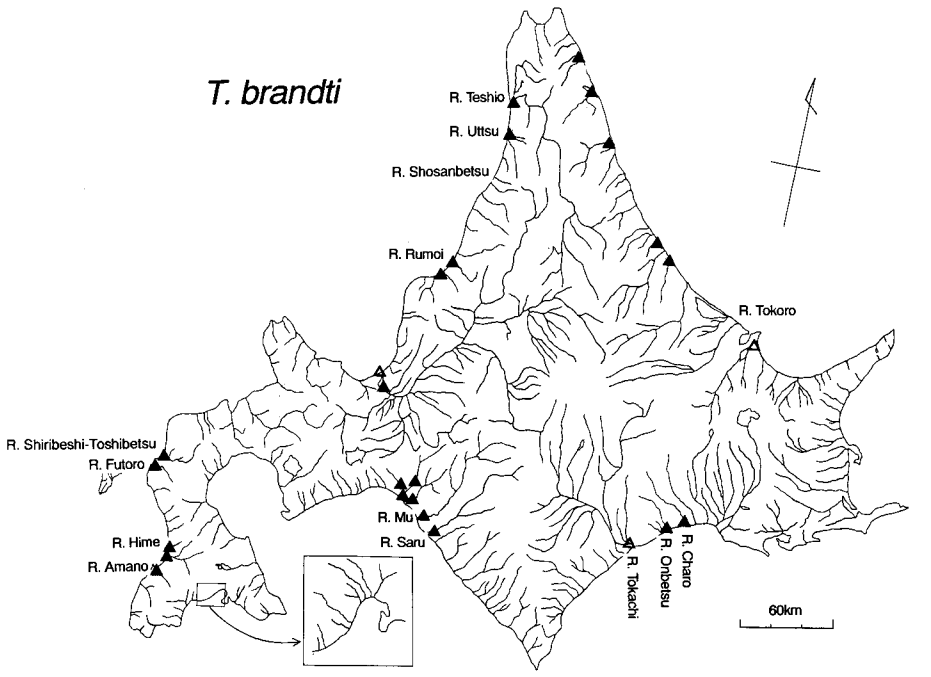

Fig. 67. Map of Hokkaido showing rivers *Tribolodon brandti* was recorded. Open marks are records in literatures.

2. Distribution in Hokkaido

T. hakonensis is widely distributed on nearly the whole region of Hokkaido (Fig. 66). *T. brandti* was recorded from the Okhotsk Sea slope of Souya, Kitami and Abashiri, from the Japan Sea slope of Teshio, Rumoi, Ishikari and Hiyama, and from the Pacific slope of Kushiro, Tokachi and Iburi (Fig. 67). *T. ezoe* was collected from a wide area of Hokkaido, but was not scored from Funka Bay, Shiretoko, Shakotan and Kameda Peninsulas (Fig. 68).

T. brandti was collected from the river mouth without exception. *T. hakonensis* and *T. ezoe* were collected in various proportions from river to river. *T. hakonensis* was predominant over *T. ezoe* in Oshima District, the Shiribeshi-Toshibetsu, Futoro, Hime and Amano Rivers. Especially in the Hime River, *T. ezoe* was not seen and the same situation is shared in many small rivers in the same District. In the river where *T. ezoe* was distributed, it tended to be caught more in the upper reaches.

T. ezoe was predominant over *T. hakonensis* in Rumoi and Hidaka Districts, the Uttsu, Shosanbetsu, Rumoi and Saru Rivers. In these rivers, *T. hakonensis* was restricted to the lower reach. The same condition was seen in the Mu River in Iburi District, the next river of the Saru River.

In Kamikawa, Abashiri, Kushiro and Tokachi Districts, both *T. hakonensis* and *T. ezoe* were recorded from the upper to lower reaches in the Teshio, Tokoro, Charo,

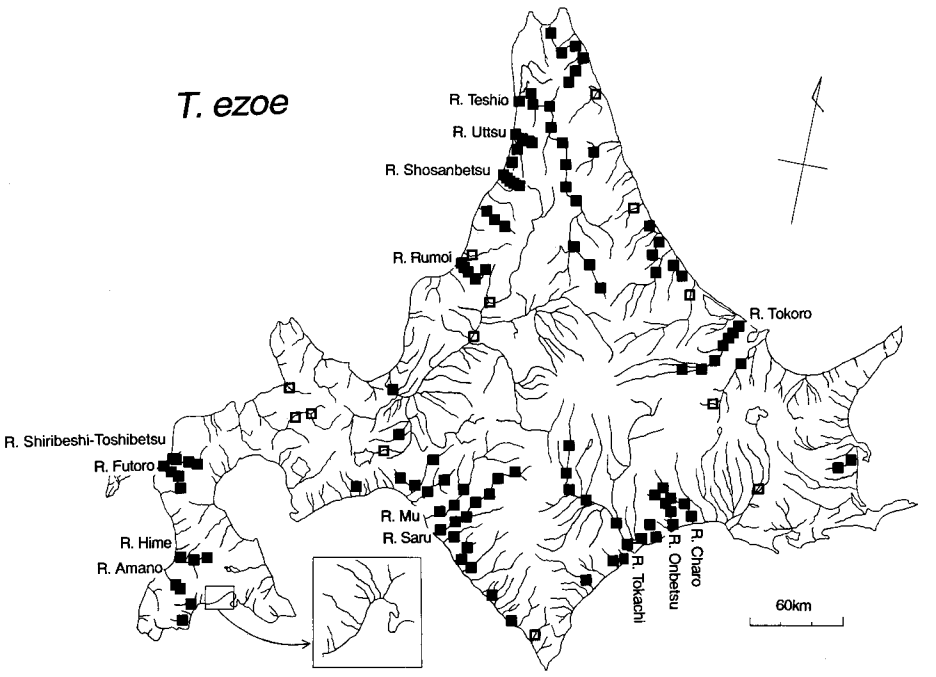

Fig. 68. Map of Hokkaido showing rivers *Tribolodon ezoe* was recorded. Open marks are records in literatures.

Onbetsu and Tokachi Rivers. However, the situation of the Charo and Tokoro Rivers may resemble that of the rivers on Oshima and Rumoi Districts, respectively.

Discussion

The genus *Tribolodon* exhibits the circum-Japan Sea distribution pattern (Nishimura, 1964). *T. hakonensis* exhibits the largest distribution within the genus, nearly covering the entire range of *Tribolodon* distribution. *T. brandti* and *T.* ezoe are distributed in the northern half of the range. *T.* sp. is restricted to the southern most area of the northern half.

Many species of so-called Siberian elements of freshwater fish in Japan (Aoyagi, 1957; Goto, 1982, 1991; Maekawa and Goto, 1982; Goto and Nakano, 1993) exhibits similar distribution patterns. However, in the primary freshwater fish groups, *Tribolodon* is the exception among the Siberian elements which invaded Honshu or more. This phenomenon strongly relates to their adaptation to changes in salinity. Even *T. ezoe* goes to the sea in Sakhalin (Gritsenko, 1974) which lives fluvial life in Japan. This study indicated that *T. ezoe* could tolerate more salinity than other cyprinids (chapter IV). During historical climatic periods as in the glacial age, *T. ezoe* might have been able to enter the sea shore water and to extend its distribution to the southern area.

In the Southern Maritime Territory, there existed no fluvial *T. hakonensis* and *T. ezoe*, although they extended to the Sakhalin-Japan side from Shanter Island to the Japanese Archipelago. In the southern most areas of the continental side, in southern Korea, fluvial type of *Tribolodon* does not appear (Uchida, 1939; Jeon and Sakai, 1984; Jeon, 1987). Though, in Japan, the more fluvial populations of *T. hakonensis* appear in the more southern area such as Kyushu Island which is located close to the Korean Peninsula. This indicates only the anadromous *Tribolodon* had migrated to the continental side of the distribution range through the sea shore in recent age. It may also be deduced from the above that the speciation events of fluvial *T. hakonensis* and *T. ezoe* had occurred at the Sakhalin-Japan side of the distribution range, even if the ancestral *Tribolodon* had had the Japan Sea origin (Nishimura, 1974; Goto, 1991). The age of speciation events are discussed in chapter IX.

The anadromous *T. hakonensis* in the continental side seemed not to yield the fluvial type. If this is also true of the anadromous *T. hakonensis* in Hokkaido, the situation must be complicated.

In Hokkaido, three species of *Tribolodon* are widely distributed, but the distribution pattern in a river varied from river to river. Three patterns existed based on the distribution relationships of *T. hakonensis* and *T. ezoe*. In some rivers, only *T. ezoe* inhabited the upper reaches, in other rivers, *T. hakonensis* dwelled mono-specifically, and in the other rivers both species inhabited the upper reaches. When *T. hakonensis* was disributed to the upper reaches of the river, it might be the fluvial type, and when *T. hakonensis* was distributed only to the lower reaches, it might be the anadromous type. The reason is uncertain why these two species coexist in some cases and not in the other cases in the upper reaches of the rivers. It may not be historical but occasional according to the river environmental parameter.

Two populations of the fluvial type of *T. hakonensis* did not always make a pair in the *T. hakonensis* cluster, and rather both types of *T. hakonensis* were very closely related each other genetically (chapter VI). The fluvial type, in contrast to the anadromous type, might be able to yield the anadromous type, and the two types, when coexisting, might hybridize and introgress each other occasionally according to the environments of the river. Or, the rebirth of the fluvial type from the anadromous type of *T. hakonensis* might be repressed by the existence of many other cyprinids in the continental rivers.

IX. Synthetic discussion

The developmental intervals of three species of *Tribolodon* were divided into eight intervals, I-V for larva, VI for juvenile, VII for young, and VIII for adult (Table 17). The subtle differences in the larval intervals were seen between *T. hakonensis* and the other species. The onset of exogenous feeding in *T. brandti* and *T. ezoe* was earlier than in *T. hakonensis*, which seemed to reflect the difference in the yolk volume between them.

The greater differentiation among species was actualized in the later intervals. Anadromous *T. hakonensis* and *T. brandti* took two years for the juvenile interval VI, while fluvial *T. hakonensis* and *T. ezoe* took three years. High salinity tolerances were expressed during this interval, especially in the two anadromous species. Throughout this interval, the upper layer preference for foraging space became expressed in *T. hakonensis*, while the lower layer preference became conspicuous in *T. brandti* and T. ezoe.

The greatest differentiation among species was exhibited during interval VII. Andromous *T. hakonensis* and *T. brandti* actually run to the sea in this interval.

Table 17. Life cycle (developmental) intervals of *Tribolodon* in Hokkaido (embryonic intervals excepted in this study).

	Larval intervals I-V	Juvenile VI	Young* VII	Adult VIII
T. hakonensis				
anadromous	30–40 days	2 years	2 years go to the sea with wintering migration	several years spawning and wintering migrations
fluvial	30–40 days	3 years	0 year	several years shorter in male
T. brandti	30–40 days	2 years	3 years go to the sea with wintering migration	several years spawning and wintering migrations
T. ezoe	30–40 days	3 years	1 year	several years shorter in male
T. sp.	?	?	?	?

* : presumed shortest years are presented in the interval VII.

The wintering migration developed during this interval in the two anadromous species, at least in Hokkaido. The length of interval VII was shorter in fluvial *T. hakonensis* and *T. ezoe* than the two anadromous species. The shortest period was 0 year in fluvial *T. hakonensis*.

The adult interval VIII might continue several years in all species, though the actual length per one individual was unknown. Fluvial *T. hakonensis* and *T. ezoe* exhibited a spawning potential more than once per spawning season, which might serve to increase their fecundity under the severe environmental conditions of a fluvial life in Hokkaido.

These life-cycle are schematically illustrated in Fig. 69. The life-cycle of *T. hakonensis* involves two types, andromouns and fluvial (Fig. 69A). Although the degree of genetic interchange between the two types is ambiguous, no evidence was obtained indicative of the different species. *T. brandti* lives the anadromous mode of life exclusively (Fig. 69B), experiencing winter migration as in anadromous *T. hakonensis* (Fig. 69A). *T. ezoe* possesses the fluvial life-cycle entirely at least in Japan (Fig. 69C). The remaining species, *T.* sp., probably lives the fluvial life (Nakamura, 1969).

The relationships of the life type, foraging space preference and geographical distribution range are rearranged by species in Table 18. It is clearly expressed in the Table that *Tribolodon* spp. divide their life space poly-dimensionally with each other. Anadromous *T. hakonensis* and *T. brandti* partition their foraging space into the upper and lower layers in the sea, while fluvial *T. hakonensis* and *T. ezoe*

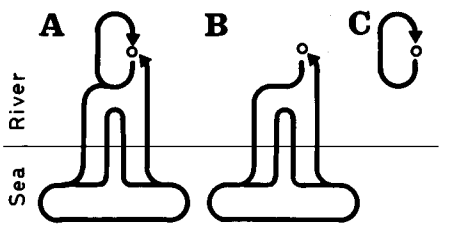

Fig. 69. Simplified life cycles of *Tribolodon hakonensis* (A, including fluvial and b anadromous cycles), *T. brandti* (B, anadromous), and *T. ezoe* and *T.* sp. (C, fluvial).

Table 18. Relationships of life type, foraging space preference and geographical distribution range in *Tribolodon*.

Foraging space preference	Life type		Goegraphical distribution range
	anadromous	fluvial	
Upper layer	anadromous *T. hakonensis*	fluvial *T. hakonensis*	full range of *Tribolodon* but fluvial type restricted to the Sakhalin-Japan side
Lower layer	*T. brandti*	*T. ezoe*	northern half of *Tribolodon* range but *T. ezoe* restricted to the Sakhalin-Japan side
Unknown (carnivorous ?)		*Tribolodon* sp.	south-most part of northern half of the Japan side

partition the space in the same manner in the river. The geographical distribution of *T. hakonensis* extends throughout the entire distribution range of *Tribolodon*, while *T. brandti* and *T. ezoe* are distributed to the northern half of the range. Moreover, the fluvial species and type are restricted to the Sakhalin-Japan side of the range, while the anadromous species and type extend to the continental side. The remaining species, *T.* sp., is unique. Its foraging space is unknown but it seems carnivorous, exceptionally in cyprinids (Kurawaka, 1992). Its distribution is narrowly restricted to the southern most part of the northern half in Japan, where it coexists with the southern most populations of *T. ezoe*.

Speciation events in *Tribolodon* may have proceeded with the partitioning of their life space as described above. However, the members of the genus did not differ in their spawning ground, and it was often observed in Hokkaido that three species of the genus, *T. hakonensis*, *T. brandti* and *T. ezoe* spawned side by side, at the same time, and on the same rapid. Most of them spawned in a spawning school of the same species, which may serve as the mechanism of premating reproductive isolation. Many fish hybridized nevertheless, especially between *T. brandti* and the other two species. However, the introgression between them would not proceed so much because of the presumed existence of the hybrid breakdown, which may act as the mechanism of postmating reproductive isolation.

The space partitioning relationships can be superimposed on the genetic relationships of *Tribolodon* spp. schematically as in Fig. 70. The speciation events of *Tribolodon* were assumed to have occurred as follows. The ancestral stock of *Tribolodon* evolved a salinity tolerance. Next, *T. hakonensis* line was branched as an upper layer forager with having two modes of life, anadromous and fluvial. Third, *T. ezoe* line was separated from the lower layer forager stock as a fluvial group. Fourth, the carnivorous species, *T.* sp., was speciated from *T. ezoe* at the southern most end of the distribution range of *T. ezoe*. At last, *T. brandti* was derived directly from the ancestral stock line as an anadromous and lower layer foraging species.

The genetic relationships investigated in this study suggest that the evolutionary rate or speed had been different among species. The founder effect and/or the bottle neck effect might affect the rate on certain lines (Nei, 1990). Therefore, the time at which each evolutionary divergence between species occurred can not be calculated using Nei's (1975) formula. Because of no other available data for

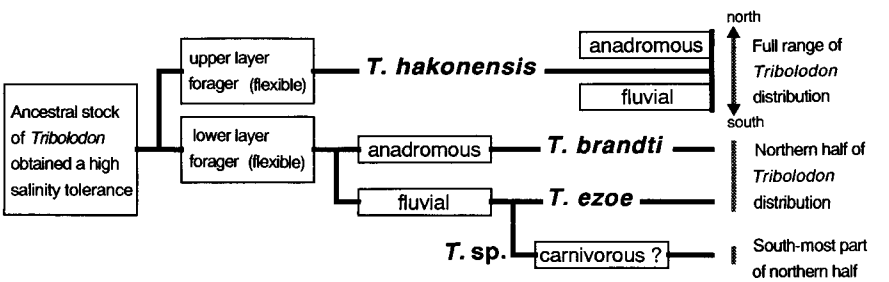

Fig. 70. Speciation events occurred along the presumed phylogeny of *Tribolodon*.

estimating the time, however, it may not be meaningless to calculate the time using the mean genetic distance among *Tribolodon* spp. The mean ND among them are 0.353 and it corresponds to about 1.8 million years ago. The ancestral stock of the genus *Tribolodon* might have been born prior to this time. Lindberg (1955) and Nishimura (1974) discussed that it had originated from the ancestral population locked in the freshwater Japan Sea Lake which had existed in the Pliocene more than 2 million years ago. Their assumption coincides well with the above estimation at first sight. However, there has been no evidence which indicated that the Japan Sea Lake was the freshwater lake in the late Pliocene, rather every evidences indicated that the Sea of Japan had been an inland sea or an inlet sea from the Pliocene to the Pleistocene, whose outlet was opened to the north (Taira, 1977;

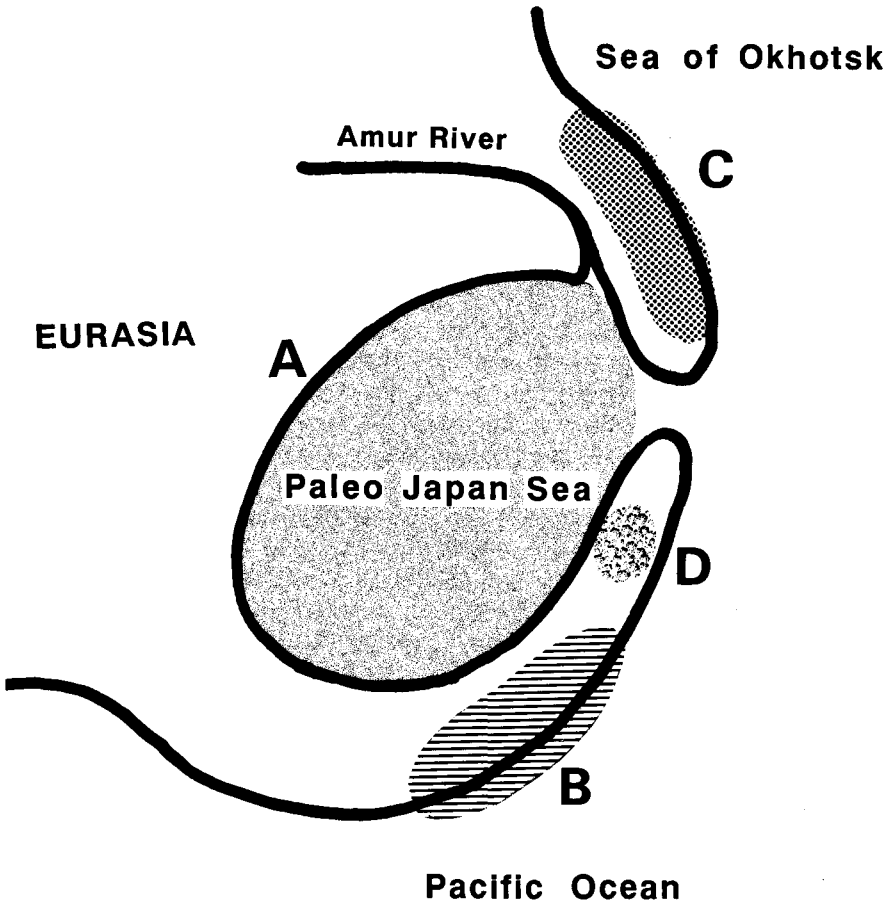

Fig. 71. Designed map of late Pliocene to early Pleistocene showing the presumed areas where the genus *Tribolodon* (A), *T. hakonensis* (B), *T. ezoe* (C), and *T.* sp. (D) originated from. *T. brandti* was presumed to derive directly from the original stock of *Tribolodon*.

Ujiie and Ichikawa, 1977).

It was well known that there arose three to four glacial epochs intermittently in the Pleistocene. In these times, at least the surface layer of the Sea of Japan was thought to become less salty (Taira, 1977; Nakajima, 1977; Goto, 1991), especially before the second latest glacial epoch (the Lith Glacial Epoch) when the river mouth of the Paleo Amur River had still opened to the north end of the Sea of Japan (Lindberg, 1955; Nishimura, 1974). Goto (1991) discussed the presence of the environment in the early glacial epoch for the ancestral *Tribolodon* to obtain salinity tolerance, where a large volume of brackish water would have existed and the cool temperature would have made the sea bio-productivity higher than the river one (Gross, 1987; Gross et al., 1988; McDowall, 1988).

Figure 71 schematically shows the areas where the genus *Tribolodon* had originated and its speciation events had occurred. The ancestral *Tribolodon* would have born in the Paleo Japan Sea where it had developed salinity tolerance. It would have spread over the Pacific coast in the following inter-glacier. The speciation event of *T. hakonensis* would have occurred at the southern Pacific side of the distribution range when the original stock of *Tribolodon* retreated to north in the next glacier, because the fluvial type was restricted to the Sakhalin-Japan side. It would have extended its range north through the repeating later interglacial and glacial epochs and at last the anadromous type of *T. hakonensis* would have reached the continental side. The speciation event of *T. ezoe* would have occurred at the north-east part of the distribution range when the original stock of *Tribolodon* moved south in the next inter-glacier, because *T. ezoe* was distributed to the northern half of the Sakhalin-Japan side. In the following glacial epoch, it would have invaded to south, and retreated to north in the following inter-glacder. At that time, *T.* sp. would have been evolved from *T. ezoe* relictly at the middle part of Japan. At last, *T. brandti* would have derived directly from the ancestral stock of *Tribolodon* as an anadromous species.

It may be possible to assume that *T. ezoe* developed in the Lith Glacial Epoch (about 0.2 million years ago) when the river mouth of the Amur River had begun to open to the Sea of Okhotsk (Lindberg, 1955; Nishimura, 1974), because if it had already existed in the Amur River earlier than that time, it might have extended its distribution to the continental side. The differentiation time could be estimated at approximately 1.3 million years from the genetic distance data between *T. ezoe* and *T brandti*. These two estimations are too different to decide which is reasonable, now. However, the mean differentiation time among *Tribolodon* spp. based on the genetic distances (1.8 million years ago) might suggest that all the speciation events of *Tribolodon* had occurred in the early Pleistocene, immediately following in the deviation of the ancestral stock of the genus.

X. Summary

In the present study, the life histories and the degree of reproductive isolation of three species, *Tribolodon hakonensis*, *T. brandti* and *T. ezoe* from Hokkaido, especially from the Mu River, were surveyed and compared. The genetic relationships and geographic distribution pattern of the four species of *Tribolodon* (includ-

ing *T.* sp.) were researched. Comprehensive speculation was made concerning the question; how and why the difference of their life histories had developed along their genetic differentiation.

The conclusions of the study are summarized as follows.

(1) The developmental intervals of three species of *Tribolodon* were divided into eight intervals, I-V for larva, VI for juvenile, VII for young, and VIII for adult.

(2) The larvae of three species of *Tribolodon* seemed to developed passing the same series of developmental intervals with each other in 30-40 days. Namely, hatched larvae migrate down into the interstices of gravels (interval I), and then actively hide themselves deep in the gravel (interval II). The larvae energe out of the gravel (interval III) when they are able to eat large food such as water fleas. As the developmental intervals proceed, the larvae acquire new feeding habits enabled by advancing swimming ability (interval IV). Finally they metamorphose (interval V) to juveniles (interval VI). The subtle difference in the larval intervals was seen between *T. hakonensis* and the other species. The beginning of exogenous feeding in *T. brandti* and *T. ezoe* was earlier than in *T. hakonensis*, which seemed to reflect the difference in the yolk volume between them.

(3) The larger differentiation among species were actualized in the later intervals. Anadromous *T. hakonensis* and *T. brandti* took two years for the juvenile interval VI, while fluvial *T. hakonensis* and *T. ezoe* took three years. The high salinity tolerance was expressed during this interval especially in the two anadrorous species. Throughout this interval, the upper layer preference for foraging space became expressed in *T. hakonensis*, while the lower layer preference became conspicuous in *T. brandti* and *T. ezoe*.

(4) The largest differentiation of life among species was exhibited from the next interval VII. Anadromous *T. hakonensis* and *T. brandti* migrated to the sea in this interval, in late spring to early summer when they were 2 years old. The wintering migration was also seen during this interval in the two anadromous species, at least in Hokkaido. The length of the interval VII was shorter in fluvial *T. hakonensis* (0 year) and *T. ezoe* (1 year) than anadromous *T. hakonensis* (2 years) and *T. brandti* (3 years).

(5) The adult interval VIII might continue several years in all species, though the actual length per individual was unknown. Fluvial *T. hakonensis* and *T. ezoe* showed a spawning potential more than once in a spawning season, which might serve to increase their fecundity under the severe environmental condition of the fluvial life in Hokkaido.

(6) Anadromous *T. hakonensis* and *T. brandti* growth patterns resulted in logistic growth curves and they grew larger than fluvial *T. hakonensis* and *T. ezoe* which developed nearly straight growth lines. The standard length (L) and age (t) relationships were represented as following equatins:

for anadromous *T. hakonensis*, $L_t=306.034/(1+e^{-0.7352832(t-3.60487)})$, $ss=114.213$
for fluvial *T. hakonensis*, $L_t=12.5964+21.4369t$, $r=0.9984$
for *T. brandti*, $L_t=347.756/(1+e^{-0.735271(t-3.86975)})$, $ss=48.8136$
for *T. ezoe*, $L_t=2.44279+22.9572t$, $r=0.9979$

(7) In the Mu River, Hokkaido, the spawning period of *T. hakonensis* was the

longest, from May through July. The next was that of *T. brandti*, lasted punctuatedly from June to July. The last and shortest was that of *T. ezoe*. The three species sometimes spawned at the same time and on the same spawning ground. At that time, *T. hakonensis* came first, *T. brandti* joined next, and *T. ezoe* spawned last, but they never mingled with several exceptional species contanimations, which might act as the pre-mating reproductive isolation mechanism. *T. brandti* spawned at the most swift and deepest part of the spawning rapid.

(8) The size of eggs ranged 2.50-3.12 (mean 2.89, SD 0.126), 2.39-2.85 (mean 2.61, SD 0.073), 2.14-2.67 (mean 2.40, SD 0.076), and 1.77-2.38 (mean 2.10, SD 0.092) mm diameter for anadromous *T. hakonensis*, fluvial *T. hakonensis*, *T. brandti* and *T. ezoe*, respectively. Relationships between number of ovarian eggs (y) and standard length (x) were as following equations:

for anadromous *T. hakonensis*, $\log y = 0.003637x + 3.222$, ($r = 0.755$)
for fluvial *T. hakonensis*, $\log y = 0.007345x + 2.277$, ($r = 0.904$)
for *T. brandti*, $\log y = 0.003401x + 3.461$, ($r = 0.648$)
for *T. ezoe*, $\log y = 0.006738x + 2.513$, ($r = 0.876$)

(9) According to the analysis of 21 loci of 12 enzymes and/or protein, two remarkable features can be mentioned. First, all species other than *T. brandti* were nearly by related to *T. brandti* at similar distances (0.245-0.304 in genetic distance, ND, and 0.421-0.464 in chord distance, CD). In other words, *T. brandti* was near to all the other species. Second, all species other than *T. hakonensis* were nearly related with each other (0.245-0.325 ND and 0.421-0.473 CD). In other words, *T. hakonensis* was distantly related to the other two species except for *T. brandti* (0.441-0.521 ND and 0.537-0.565 CD). Although the degree of genetic interchange between the two types of *T. hakonensis* is ambiguous, no evidence was obtained indicating a different species.

(10) Based on the cluster analysis, phylogenetic relationships of *Tribolodon* is presumed to be as follows. *T. hakonensis* line branched first from the ancestral *Tribolodon* stock, *T. ezoe-T.* sp. line separated next, *T. ezoe* and *T.* sp. were divided at last, and *T. brandti* was derived directly from the ancestral stock with less allozymic changes than the other species.

(11) The intense allozyme research on the Mu River revealed that more than 10% of 0+fish were hybrids. However, only a few hybrids between *T. hakonensis* and *T. ezoe* were collected. All the other hybrids were between *T. brandti* and the ther two species. Approximately 84% of the hybrids had the mtDNA haplotype of *T. brandti*, which indicated this species was the mother species of hybridization in the most cases. This result was consistent with their genetic relationships that *T. hakonensis* and *T. ezoe* were more closely related to *T. brandti* than they were related with each other. It was assumed from the field observation of spawning schools that these hybridizations might be provoked by some males of *T. hakonensis* and *T. ezoe* chasing the spawning school of *T. brandti*.

(12) A simulation analysis of hybridization suggested that the hybrid breakdown prevented the introgression among species in *Tribolodon*, which might serve as the post-mating reproductive isolation mechanism.

(13) The genus *Tribolodon* exhibits the circum-Japan Sea distribution pattern.

T. hakonensis is distributed widest in the genus covering nearly the entire *Tribolodon* distribution range, but the fluvial type are restricted to the Sakhalin-Japan side of the range. On the other hand, *T. brandti* is distributed to the northern half of the range, and *T. ezoe* is restricted to the northern half of the Sakhalin-Japan side of the range. *T.* sp. is distributed only to the southern most area of the northern half of the Japan side. This may indicate that the speciation events of fluvial *T. hakonensis*, *T. ezoe* and *T.* sp. had occurred at the Sakhalin-Japan side of the distribuion range, even if the ancestral *Tribolodon* itself had had a Japan Sea origin.

(14) The speciation events in *Tribolodon* must have accelerated with the partitioning of their life space vertically (upper layer or lower layer), horizontally (sea or river), as well as geographically.

(15) Consequently, the speciation of *Tribolodon* was assumed to have proceeded as follows. The ancestral *Tribolodon* evolved in the Paleo Japan Sea where it developed a salinity tolerance. It might have spread over the Pacific coast in the following inter-glacier. Next, *T. hakonensis* line evolved as an upper layer forager and having two modes of life, anadromous and fluvial, at the southern Pacific side of the distribution range. It might extend its range to north through the repeating later inter-glacial and glacial epochs, and at last the anadromous type of *T. hakonensis* reached the continental side. Third, *T. ezoe* line was separated from the lower layer forager stock as a fluvial group at the north-east part of the distribuion range. It might have migrated south in the following glacial epoch, and retreat north in the next inter-glacier. Fourth, when *T. ezoe* retreated north, the carnivorous species, *T.* sp., was speciated from *T. ezoe* relictly at the southern-most end of the distribution range of *T. ezoe*. At last, *T brandti* was derived directly from the ancestral stock line as an anadromous and lower layer foraging species.

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