

Genetic Distance between Populations of the Cyprinid Fish *Tribolodon hakonensis*

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Introduction

In parallel with molecular phylogeny of echinoderms, we have been studying molecular phylogeny of fishes which have rich species diversity. Until now, we reported molecular phylogeny of some fish groups by allozyme or mitochondrial DNA analysis: genus *Onchorhynchus* (Kitano *et al.*, 1997); family Hexagrammidae (Matsuoka *et al.*, 2000); order Clupeiformes (Asanuma and Matsuoka, 2002); suborder Percoidei (Matsuoka, 2003); family Osmeridae (Matsuoka and Kitano, 2003); two body color types of *Sebaster inermis* (Matsuoka and Sugiyama, 2005). However, we have not yet reported molecular phylogeny of the representative family Cyprinidae including many fresh-water fish species in Japan.

Tribolodon hakonensis is one of common species of the Cyprinidae in Japan and widely distributed from Hokkaido to Kyusyu. It is also distributed in the southern part of Korean Peninsula and Sakhalin in the Asian Continent. The species shows the remarkable variation in the morphology and ecology. For example, the specimens from river have small body size, but those from large lake such as Lake Biwa in Japan and seas show large body size. Further, the species diversifies ecologically and inhabits in the strong acidic waters (pH is about 3) of the lake of Usoriko in Shimokita Peninsula of Aomori Pref. Although it shows wide geographical distribution, genetic differentiation between different local populations of the species has not yet been examined at molecular level. We consider that such population genetic study is important and valuable to clarify the speciation of fish. As a first step of such population genetic studies, we investigated the extent of genetic differentiation between two local populations of *Tribolodon hakonensis*: one is population from large lake of Lake Biwa in the middle part of Japan and the other from river of Aomori Pref. in northern Japan. It is well known that population from Lake Biwa is characteristic morphologically when compared with other local populations. Nakamura (1969) reported that population of *T. hakonensis* from Lake Biwa shows the characteristic morphology: Generally, in many local populations of the species, the length of lower jaw is shorter than that of upper jaw. However, population from Lake Biwa has the projecting lower jaw, and thus the upper and lower jaws show the same length and the snout is sharp-pointed, which are not observed in other local populations (Fig. 1). Furthermore, population from Lake Biwa has more slender body. Therefore, it is interesting to investigate the extent of genetic differentiation between populations from Lake Biwa and other populations at molecular level. As the out-group in analysis of two populations of *T. hakonensis*, we adopted the cyprinid fish *Phoxinus lagowskii steindachneri*. It is also one of common species in Japan and widely distributed in Honshu of Japan, Korea, China, Mongolia and Siberia. In the upper stream of many rivers in Japan, the two cyprinid species are sympatric.

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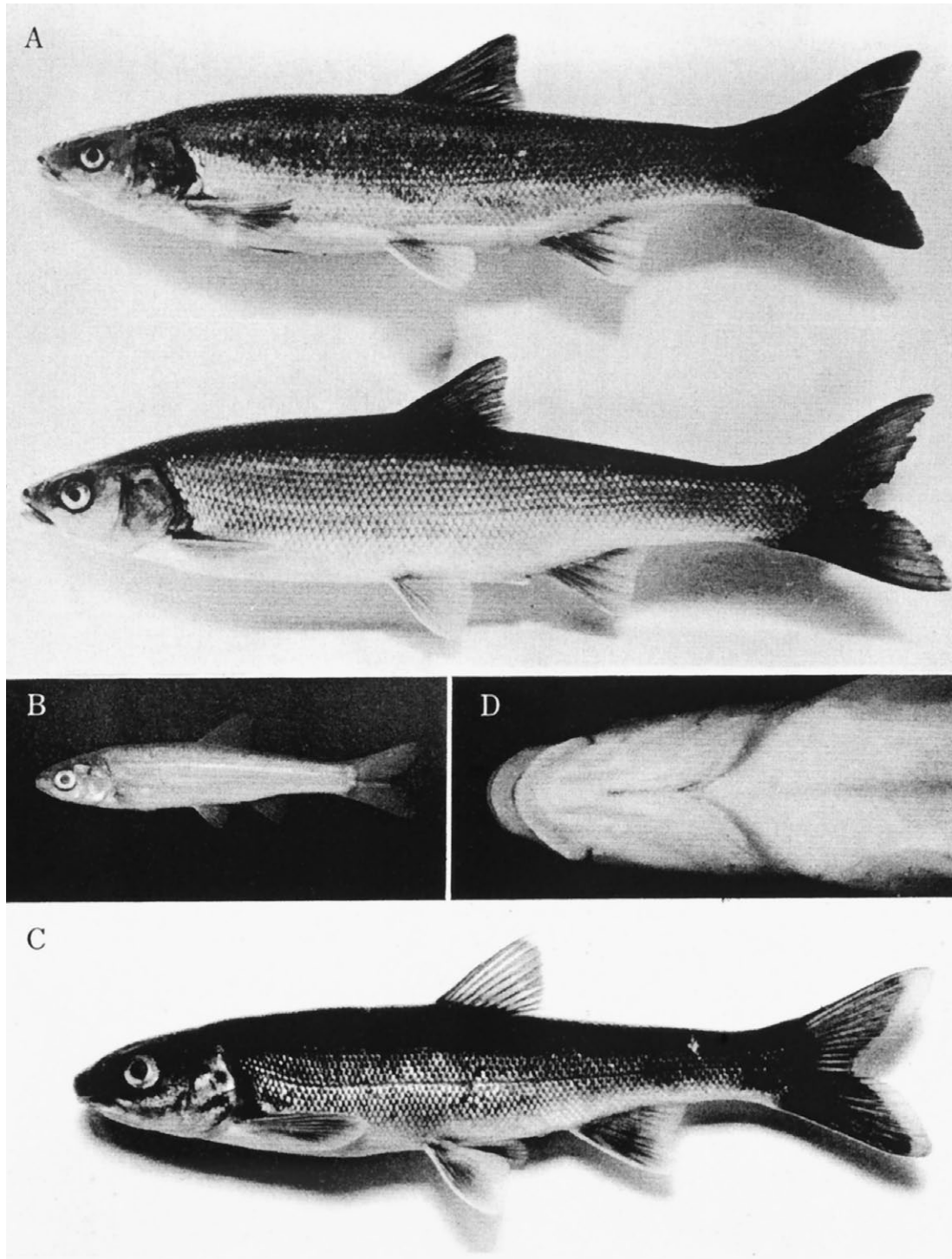


Fig. 1. *Tribolodon hakonensis* from Lake Biwa. A: Adult fish (upper is male and lower female) from Lake Biwa, B: Fry from Lake Biwa, C: Adult fish of other populations, and D: Ventral view of head of adult fish shown in C. This figure was quoted from Nakamura (1969).

In this paper, we report the genetic relationship of two local populations (from Lake Biwa and river in Aomori Pref.) of *T. hakonensis*, and that of two cyprinid fish, *T. hakonensis* and *P. lagowskii steindachneri* inferred from allozyme variation.

Materials and Methods

Tribolodon hakonensis was collected from two different localities: Lake Biwa in Shiga Pref. at the central part of Japan and the river of Hirakawa at Ohwani-machi in Aomori Pref. of northern Japan. *Phoxinus lagowskii steindachneri* of the same family Ciprinidae was collected from the river of Shimadagawa at Ohwani-machi in Aomori Pref. The number of individuals examined was 10 for population from Lake Biwa and 8 for population from Hirakawa in *T. hakonensis*, and 6 for *P. lagowskii steindachneri*. The two species were collected by fishing. After collection, the whole bodies were stored at -40°C until use. Protein electrophoresis was performed on 7.5% polyacrylamide gel as described in Matsuoka and Hatanaka (1991): The muscle was cut off from specimens, and about 0.2g muscle was individually homogenized with 3 vols. of 20mM phosphate buffer containing 0.1M KCl and 1mM EDTA (pH 7.0) by using Potter-Elvehjem type homogenizer in an ice water bath. The tissue extract was centrifuged at 10,000 rpm for 5 min and the clear supernatant was used for protein electrophoresis. Electrode buffer was Glycine-tris buffer, pH 8.3. After electrophoresis, allozyme variation of the following eight different enzymes was analyzed: alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (G6PD), lactate dehydrogenase (LDH), nothing dehydrogenase (NDH), xanthine dehydrogenase (XDH), α -glycerophosphate dehydrogenase (α -GPDH), fumarase (FUM) and superoxide dismutase (SOD). Allozyme analysis was carried out as described in Matsuoka and Hatanaka (1991).

Results and Discussion

In previous studies, we indicated that allozyme electrophoresis is much effectual method for distinction or identification of sibling species or morphologically very similar species in echinoderms. For example, the sea-urchin *Echinometra mathaei* which is common species in the seas of southern Japan is much variable in morphology and ecology, and there exist four different types (Type-A, B, C and D) within the sea-urchin from Okinawa. We investigated the problem whether these four types are distinct species by allozyme analysis. The results revealed that they are genetically differentiated to one another and four types are distinct species (Matsuoka and Hatanaka, 1991). Further, we reported the allozyme study showing that the common starfish *Asterias amurensis* from Japanese waters might consists of three genetically different species (Matsuoka and Hatanaka, 1998). More recently, we also demonstrated that the primitive sea-urchin *Asthenosoma ijimai* from Japan consists of two distinct species (one is *A. ijimai* and the other from Okinawa is *A. ijimai R.*) (Matsuoka *et al.*, 2004). As described above, we showed that allozyme analysis is much effective in distinction of sibling species.

Recently, mtDNA analysis has often been used in the field of molecular phylogeny. In general, it is considered that protein electrophoresis is not more sensitive than mtDNA analysis. However, in comparison between allozyme and mtDNA analysis, Nei (1987) suggested that the resolving power of the latter is not necessarily higher than that of the former. According to the estimation of Nei (1987), enzyme electrophoresis is expected to survey about 100 nucleotides per locus. If we examined 30 genetic loci by electrophoresis, it is equivalent to studying 3,000 base pairs at mtDNA level. Therefore, the resolving power of allozyme analysis is not lower than mtDNA analysis. For example, Nei and Roychoudhury (1993) succeeded in elucidation of evolutionary relationships of human populations by allozyme study, and the result was well consistent with mtDNA study by Cann *et al.*, (1987). Furthermore, Murphy *et al.* (1996) claimed that in phylogenetic studies many molecular characters should be used and that the enzyme loci are the important molecular characters. The number of

Table 1. Allele frequencies at six polymorphic loci in two populations of *T. hakonensis*

Locus	Allele	Th-B	Th-A
<i>Adh</i>	a	0.44	0.43
	b	0.56	0.57
<i>G6pd-2</i>	a	0.50	0.37
	b	0.50	0.63
<i>α-Gpdh-1</i>	a	0.60	0.50
	b	0.40	0.50
<i>Ldh-1</i>	a	0.78	0.50
	b	0.22	0.50
<i>Ldh-2</i>	a	0.45	0.50
	b	0.55	0.50
<i>Ndh</i>	a	0.40	0.33
	b	0.60	0.67

Th-B=population from Lake Biwa of *T. hakonensis*,

Th-A=population from the river in Aomori Pref. of *T. hakonensis*.

Table 2. Genetic identities (above diagonal) and genetic distances (below diagonal) among three populations of *T. hakonensis* and *P. lagowskii steindachneri*

Populations	1	2	3
1. Th-B	-	0.985	0.489
2. Th-A	0.015	-	0.546
3. Pls	0.715	0.606	-

Genetic identities (*I*) and genetic distances (*D*) were calculated by the method of Nei (1972). Th-B=population from Lake Biwa of *T. hakonensis*, Th-A=population from the river in Aomori Pref. of *T. hakonensis*, Pls=*P. lagowskii steindachneri*

molecular characters adopted in protein electrophoresis is more enough than that of mtDNA study. Protein electrophoresis is one of powerful techniques for estimating the phylogenetic relationships.

By allozyme analysis, we attempted to clarify the problem whether population from Lake Biwa of *T. hakanensis* (it shows characteristic external morphology as evident from Fig. 1) is genetically differentiated from other local population. The out-group was *P. lagowskii steindachneri* of the same family Cyprinidae which is sympatric with *T. hakonensis* in many areas in Japan. From allozyme variation of eight different enzymes, 12 genetic loci were scored: *Adh*, *G6pd-1~3*, *Ldh-1~2*, *Ndh*, *Xdh*, *α -Gpdh-1~2* *Fum* and *Sod*. Of these 12 genetic loci, six loci (*Adh*, *G6pd-2*, *α -Gpdh-1*, *Ldh-1~2* and *Ndh*) were polymorphic, and the remaining six loci were monomorphic. Table 1 shows the allele frequencies at six polymorphic loci of a total 12 genetic loci in two populations of *T. hakonensis*. To quantify the extent of genetic differentiation between two populations, genetic identities (*I*) and genetic distances (*D*) were calculated from allele frequencies data in 12 genetic loci by the method of Nei (1972). Table 2 shows the matrix of *I* and *D* values. As a result, the genetic identity between two local populations of *T. hakonensis* was $I=0.985$ and the genetic distance $D=0.015$. The high *I* value was comparable to *I* values observed between conspecific local populations in many other animals (Thorpe, 1982). Furthermore, as evident from Table 1, the statistically significant differences of allele frequencies between two local populations were not observed in any loci, and the diagnostic loci showing the difference of allele composition were not found. In general, sibling species or very closely related

species show the significant differences of allele frequencies in some genetic loci and have some diagnostic loci showing the different allele composition (Matsuoka and Hatanaka, 1991). The present allozyme study indicated that two local populations of *T. hakonensis* from Lake Biwa and river in Aomori Pref. were little genetically differentiated. The similar allozyme results were also obtained in two different body color types (Black-type and Redbrown-type) of the fish *Sebastes inermis* (Matsuoka and Sugiyama, 2005).

On the other hand, the average genetic identity value between two different species, *T. hakonensis* and *P. lagowskii steindachneri* was $I=0.518$. In general, the I values between confamilial different genera are in the range of $I=0.20 \sim 0.35$ in many animals (Thorpe, 1982). The present allozyme study showed that the I value ($I=0.518$) between *T. hakonensis* and *P. lagowskii steindachneri* was comparable to I values observed between different species belonging to same genus. Namely, they are at congener's level, though taxonomic system classified them into two distinct genera.

The Lake Biwa is a treasury of fresh-water fish in Japan and has rich species diversity. However, during the last about 20 years, the number of individuals of some species such as *Plecoglossus altivelis altivelis* and *Carassius auratus grandoculis* endemic to Lake Biwa (they are delicious edible fish) have extremely decreased by the introduced fish bluegill (*Lepomis macrochirus*) and blackbass (*Micropterus salmoides*) from North America which feed eggs or young fish of these endemic species ferociously. These introduced fish have strong reproductive power and ecological predominance, and thus the number of individuals (population size) is increasing drastically. As a result, the ecological system of Lake Biwa drastically changed by these introduced fish. The number of individuals of common species *T. hakonensis* has also been decreasing by the introduced fish in recent years, and thus it is more difficult to collect specimens. Under such situation, population genetic study of the species at molecular level would provide some useful information for elucidating the phylogeny or evolution of fresh-water fish.

In conclusion, the present allozyme study indicated that population from Lake Biwa of *T. hakonensis* showing characteristic morphology is little differentiated genetically from population from river in Aomori Pref. in northern Japan, and that *T. hakonensis* and *P. lagowskii steindachneri* might be classified as congeners on account of the high genetic identity verified by this allozyme study. Further, the evolutionary rate at morphological level in fish might be faster than that in other animal groups when considered the relation between genetic identity and taxonomic rank (morphological differences).

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Abstract

Tribolodon hakonensis is one of common cyprinid fish species in Japan and widely distributed in eastern Asian Continent. The local populations of the species in Japan show remarkable variation in morphological and ecological characters. In particular, population from Lake Biwa in Shiga Pref. in middle part of Japan is characteristic at morphological viewpoint: The population has the projecting lower jaw and slender body which are not observed in other local populations. We examined the extent of genetic differentiation between two local populations of *T. hakonensis* from Lake Biwa and the river in Aomori Pref. of northern Japan by allozyme analysis. The out group was *Phoxinus lagowskii*

steindachneri of the same family Cyprinidae. Twelve genetic loci were detected by electrophoresis of eight different enzymes. From allele frequencies data in 12 genetic loci scored, genetic identity (I) and genetic distance (D) were calculated by the method of Nei (1972). The allozyme study indicated the followings: (1) The genetic identity ($I=0.985$) between two local populations was much high value and comparable to I values between conspecific local populations in many other animals, but was not equivalent with the I values observed between sibling species or very closely related species. (2) The statistically significant differences of allele frequencies between two populations were not found in any loci. (3) The diagnostic loci which show the difference of allele composition were not observed. (4) The I value between *T. hakonensis* and *P. lagowskii steindachneri* was $I=0.518$ and equivalent with those between congeners in many other animals. These allozyme results demonstrated that two populations of *T. hakonensis* are little genetically differentiated, and that two cyprinid fish, *T. hakonensis* and *P. lagowskii steindachneri* might be classified as congeners at molecular level. The present study also suggested that fish might have faster evolutionary rate than other animals at morphological level, though the molecular evolutionary rate is constant.

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ウグイ地域集団間の遺伝的距離

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ウグイ (*Tribolodon hakonensis*) は、日本では北海道から九州までの河川、湖、海洋に広く分布するコイ科魚類の普通種である。本種は形態・生態学的に地理的変異が著しく、特に琵琶湖集団は他の集団と比較して形態学的に特徴的である（上顎と下顎との先端はほぼ同長で吻端が尖る、体型が細長いなど）。本研究では、このウグイ琵琶湖集団が本州最北端の青森県河川集団と、どの程度の遺伝的分化を遂げているのか？という集団遺伝学の問題をアロザイム分析により調査した。アウトグループには、河川上流部でウグイとよく混生している同じコイ科のアブラハヤ (*Phoxinus lagowskii steindachneri*) を用いた。8酵素の電気泳動によるアロザイム分析から12酵素遺伝子座が検出された。12遺伝子座における対立遺伝子頻度から、Nei (1972) の遺伝的類似度 (I) と遺伝的距離 (D) を算出した。その結果、(1) ウグイの2地域

集団間の遺伝的類似度は $I=0.985$ と極めて高く(遺伝的距離は $D=0.015$ と小さく)、(2) 2地域集団間で対立遺伝子頻度に統計的有意差を示す遺伝子座は見られず、(3) 対立遺伝子組成が全く異なるDiagnostic Lociも観察されなかった。これらの結果より、ウグイ2地域集団は形態学的には異なるが、ほとんど遺伝的分化を遂げていないことが判明した。すなわち琵琶湖集団の形態学的差異は種内変異であると推察できる。またウグイとアブラハヤは、形態レベルからの分類体系では同科別属に分類されているが、2種間の遺伝的類似度は $I=0.518$ と比較的高く、他動物の同属別種間での値と同等であった。これらの結果は、魚類は他の動物群と比較して表現型レベルの進化速度が速いことも示唆している。

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