生物圏科学
Biosphere Sci.
50：15－23 (2011)

Estimation of geographical distribution limits between two subspecies of white-spotted charr, *Salvelinus leucomaenis imbrius* and *S. l. pluvius*, on the basis of RAPD analysis

Koichiro Kawai, Takanori Inoue, Hidetoshi Saito and Hiromichi Imabayashi

Graduate School of Biosphere Science, Hiroshima University, Kagamiyama 1-4-4, Higashi-Hiroshima, Hiroshima 739-8528, Japan

Abstract We estimated the distribution limits of the 2 subspecies of the white-spotted charr, *Salvelinus leucomaenis* ('Iwana'), *S. l. pluvius* ('Nikkoiwana') and *S. l. imbrius* ('Gogi') by examining the distribution of specific genetic types to Nikkoiwana or Gogi in the rivers flowing into the Sea of Japan on the basis of Random Amplified Polymorphic DNA (RAPD). A total of 16 DNA fragments was amplified. Seven to 14 bands were detected from an individual. There were no common bands only to the Nikkoiwana or Gogi. Fifteen and 9 haplotypes were recorded for the Nikkoiwana and Gogi, respectively. Among these, only 2 haplotypes were common to both subspecies. In the intermediate region where both the species were possible to be distributed, 24 haplotypes were detected, among which 9 and 5 types were Nikkoiwana- and Gogi-specific, respectively. Nikkoiwana-specific types were distributed in westernmost to the Hino River, Tottori Prefecture, whereas Gogi-specific types were distributed in easternmost to the Katsuta River, Tottori Prefecture. For the Hino River Basin, 17 haplotypes were detected, among which 7 and 3 types were Nikkoiwana- and Gogi-specific, respectively. In a cladogram, there were no large clades comprising only Nikkoiwana- or Gogi-specific haplotypes. These results suggest westward and eastward range expansions for the Nikkoiwana and Gogi, respectively, and the existence of Mt. Daisen Mountain Mass as a barrier to expansion of both subspecies.
Keywords: distribution, Gogi, Nikkoiwana, RAPD, *Salvelinus*

INTRODUCTION

Three subspecies of the white-spotted charr, *Salvelinus leucomaenis* (Pallas) (called 'Iwana'); *S. l. leucomaenis* (Hilgendorf) (called 'Amemasu'), *S. l. pluvius* (Hilgendorf) (called 'Nikkoiwana') and *S. l. imbrius* (Jordan et McGregor) (called 'Gogi'), are distributed in the rivers flowing into the Sea of Japan (Hosoya, 2000). The taxonomic stata of the 3 subspecies are still controversial (Oshima, 1961; Inamura & Nakamura, 1962; Imanishi, 1967; Miyaji et al., 1986; Kimura, 1989). Amemasu is distinguishable from other two subspecies in possession of large white spots along the body side (Miyaji et al., 1986; Hosoya, 2000), and Gogi is distinguishable from others in possession of clear white spots on the dorsal surface of the snout (Miyaji et al., 1986; Hosoya, 2000). However, Yamamoto et al. (2004) reported that most of genetic variance is distributed within the subspecies and each population of white-spotted charr, rather than each subspecies, must be treated as an evolutionary significant unit on the basis of mtDNA sequence analysis and that this may be the results of secondary genetic exchange via seaward migration in the glacial periods after the genetic isolation in the previous interglacial periods. Further, Yamamoto et

Accepted on September 9, 2011

al. (2004) also reported that the distributions of 4 subspecies, including *S. l. japonicus* (Oshima) (called 'Yamatoiwana'), were not in accordance with the patterns of some clades in the genetic tree.

For the Sea of Japan side, Amemasu is distributed in the rivers southernmost to the Mogami River, Yamagata Prefecture, and Nikkoiwana distributed in the rivers westernmost to the Hino River, Tottori Prefecture, whereas Gogi is distributed in the rivers easternmost to the Hii River, Shimane Prefecture (Kimura, 1989). However, the distribution limits of these subspecies are also still controversial. Imanishi (1967) estimated the eastern limit of the Gogi in the Hakuta River, Shimane Prefecture. There is a description of the Gogi-like charr in the Yata River, Hyogo Prefecture (Kobeshinbun, 1974).

In this study, we focused in the charr populations distributed in the rivers flowing into the Sea of Japan side from the Tohoku to San-in Region, divided the rivers into 3 categories, 'Nikkoiwana Region', 'Gogi Region' and the intermediate region, examined the genetic relationships on the basis of Random Amplified Polymorphic DNA (RAPD) analysis, and estimated the distribution limits of the both subspecies on the basis of haplotype compositions in the intermediate region. For this region, moreover, we examined the samples from as many branches as possible of the Hino River, due to its central existence.

MATERIALS AND METHODS

Samples

We collected charr samples in the rivers flowing into the Sea of Japan from the Omono River in the Akita Prefecture to the Takatsu River in the Shimane Prefecture. We categorized all the rivers into 3 regions: the Omono to Kitagawa River in the Fukui Prefecture ("Nikkoiwana Region"), the Hii to Takatsu River in the Shimane Prefecture ("Gogi Region") and the Maruyama River in the Hyogo Prefecture to Iinashi River in the Shimane Prefecture ("Intermediate Region") because of no refuting reports to date (Fig. 1). For the samples collected in the Nikkoiwana Region, we excluded "Amemasu" according to the description in Hosoya (2000).

We performed a sampling 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 portable aeration system. After killing by bleeding, we measured samples for body sizes, dissected the liver out and stored it in an Eppendorf tube at -20℃ until use.

RAPD-PCR

We prepared a DNA template using GenomicPrep™ Cells and Tissue DNA Isolation Kit (Amerscham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

We used 50ng of prepared DNA as a template. The sequence of an oligonucleotide primer used was 5'-GGTGCGGGAA-3' (RAPD Analysis Primer Set 01, Amerscham Biosciences, Piscataway, NJ, USA).

PCR was performed with a DNA thermal cycler (TR-100, Taitec, Tokyo, Japan) in the following conditions using Ready-To-Go RAPD analysis beads (Amerscham Biosciences, Piscataway, NJ, USA); preheated at 95℃, 1 min→ (95℃, 1 min→36℃, 1 min→72℃, 2 min) ×45cycles→ stretched at 72℃, 7 min. Electrophoresis was performed in 1.5% agarose gel at 100V for 3 hrs. After electrophoresis, gel was stained with ethidium bromide solution, bands were sequentially numbered in order of electrophoresis and band composition was compared among the samples.

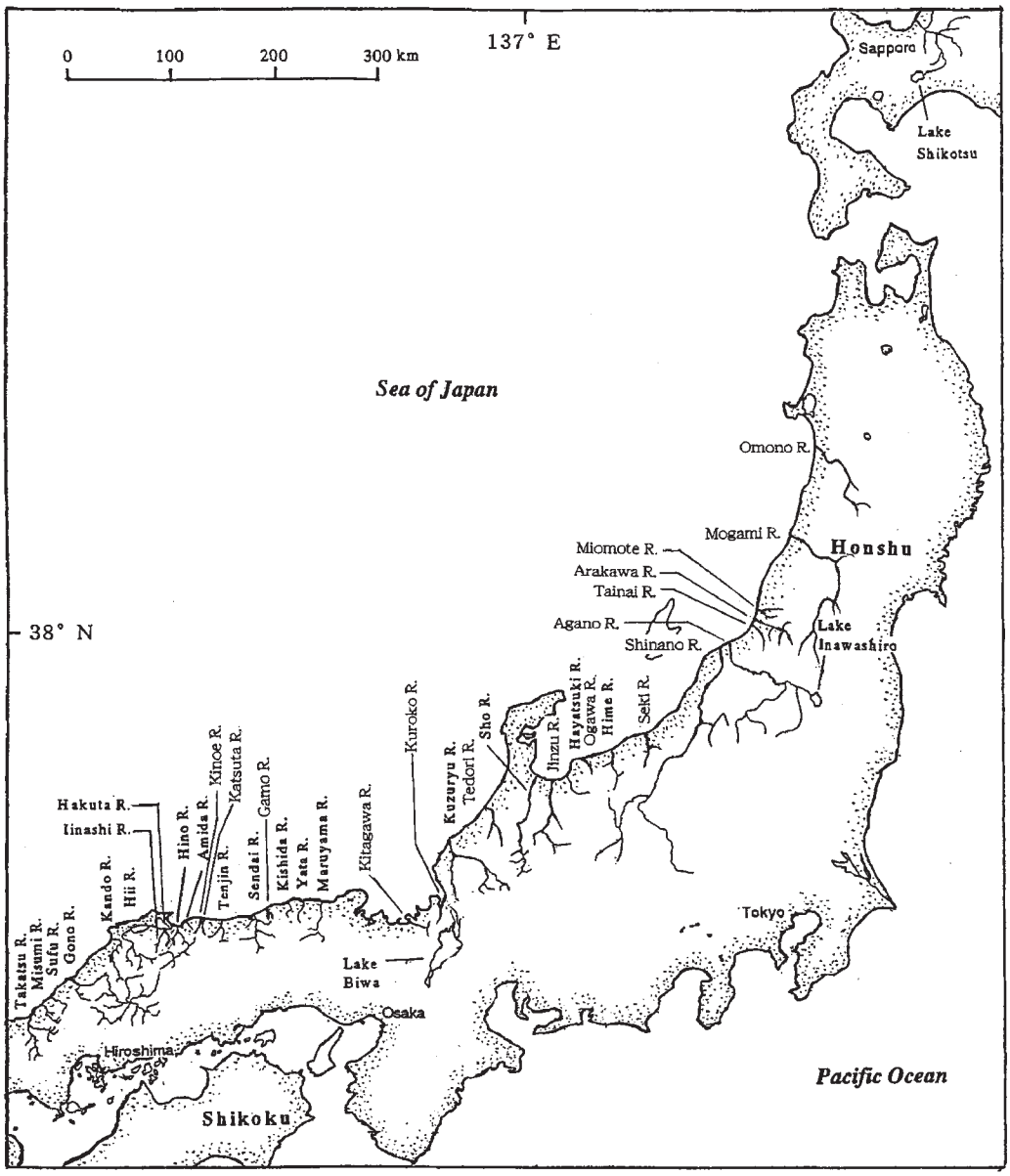

Fig. 1. Map of the 35 rivers flowing into the Sea of Japan.

Dendrogram

Genetic distance between the individuals was calculated with PAUP 4.0b (Swofford, 2000) and a dendrogram was constructed by maximum parsimony method using PAUP 4.0b.

RESULTS

A total of 135 samples was collected from 35 rivers. Total and body length was in the range of 8.8-27.8 and 7.3-23.2cm, respectively. Body weight was in the range of 5.3-187 g.

Table 1 Band compositions of 30 haplotypes.

	Haplotype.																												
Band No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1							○				○	○				○	○								○	○		○	○	
2							○				○						○									○			○	
3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
4			○						○		○		○		○	○			○										○	○
5	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
6	○	○		○	○			○	○	○	○		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
7	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
8	○				○	○	○	○	○	○		○				○	○	○	○				○			○	○	○		○
9			○	○	○	○	○	○	○		○		○		○	○	○	○	○		○	○	○			○	○	○	○	
10					○			○					○	○						○	○	○					○			
11	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○		○	○	○	○	○		○	○	○		○	○	○
12			○	○			○	○	○	○	○		○	○	○	○		○	○		○	○		○	○					○
13							○	○		○	○				○					○		○			○		○			○
14			○		○						○			○	○	○	○	○	○	○	○	○	○		○	○	○			○
15	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
16	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
No. bands	8	7	10	9	11	8	12	12	11	10	14	8	11	10	12	13	11	11	12	10	11	12	9	8	11	12	11	10	11	12

Haplotyping

A total of 16 DNA fragments was amplified and 30 haplotypes were recognized on the basis of band composition (Table 1). Seven to 14 bands were detected from an individual. Bands 3, 5, 7, 15 and 16 were common to all the individuals and bands 6 and 11 were usually detected in all the individuals. In contrast, bands 8 and 14 were detected in about a half of individuals and bands 1, 2 and 10 were detected only in a small part of individuals.

Haplotype composition

A total of 26 haplotypes was observed in all the rivers excepting the Hino River (Table 2).

Fifteen haplotypes were recorded for the Nikkoiwana Region. Among these, only one sample was collected for types 7, 10, 12, 13 and 14 whereas multiple samples were collected for other types. Nine haplotypes were recorded for the Gogi Region, among which only 2 types, 4 and 14, were common to both subspecies. Other types, 16-22 were specific to the Gogi. In the intermediate region excepting the Hino River, 13 haplotypes were detected, among which 4 (2, 9, 11 and 15) and 3 (18, 19 and 22) types were Nikkoiwana- and Gogi-specific, respectively, and types 23-26 were detected only in this region. The Nikkoiwana-specific types were distributed in the westernmost to the Amida River whereas Gogi-specific types were distributed in

Table 2 Haplotype compositions in all the rivers excepting the Hino River.

River	No.Indiv.	Haplotype
Nikkoiwana Region		
Omono	1	*1*
Mogami	2	*2*,*3*
Miomote	3	4,*5*,*5*
Arakawa	3	*1*,*1*,*6*
Tainai	3	4,4,*5*
Agano	2	*6*,*7*
Shinano	2	*3*,*8*
Sekigawa	3	4,*9*,*10*
Himekawa	2	*11*,*11*
Ogawa	3	4,*11*
Hayatsuki	1	*2*
Jinzu	1	*2*
Sho	1	*11*
Tedori	3	4,*8*,*12*
Kuzuryu	3	*2*,*9*,*13*
Kuroko	2	14,*15*
Kitagawa	1	*15*
Gogi Region		
Hii	2	**16**,**17**
Kando	3	**18**,**19**,**19**
Gonogawa	3	4,4,4
Sufu	2	**18**,**20**
Misumi	2	14,**21**
Takatsu	1	**22**
Intermediate region		
Maruyama	3	*11*,*15*,23
Yata	2	*2*,24
Kishida	3	*2*,*11*,*11*
Gamo	3	4,*11*,*11*
Sendai	2	*2*,14
Tenjin	1	4
Katsuta	3	*9*,**18**,**18**
Kinoe	3	*9*,**22**,**22**
Amida	2	*2*,*2*
Hakuta	3	24,24,25
Iinashi	3	**19**,26,26

1-3, *5-13*, *15*, Nikkoiwana-specific; **16-22**, Gogi-specific; 4, 14, common to Nikkoiwana and Gogi; 23-26, specific to intermediate region.

the easternmost to the Katsuta River. For the Hino River Basin, 17 haplotypes were detected, among which 7 (2, 5, 6, 8, 12, 13 and 15) and 3 (16, 18 and 21) types were Nikkoiwana- and Gogi-specific, respectively, and 4 (27, 28, 29 and 30) were detected only in this river basin (Fig. 2). Nikkoiwana-specific and Gogi-specific types were distributed all over. However, a common type 4 was the most dominant in the branches flowing into the main flow from the right bank whereas a Gogi-specific type 18 was the most dominant in those from the left bank.

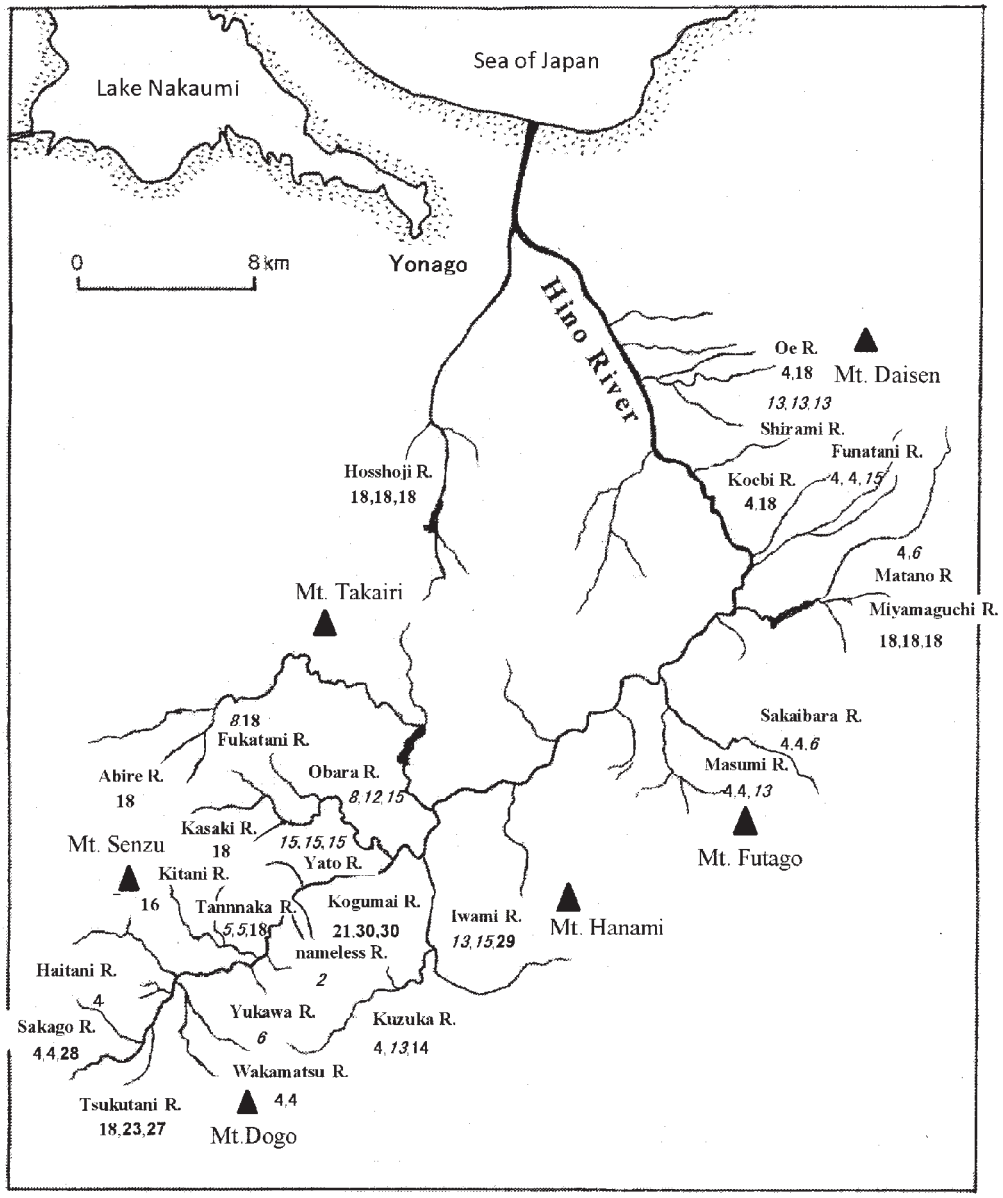

Fig. 2. Haplotype distribution in the Hino River Basin.
2, *3*, *5-13*, *15*, Nikkoiwana-specific; **16-21**, Gogi-specific; 4, 14, common to Nikkoiwana and Gogi; 23-30, specific to intermediate region.

Dendrogram

There were no large clades comprising only Nikkoiwana- specific or Gogi- specific haplotypes (Fig. 3). However, some clades comprised only Nikkoiwana-specific and common haplotypes to both subspecies, or Nikkoiwana-specific and intermediate region-specific haplotypes.

DISCUSSION

While some problems have been reported, RAPD analysis is still useful to genetic analysis of various populations or sibling species group (Miyanohara et al., 1999). In this study, distribution limits of two subspecies, Nikkoiwana and Gogi, were estimated by haplotype distribution on the basis of combination of RAPD-PCR products.

In the intermediate region, Nikkoiwana-specific haplotypes were detected westernmost to the Hino River, which is in accordance with Kimura (1989). This is also compatible to the report of Yamamoto et al. (2004) on the restricted distribution of a clade of haplotypes to the Kuzuryu (Fukui Pref.) and Tenjin (Tottori Pref.) Rivers. On the other hand, Gogi-specific haplotypes were detected easternmost to the

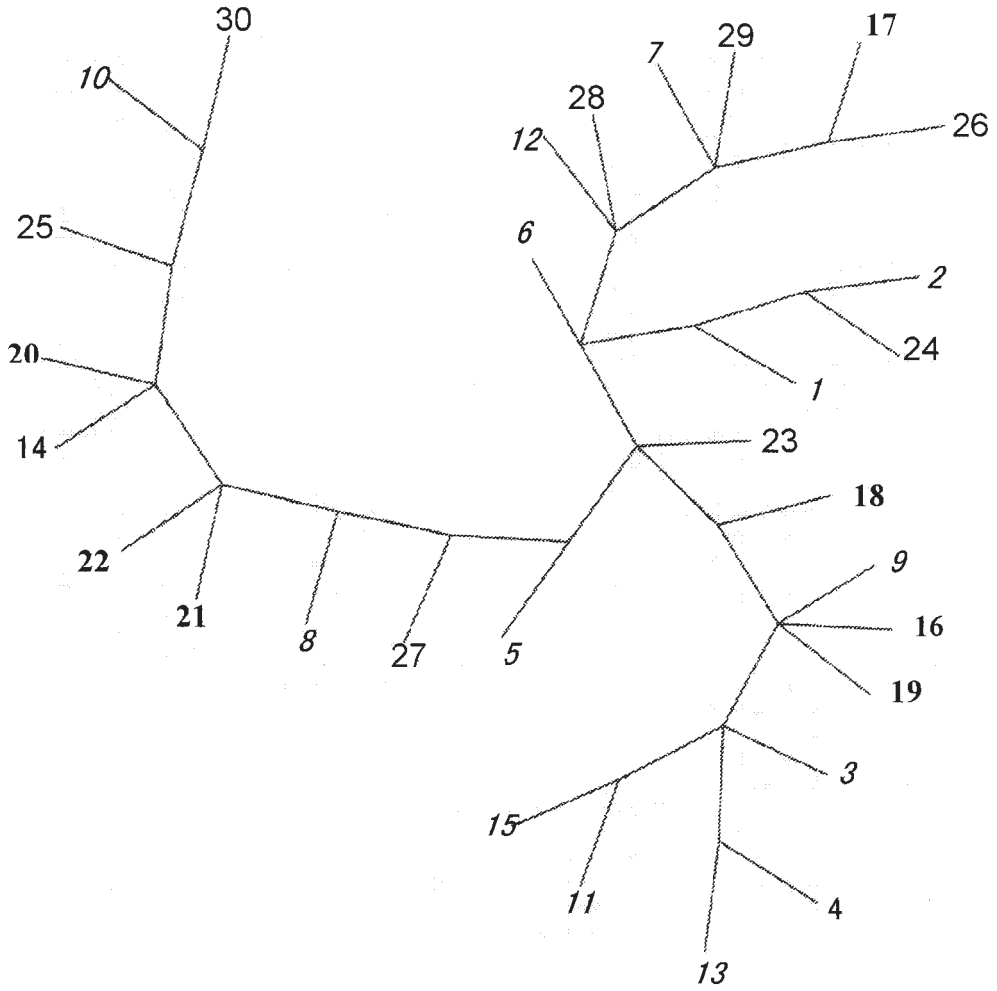

Fig. 3 A dendrogram of 30 haplotypes.

Katsuta River, which is incompatible to Kimura (1989). The discrepancy might be attributable to the difference of repertoire of investigated rivers between Kimura (1989) and the present study.

The most important results in this study is that the co-existence of Nikkoiwana and Gogi in the Hino River Basin. Besides, a common type was the most dominant in the branches flowing into the main flow from the right bank whereas a Gogi-specific type was the most dominant in those from the left bank. A strange thing is the reason why the co-existence of 2 subspecies had occurred in the Hino River Basin. Topographic factors are possible to be involved in this phenomenon. Kikko et al. (2008) suggested that white-spotted charr 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. Around the Hino River Basin, a mountain mass surrounding Mt. Daisen might have provided a barrier to the range expansion of both subspecies via topographical factors. The Daisen Volcano was estimated to have been highly active in the Pleistocene (0.02-0.9MyBp) (Kurasawa and Tsukumi, 2003). Gogi and Nikkoiwana were estimated to have been derived from an ancestral charr species in this period (Numachi, 1975). Yamamoto et al. (2004) estimated that an ancestral species to the white-spotted charr enlarged the distribution range south and westwards every glacial period via seaward migration. On the other hand, Sato (1981) reported the distribution of *Salvelinus* species in the rivers in the North Korea flowing into the Sea of Japan. In this study, furthermore, the haplotypes 20-22 (Takatsu, Misumi and Sufu Rivers in the western part of the Shimane Pref.) were estimated to be phylogenically different from the haplotypes 16, 18 and 19 (Hii and Kando Rivers in the eastern part of the Shimane Pref.) in the dendrogram. Collectively, a hypothesis might occur. Thus, the 2 subspecies might have invaded into the San-in area in the opposite directions, i.e., westward for Nikkoiwana and eastward for Gogi, like as the invasion of temperate cyprinid fishes in glacial periods from the Eurasia via continental bridges (Mizuno, 1987), respectively. Further studies on the distribution of Gogi or relatives in the Korean Peninsula should be undertaken for conclusion of this issue.

There were no clades constructed only by Gogi- or Nikkoiwana-specific haplotypes in this study. This is compatible to the results of a clade of haplotypes patchily distributed widely from the Shinano to Takatsu Rivers in Yamamoto et al. (2004). However, some clades comprised only Nikkoiwana-specific and common haplotypes to both subspecies, or Nikkoiwana-specific and intermediate region-specific haplotypes. These suggest an incomplete genetic differentiation between 2 subspecies. This is supported by the results of most of genetic variance distributed within the subspecies rather than between the subspecies in Yamamoto et al. (2004). On the other hand, the isolation level of the Gogi has been reported to be higher than that of the Nikkoiwana (Kawai et al., 2007). Further studies should be undertaken on much more charr samples from more extensive repertoire of river basins.

REFERENCES

Hosoya K (2000) Salmonidae. In “Nihonsan gyorui kensaku” Ed by Nakabo T, Tokai University Press, Tokyo, pp 256-261 (in Japanese)

Imanishi K (1967) Chars, the genus *Salvelinus*, its distribution in Japan. Shizen: 3-46 (in Japanese)

Inamura A, Nakamura M (1962) Distribution and variation of fishes in the genus *Salvelinus* in Japan. Rep Res Inst Nat Resource 58/59: 64-78 (in Japanese)

Kawai K, Imayoshi Y, Imabayashi H (2007) Differences in allele frequency at ME-1 Locus among four subspecies of Japanese white-spotted char *Salvelinus leucomaenis*. Fish Sci 73: 1398-1400

Kikko T, Kuwahara M, Iguchi K, Kurumi S, Yamamoto S, Kai Y, Nakayama K (2008) Mitochondrial DNA

population structure of the white-spotted charr (*Salvelinus leucomaenis*) in the Lake Biwa water system. Zool Sci 25: 146-153

Kimura S (1989) *Salvelinus leucomaenis* f. *imbrius*. In "Japanese freshwater fishes" Ed by Kawanabe H, Mizuno N, Yama To Keikoku-sha, Tokyo, pp128-131 (in Japanese)

Kobeshinbun (1974) River fish. In "Hyogotanken Shizen-hen" Ed by Kobeshinbun, Kobeshinbunsha, Kobe, pp 339-343 (in Japanese)

Kurasawa H, Tsukumi M (2003) Daisen Volcano. In "Physical Geography Encyclopedia New Edition" Ed by Physical Geography Encyclopedia New Edition Editorial Board, Heibonsha, Tokyo, p 747 (in Japanese)

Miyaji D, Kawanabe H, Mizuno N (1986) Genshoku nihon tansuigyorui zukan. Hoikusha, Osaka (in Japanese)

Miyanohara M, Iwatsuki Y, Sakai M (1999) Analysis of the Okinawa and Miyazaki populations of the common silver-biddy Gerres oyena using random amplified polymorphic DNA (RAPD) techniques, Fish Sci 65: 177-181

Mizuno N (1987) Establish of Japanese fresh water fish fauna. In "Japanese freshwater fish -its distribution, variation and speciation-" Ed by Mizuno N, Goto A, Tokai University Press, Tokyo, pp 232-244 (in Japanese)

Numachi K (1975) *Isozyme*. In "Suisangaku Series 9, Biochemical Classification of Fish species Ed by Japanese Society of Fisheries Science, Koseishakoseikaku, Tokyo, pp 49-64 (in Japanese)

Oshima M (1961) Studies on Japanese chars. Chojushuho 18: 3-70 (in Japanese)

Sato K (1981) *Salvelinus* in the Korean Peninsula. In "Special on freshwater fishes; *Salvelinus*" Ed by Kimura E, Association for Freshwater Fish Preservation, Osaka, pp 86-87 (in Japanese)

Swofford DL (2000) PAUP: Phylogenetic analysis using parsimony. Version 4. Sinauer Associates Sunderland

Yamamoto S, Morita K, Kitano S, Watanabe K, Koizumi I, Maekawa K, Takamura K (2004) Phylogeography of white-spotted charr (*Salvelinus leucomaenis*) inferred from mitochondrial DNA sequences. Zool Sci 21: 229-240

RAPD 分析に基くイワナ2亜種，ゴギとニッコウイワナの地理的分布境界の推定

河合幸一郎・井上貴智・斎藤英俊・今林博道

広島大学大学院生物圏科学研究科　〒739-8528　東広島市鏡山1-4-4

要　旨　RAPD 分析に基づき，日本海流入河川のイワナ2亜種，ゴギとニッコウイワナに特異的な遺伝子型の分布を調べることにより，両者の分布境界を推定した。計16断片が増幅され，1個体から7-14バンドが検出されたが，ゴギあるいはニッコウイワナにのみ特異的なバンドは見られなかった。ゴギ，ニッコウイワナ各々9，15のハプロタイプが見られ，これらのうち，2タイプのみが両亜種共通であった。両者が混棲する可能性がある中間域では24ハプロタイプが見られ，これらのうち，5，9タイプがそれぞれゴギ，ニッコウイワナ特異的であった。ゴギ特異的タイプは鳥取県勝田川が東限，ニッコウイワナ特異的タイプは鳥取県日野川が西限であった。日野川水系では17ハプロタイプが見られ，それらのうち，3，7タイプがそれぞれゴギ，ニッコウイワナ特異的であった。クラドグラムではゴギあるいはニッコウイワナ特異的タイプのみからなる大きなクレードは形成されなかった。これらは，ゴギは東方へ，ニッコウイワナは西方へ分布を広げ，その際に大山山塊が両者にとって分布拡大の障壁となったことを示唆する。

キーワード：分布，ゴギ，ニッコウイワナ，RAPD，イワナ属