

# Genetic relationships of char distributed in the watershed border between three river basins in the eastern Chugoku Region, Japan.

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**Abstract:** Genetic relationships among a subspecies of white-spotted char, *Salvelinus leucomaenis pluvius*, distributed in the border area of three river basins in the eastern Chugoku region of Japan (the Sendai River, flowing into the Sea of Japan, and the Yoshii and Chigusa rivers, both flowing into the Seto Inland Sea), were investigated based on mitochondrial DNA cytochrome-b gene sequences. A total of 7 haplotypes were recognized and there were 8 polymorphic sites in a 447 bp fragment. Some haplotypes were shared by fish in two adjacent basins across passes, and some were confined to restricted basins. One haplotype estimated to be the oldest could be connected to ‘Gogi’, *S. leucomaenis imbrius*. Another haplotype, estimated to have derived in the most recent period, was observed in more than half the samples. There was a significant positive correlation between the geographic and genetic distances. These results suggest that genetic distance was more strongly determined by geographic distance than by river-basin location, and may support the possibility of range expansion of char by taking advantage of geologic events.

**Keywords:** char, distribution, genetic tree, Nikkoiwana, *Salvelinus*

## I. Introduction

Two subspecies of white-spotted char, *Salvelinus leucomaenis* (Pallas) (called ‘Iwana’); *S. l. pluvius* (Hilgendorf) (called ‘Nikkoiwana’) and *S. l. imbrius* (Jordan et McGregor) (called ‘Gogi’), are distributed in the rivers originating from the Chugoku Mountains (Hosoya, 2000). Some rivers flow into the Sea of Japan, and some flow into the Seto Inland Sea. Char is usually distributed in all the rivers flowing into the Sea of Japan, whereas it is distributed only in some limited basins flowing into the Seto Inland Sea. The origin of char distributed in the rivers flowing into the Seto Inland Sea is controversial (Takeshita, 1988; Oshima, 1961). The taxonomic ranks and distribution limits of the 2 subspecies are also controversial (Oshima, 1961; Inamura & Nakamura, 1962; Imanishi, 1967; Miyaji et al., 1986; Kimura, 1989; Taki et al., 2005). Gogi is distinguishable from Nikkoiwana in possession of clear white spots on the dorsal surface reaching to the end of snout (Miyaji et al., 1986; Hosoya, 2000). Nikkoiwana is distributed in the rivers westernmost to the Hino River, Tottori Prefecture, in the Sea of Japan side, and in the rivers westernmost to the Chigusa River, Hyogo

Prefecture, or Yoshii River, Okayama Prefecture, in the Seto Inland Sea side, whereas Gogi is distributed in the rivers easternmost to the Hii River, Shimane Prefecture, or Yata River, Hyogo Prefecture, in the Sea of Japan side, and in the rivers easternmost to the Chigusa River, Hyogo Prefecture, in the Seto Inland Sea side (Imanishi, 1967; Anonymous, 1974; Kimura, 1989; Taki et al., 2005).

In this study, we focused on the Nikkoiwana populations distributed in the border area among the Sendai, Yoshii and Chigusa Rivers. The genetic relationships among the char samples were examined based on the DNA sequences in mitochondrial cytochrome *b* region, and the origin was discussed based on the haplotype distribution and topographic features in this area.

## II. Materials and Methods

### 1. Samples

Char samples were collected in the Sendai River Basin flowing into the Sea of Japan in the Tottori Prefecture and in the Yoshii and Chigusa River Basins flowing into the Seto Inland Sea, in the Okayama and

Hyogo Prefecture, respectively (Fig. 1). Three sampling sites were set in the Yoshii and Chigusa River Basins, and 11 sites set in the Sendai River basin. Char was identified to the subspecies, according to the description in Hosoya (2000).

Sampling was performed by fishing using earthworm as a main bait at as upper reaches as possible for collection of native fish only. Samples were transported to the laboratory as a live form using a potable aeration system. After killing by bleeding, samples were measured for body sizes, liver and a part of caudal fin were dissected out and stored in an Eppendorf tube at  $-20^{\circ}\text{C}$  until use.

## 2. PCR

Template DNA was prepared from the liver or fin samples using DNeasy Tissue Kit (Quiagen, Tokyo, Japan), according to the manufacturer's instruction.

The cytochrome *b* region of mitochondrial DNA was partially amplified by PCR with a mixture of a template DNA (50 ng) and primers H15915 (5'-ACCTC

CGATCTYCGGATTACAAGAC-3'; Aoyama et al., 2000) and L15285 (5'-CCCTAACCGVTTCTTYGC-3'; Inoue et al., 2000) by using the TaKaRa PCR Amplification kit (TaKaRa, Ohtsu, Japan) in a thermal cycler (Mastercycler personal; Eppendorf, Hamburg, Germany) using the following protocol: preheating at  $94^{\circ}\text{C}$  for 11 min, followed by 30 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s → annealing at  $55^{\circ}\text{C}$  for 30 s → extension at  $72^{\circ}\text{C}$  for 1 min and a final extension at  $72^{\circ}\text{C}$  for 7 min. PCR products was purified using NucleoSpin Gel and PCR cleanup (Takara, Ohtsu, Japan)

Sequencing was performed directly with the Genetic Analyzer 3130xl (Applied Biosystem, CA, USA) in the Genetic Research Center of Hiroshima University.

## 3. Dendrogram

Alignment was performed by Clustal W (Thompson et al., 1994) and genetic tree was constructed by NJ methods using Kimura-Tajima parameter as a distance by MEGA 6.

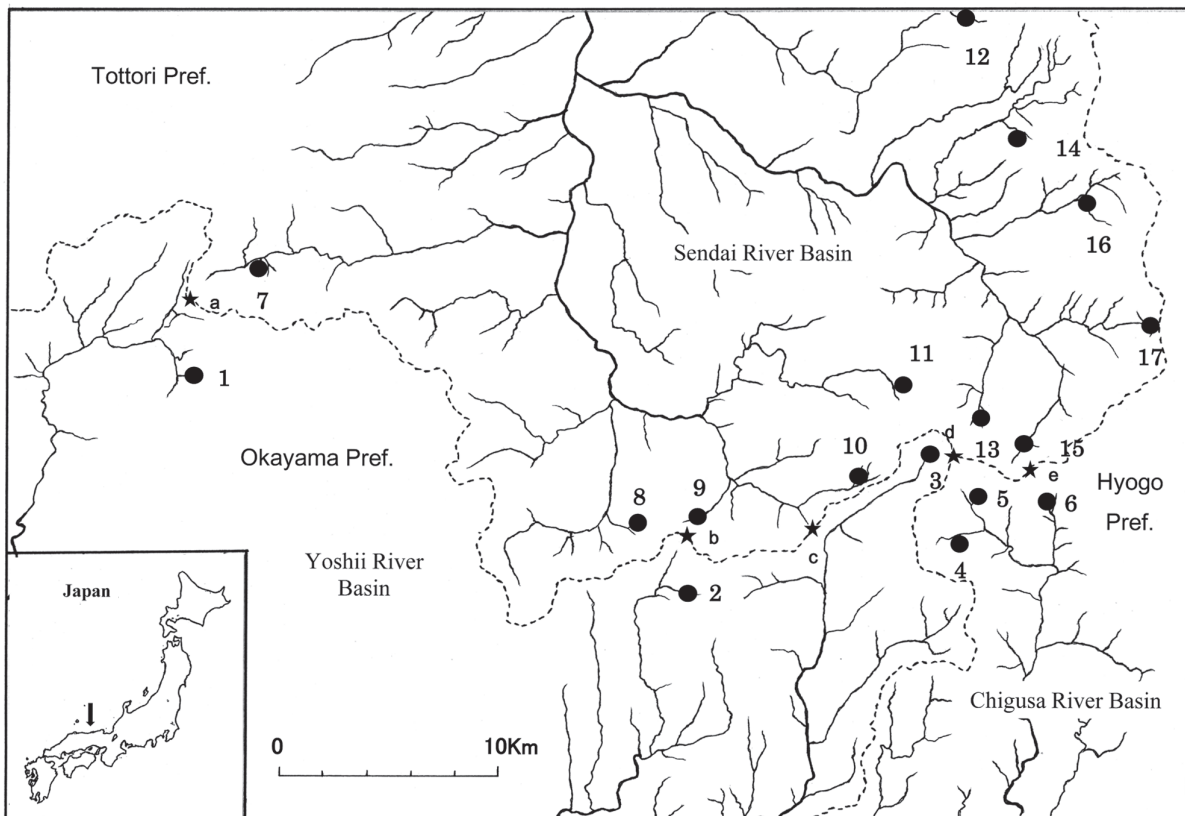


Fig. 1 Map of the border area of Sendai, Yoshii and Chigusa River basins, showing 17 sampling sites. a, Tatsumi Pass, 786m; b, Ute Pass, 620m; c, Shidosaka Pass, 454m; d, Enami Pass, 1110m; e, Odori Pass, 1031m.

### III. Results

A total of 44 samples was collected. Total and body length were in the range of 6.8-24.0 and 5.8-20.5cm, respectively. Body weight was in the range of 2.9-87.7 g.

#### 1. Haplotyping

A total of 7 haplotypes was recognized (Table 1). There were 8 polymorphic sites in 447bp fragment.

Table 1 Sequence variation in 447bp fragment of Cyt.b.

Haplotype	Nucleotide position numbers							
	-	51	111	132	159	276	318	349
1	-	C	G	G	A	A	G	G
2	G	C	A	G	A	A	A	G
3	G	C	G	G	T	A	A	G
4	G	C	G	G	A	A	G	G
5	G	C	G	G	A	G	A	G
6	G	C	G	G	A	A	A	A
7	G	A	G	A	A	A	A	G

#### 2. Genetic relationship among haplotypes

Haplotype 5 was estimated to be the oldest lineage in the tree and related to Gogi (Fig. 2). Haplotype 7 was estimated to be a middle lineage. In contrast, haplotypes 1 and 4 were estimated to be the newest.

#### 3. Haplotype composition

Haplotype 4 was the dominant, accounted for more

than 1/2, and was distributed in all the 3 basins. Haplotypes 3,5 and 7 comprised multiple samples (Table 2). Haplotypes 1, 2 and 6 comprised single sample. Haplotype 3 was distributed in the Yoshii and Sendai River Basins. Haplotypes 1 and 2 were distributed only in the Yoshii River Basin whereas Haplotypes 5, 6 and 7 were distributed only in the Sendai River Basin.

Table 2 Haplotype composition for 17 stations in 3 river basins.

River	Branch	Haplotype							Total	
		Sta.	1	2	3	4	5	6		7
Yoshii	Endo	1		1	2					3
	Kajinami	2	1			3				4
	Yoshino	3				4				4
Chigusa	Nabegatani	4				4				4
	Amagoya	5				2				2
	Kochi	6				2				2
Sendai	Saji	7		2	1				1	4
	Oya	8				3				3
	Shiratsubo	9				1				1
	Komagaeri	10				3				3
	Ashizu	11				1			2	3
	Shito	12						1		1
	Yoshikawa	13							1	1
Kurumino	14			1		1			2	
Kaji	15				1			2	3	
Tsukuyone	16				1				1	
Ochori	17					1	2		3	
Total			1	1	6	26	4	1	5	44

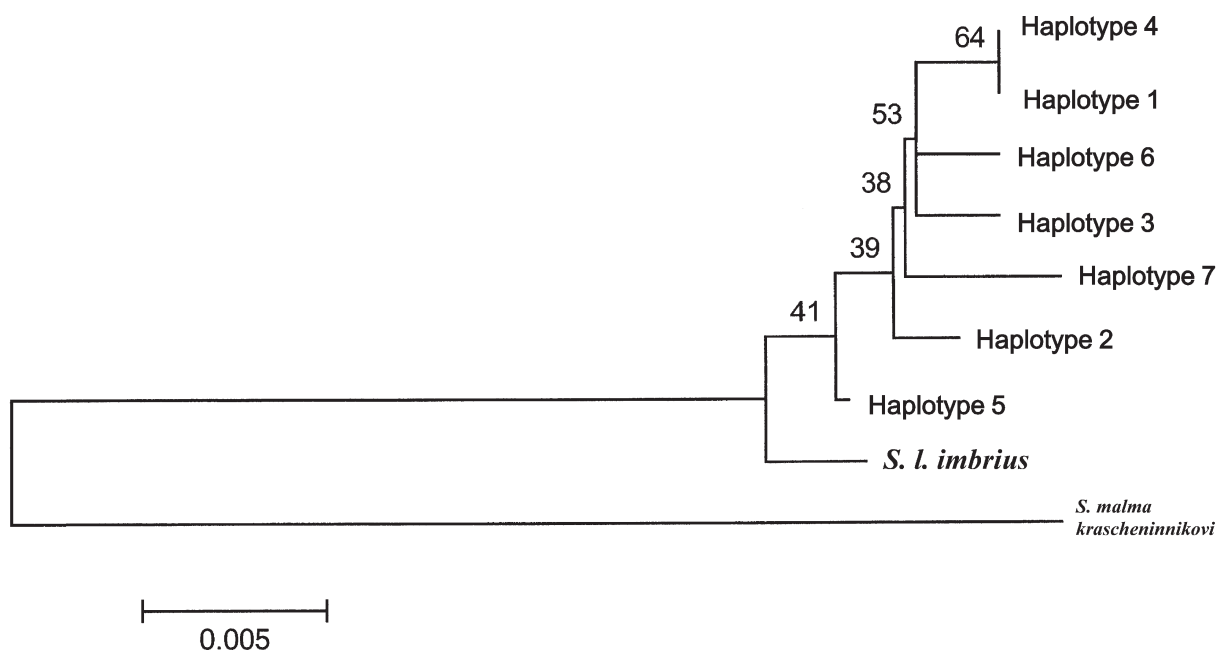


Fig.2 Genetic relationships among 7 haplotypes. *S. l. imbrius* (Takatsu River, Shimane Pref.; Hap-1 by Yamamoto et al., 2004) and *S. malma krascheninnikovi* (Churui River, Hokkaido) were used as outgroups.

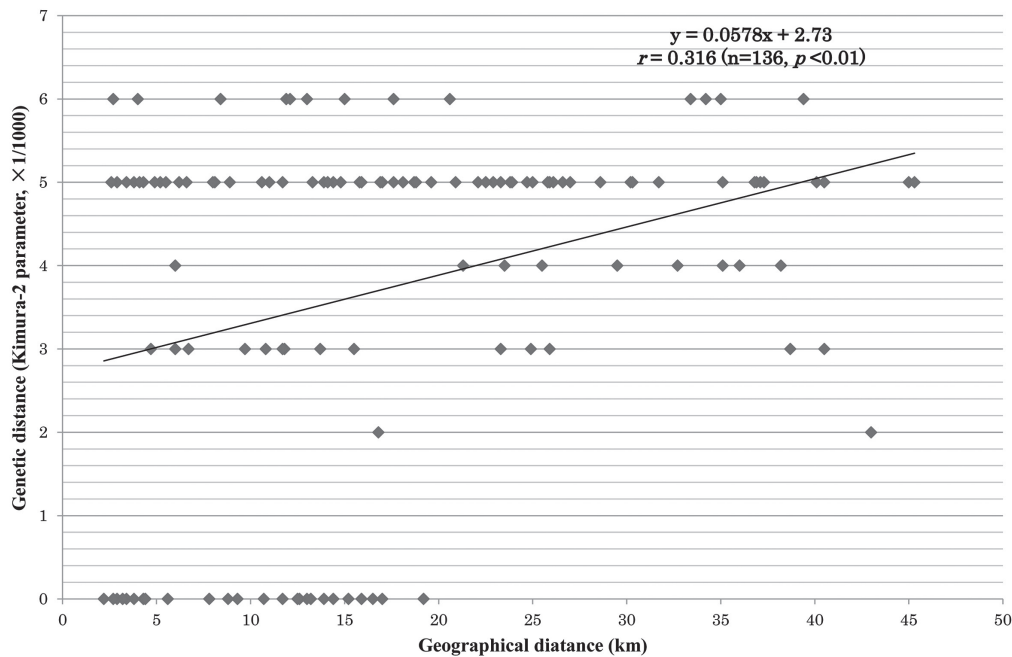


Fig.3 Relationship between geographical and genetic distances.

#### 4. Relationships between geographical and genetic distances

There was a significant positive correlation between geographical and genetic distances (Fig. 3)

#### IV. Discussion

The origin of char distributed in the rivers flowing into the Seto Inland Sea still remains unclear. In this study, genetic relationships among char samples distributed in the border area of 3 basins, the Sendai, flowing into the Sea of Japan, and the Yoshii and Chigusa Rivers, both flowing into the Seto Inland Sea, were investigated. Some haplotypes were shown to be shared by 2 adjacent basins across the loose and low passes, and some were confined to restricted basins.

Haplotype 5, distributed only in the eastern branch, the Hatto River of the Sendai River Basin, was estimated to be the oldest lineage in the tree and the most closely related to Gogi, and haplotype 4, distributed in all 3 basins and accounting for more than 1/2 of all samples, was estimated to be the newest among 7 types. On the other hand, Gogi is considered to have derived from an ancestral species at the first among 4 subspecies (Numachi, 1975). These might suggest that Nikkoiwana had derived from Gogi.

On the other hand, Yamamoto et al. (2004) have reported 29 haplotypes of Japanese white-spotted char.

Hap-1 (AB111031) was the closest to our Hap. 5 among 7 haplotypes. Hap-7 or -10; Hap-14 or -20; Hap-15 or -21; Hap-16; and Hap-17 or -19 accorded with our Hap. 3; Hap. 1 or Hap. 4; Hap. 5; Hap. 2; and Hap. 7, respectively. Besides, a haplotype, Hap. 6 was recorded firstly by this study. Haplotype 3 was shared by Sta.1 in the Yoshii River and Sta.7 in the Sendai River which face across a loose pass. Similarly, haplotype 4 was shared by Sta. 10 in the Sendai River, Sta. 3 in the Yoshi River and Sta.5 in the Chigusa River which also face across the passes. These results are compatible to those in our previous study (Kawai et al., 2006). This suggests a possibility of an invasion of char from the Sendai River to Yoshii and Chigusa River Basins, taking advantage of geologic events such as stream capture caused by fault activities or volcanic stemming, and highland marshes (Obata, 1991). Indeed, some passes across the two basins, the Sendai and Yoshii Rivers, are rather low in altitude, i.e., 454m and 620m, for the Shidozaka and Ute Passes, respectively. Besides, there are some marsh-like topography around the border area between the Yoshii and Chigusa Basins by our observation. Furthermore, there is a significant positive correlation between geographical distance and genetic distance of char in this study. These suggest that genetic distance be determined more strongly by geographical distance than by river basin, and also support some possibilities of range

expansion of char, taking advantage of various geologic events .

River capture can occur by several causes (Hattori and Tanaka, 1999). Indeed, there are some wind gap, one-side pass, elbow and mark of ancient lakes in studied areas (Imamura et al., 1984). Kikko et al. (2008) suggested that white-spotted char dispersed into the northern inlet rivers of Lake Biwa from adjacent inlet rivers of the Sea of Japan by watershed exchanges in the glacial periods of the Pleistocene. Besides, Katayama and Fujioka (1966) also suggested a possibility of invasion of Gogi from the Takatsu River Basin in the Sea of Japan side to the Nishiki River Basin in the Seto Inland Sea side taking advantage of river capture. On the other hand, Takeshita (1988) investigated the distribution of char in the Chugoku Region, and confirmed an artificial stocking of char from the Sea of Japan side to the Seto Inland Sea side in some areas. However, he stated that further study should be performed before conclusion of the origins of all the distribution in the rivers flowing into the Seto Inland side as the Sea of Japan side rivers.

In this study, char samples identified as Nikkoiwana, according to Hosoya (2000), were used for examination, although the identification basis seems to be somewhat ambiguous. On the other hand, the distribution limits of Gogi and Nikkoiwana are also still controversial (Imanishi, 1967; Anonymous, 1974; Kimura, 1989; Taki et al., 2005). However, a haplotype 4 was shown to be widely distributed in all 3 basins in this study, suggesting the distribution of Nikkoiwana in eastern Chugoku Region in both the Sea of Japan and the Seto Inland Sea sides. In our previous study, on the other hand, there was a common haplotype to these 2 subspecies in the Hino River, situated about 80km western to the Sendai River (Kawai et al., 2012). Furthermore, Hap-21 in Yamamoto et al. (2004) was shared by Gogi and Yamatoiwana, *S. leucomaenis japonicus* Oshima. Therefore, further studies should be conducted before a conclusion on the distribution limit and genetic separation level between these subspecies.

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