論文 Article

Distribution of two subspecies of white-spotted charr, *Salvelinus leucomaenis imbrius* and *S. l. pluvius*, in the rivers flowing into the Sea of Japan, and their genetic relationships, based on mitochondrial DNA sequense

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Abstract: The distribution of 2 subspecies of white-spotted charr, *Salvelinus leucomaenis imbrius* (“Gogi”) and *S. l. pluvius* (“Nikko-iwana”), in the rivers flowing into the Sea of Japan was investigated and their genetic relationships were examined based on their cytochrome *b* sequence of mitochondrial DNA. Nikko-iwana was distributed from the Omono to Hakuta Rivers whereas Gogi was distributed from the Tenjin to Sufu Rivers. Both subspecies were co-distributed in the Hino River. Nikko-iwana was distributed in the eastern area whereas both subspecies were distributed in the western area and more than three-fourth of the samples comprised of Gogi in the left branches. Fourty six haplotypes were observed. Thirty-one and 18 haplotypes were recorded for Nikko-iwana and Gogi, respectively. Among these, haplotypes 1 and 29 were common to both subspecies. Nineteen haplotypes were recorded from the Hino River Basin, among which 11 and 10 haplotypes were recorded for Nikko-iwana and Gogi, respectively. Three types of clades, comprising only Nikko-iwana, only Gogi, and both subspecies, were observed. Multiple subspecies-specific clades were observed for both subspecies. These data suggest that both Gogi and Nikko-iwana have multiple origins, and may have tried to expand their ranges during different glacial periods. Furthermore, Gogi was estimated to have been derived and evolved from an ancestor in the stream-like environment in the peneplain-like topography of the western Chugoku Mountains.

Keywords: Charr, Genetic relationship, Gogi, Nikkoiwana, *Salvelinus*

Ⅰ. Introduction

Three subspecies of the white-spotted charr, *Salvelinus leucomaenis* (Pallas) (called “Iwana”), including *S. l. leucomaenis* (Hilgendorf) (called “Amemasu”), *S. l. pluvius* (Hilgendorf) (called “Nikko-iwana”) and *S. l. imbrius* (Jordan et McGregor) (called “Gogi”), are known to be distributed in the rivers flowing into the Sea of Japan (Hosoya, 2000). The taxonomic status of the 3 subspecies have been controversial (Oshima, 1961; Inamura & Nakamura, 1962; Imanishi, 1967; Miyaji et al., 1986; Kimura, 1989). Amemasu is rather easily distinguishable from the other 2 subspecies due to the large white spots along its body side (Miyaji et al., 1986; Hosoya, 2000), and Gogi can be distinguished from the others by clear white spots on the dorsal surface of its snout (Miyaji et al., 1986; Hosoya, 2000). However, Yamamoto et al. (2004) reported that the distributions of the 3 subspecies, and *S. l. japonicus* (Oshima) (called “Yamato-iwana”), in the rivers of the Tokai Region flowing into the Pacific Ocean were not in accordance with the patterns of certain clades in the genetic tree, when examined for by their mitochondrial DNA sequences.

With regard to the habitat adjacent to the Sea of Japan, Amemasu is distributed in the rivers southernmost to the Mogami River, Yamagata Prefecture, and Nikko-iwana is distributed in the rivers westernmost to the Hino River, Tottori Prefecture (Kimura, 1989). However, the distribution limit of Gogi remains controversial. Imanishi (1967) estimated that the eastern limit of Gogi is the Hakuta River, Shimane Prefecture. A Gogi-like charr has been described in the Yata River, Hyogo Prefecture (Anonymous, 1974). According to Kimura (1989), Gogi is distributed in the rivers easternmost to the Hii River, Shimane Prefecture, which is located western to the Hakuta River.

In the present study, we focused on the charr populations of the Hino River, Tottori Prefecture because our preliminary study on snout spot patterns suggested the co-distribution of Gogi and Nikko-iwana. Furthermore,

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Gogi populations were collected from the western rivers flowing into the Sea of Japan to the Hino River and Nikko-iwana populations were collected in the eastern rivers to the Hino River. Through the examination of the cytochrome *b* gene sequence of the mitochondrial DNA, the genetic relationships of these charr samples were analyzed. Furthermore, the distribution limits of both subspecies were estimated, and the origin of Gogi is discussed from different viewpoints.

Ⅱ. Materials and methods

1. Samples

We collected charr samples from the rivers flowing into the Sea of Japan, including the Omono River in Akita Prefecture in the north to the Sufu River in Shimane Prefecture in the south (Fig. 1). We performed sampling at the highest possible upper reaches for collection of native fish only using earthworm as a main bait. Samples were transported alive to the laboratory using a potable aeration system with gentle cooling. Samples were identified by the description in Hosoya (2000) and Amemasu samples were removed. Additionally, fish with remarkably worn fins or completely white, which are general features of cultured or introduced fish, were excluded. After killing by bleeding out, we measured the body sizes of the samples, dissected the liver and stored it in an Eppendorf tube at -20℃ until analysis.

2. DNA preparation

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

3. PCR

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'-ACCTCCGATCT YCGGATTACAAGAC-3'; Aoyama et al., 2000) and L15285 (5'-CCCTAACCGGVTTCTTYGC-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℃ for 11 min, followed by 30 cycles of denaturation at 94℃ for 30 s → annealing at 55 ℃ for 30 s → extension at 72 ℃ for 1 min and a final extension at 72℃ for 7 min.

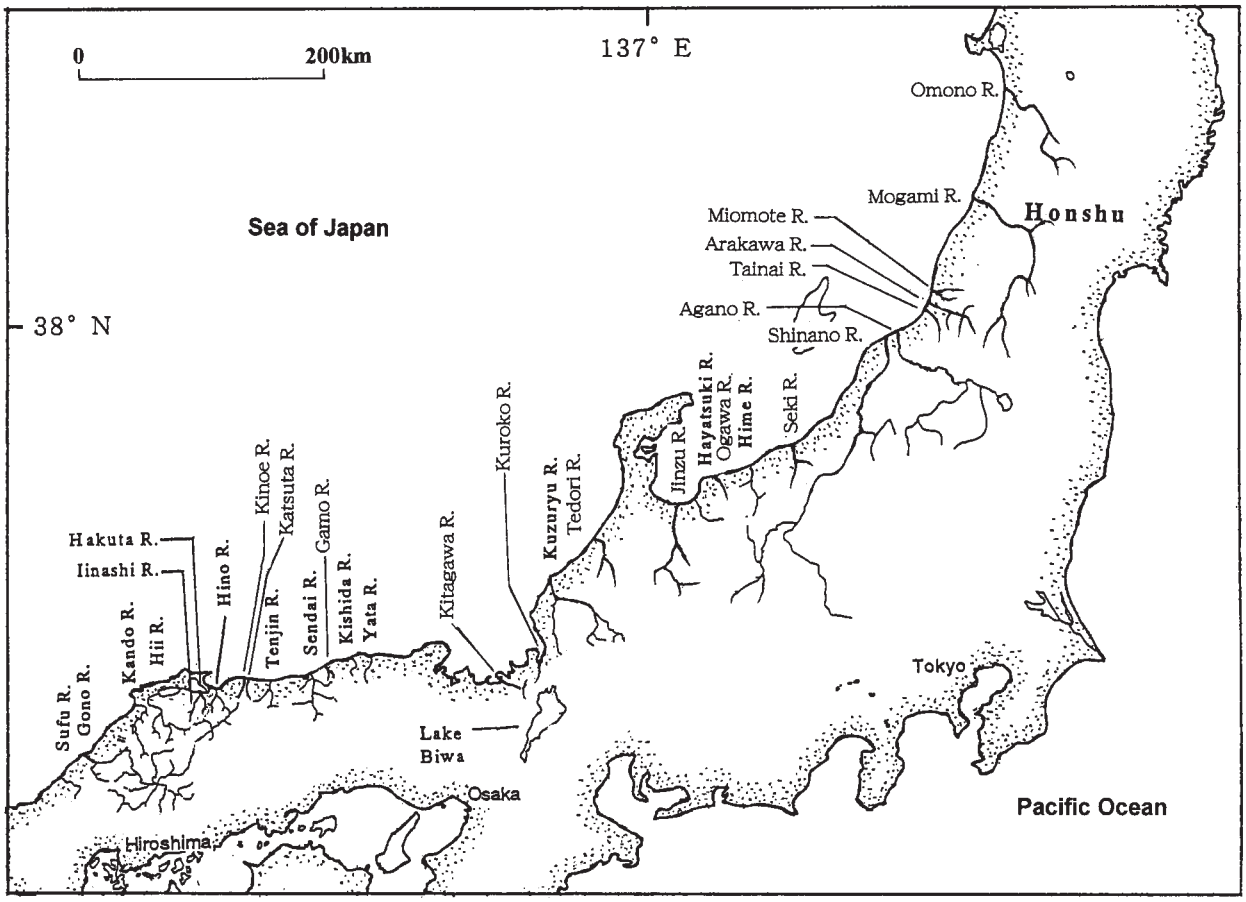

Fig. 1. Map of the 30 rivers flowing into the Sea of Japan.

4. Sequencing

Sequencing was performed directly with the Genetic Analyzer 3130xl (Applied Biosystem, CA, USA). Multiple alignment was performed with ClustalW software (Thompson et al., 1994).

5. Dendrogram

A dendrogram was constructed by the maximum parsimony method using PAUP 4.0 (Swofford, 2000).

Ⅲ. Results

A total of 109 fish samples were collected from 30 rivers. Total and body lengths ranged from 9.2-24.9 and 7.3-22.3 cm, respectively. Body weights were in the range of 6.1-134 g.

1. Distribution ranges of the 2 subspecies

Nikko-iwana was distributed from the Omono River in the Tohoku Region to the Hakuta River in the San-in Region, whereas Gogi was distributed from the Tenjin to Sufu Rivers in the San-in Region (Table 1).

Both subspecies were present in the Hino River. Only Nikko-iwana was found in the eastern branches, whereas both subspecies were found in the western branches. About three-fourth of the samples comprised of Gogi in the left branches (Fig. 2).

2. Haplotype distribution

A 568 bp region in the alignment of the mitochondrial cytochrome *b* genes was sequenced. A total of 26 nucleotide positions were polymorphic, and 46 haplotypes were identified (Table 1).

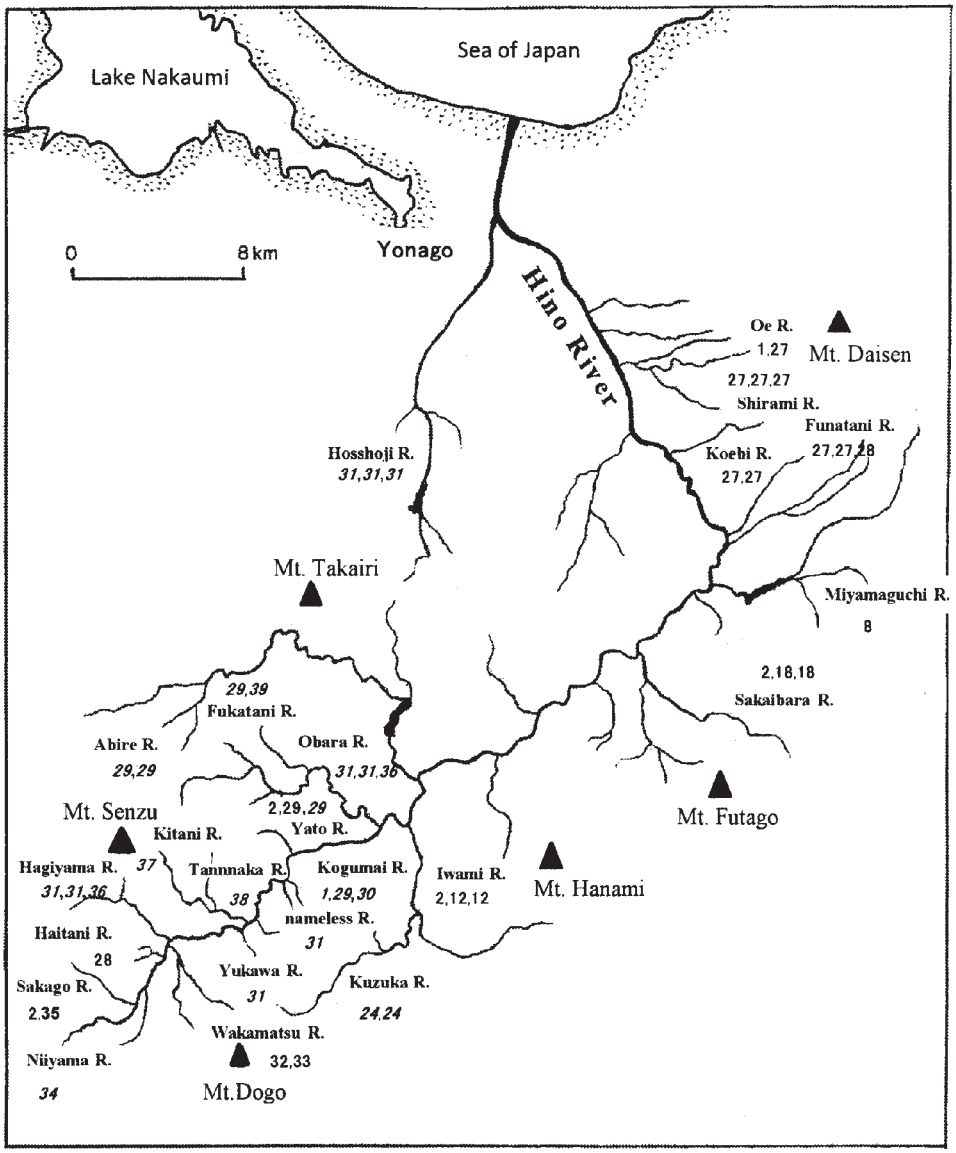

Fig. 2. Haplotype distribution in the Hino River Basin. The numbers in roman mean Nikko-iwana and those in italics mean Gogi.

Table 1 Haplotype compositions of 30 rivers. The numbers in roman mean Nikkoiwana and those in italics mean Gogi.

Haplotype	Tohoku Region		Hokuriku Region															San-in Region													
	Omono	Mogami	Miomote	Arakawa	Tainai	Agano	Shinano	Sekigawa	Himekawa	Ogawa	Hayatsuki	Zinzu	Tedori	Kuzuryu	Kuroko	Kitagawa	Yatagawa	Kishida	Gamo	Sendai	Tenjin	Katsuta	Kinoe	Hinogawa	Hakuta	Iinashi	Hiigawa	Kando	Gono	Sufu
1	1					1						1					1			1				1,*1*						
2		1		1	1	1									1									4	2					
3			1																											
4				1																										
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41																										*1*				
42																												*2*		
43																													*1*	*1*
44																													*2*	
45																													*1*	
46																														*1*

Thirty-one and 18 haplotypes were recorded for Nikko-iwana and Gogi, respectively. Among these, only haplotypes 1 and 29 were common to both subspecies. Haplotype 2 was the most widely distributed and the most dominant. Haplotypes 1 and 8 were also widely distributed. Haplotypes 29 and 31 were the second most dominant haplotypes but were distributed only in the western San-in Region.

Nineteen haplotypes were recorded in the Hino River Basin. Among these, 11 and 10 haplotypes were recorded for Nikko-iwana and Gogi, respectively. Nikko-iwana-specific haplotype 27 and Gogi-specific haplotype 31 were the most dominant, and were distributed only in the eastern and western regions, respectively (Fig. 2).

3. Dendrogram

Three types of clades were seen: clades comprising only Nikko-iwana, only Gogi and both subspecies (Fig. 3). Multiple subspecies-specific clades were observed for both Nikko-iwana and Gogi. One of the clades (haplotypes 1-24-26-27-29-39) comprised Gogi-, Nikko-iwana-specific and common haplotypes. A Nikko-iwana clade (2-3-6-11-14) comprising samples from the Tohoku to San-in Regions, was rather distantly related to another clade (4-8-9-10-15-16) comprising samples from the Hokuriku to San-in Regions. Similarly, a Gogi clade (30-31-34-36-41) comprising samples from the Hino and

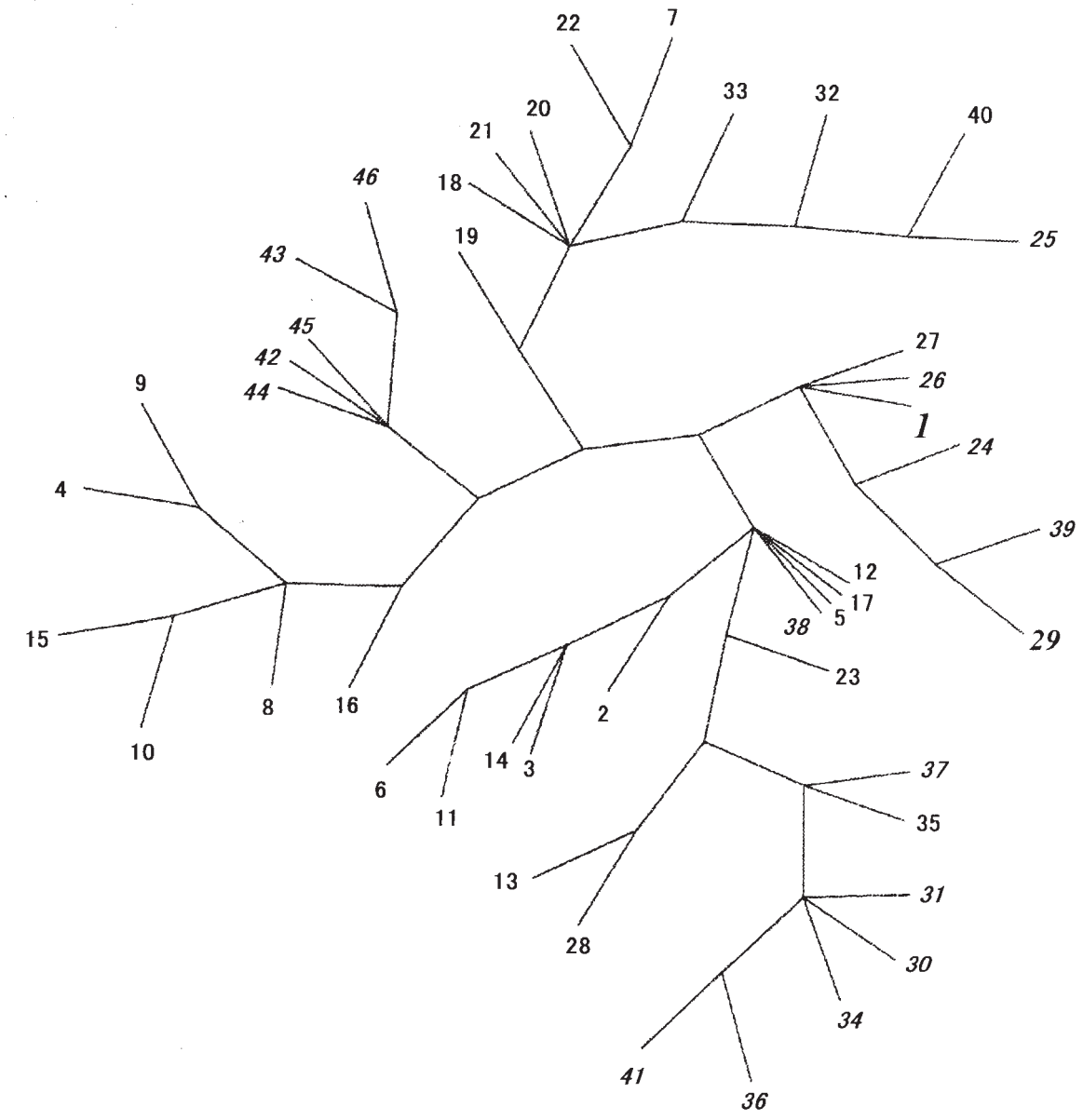

Fig. 3. A dendrogram of 46 haplotypes of Nikko-iwana and Gogi based on cytochrome *b* using PAUP. **2–23**, **27**, **28**, **32**, **33**, **35**, **40**, Nikkoiwana-specific; ***24–26***, ***30***, ***31***, ***34***, ***36–39***, ***41–46***, Gogi-specific; ***1***, ***29***, common to Nikkoiwana and Gogi.

Iinashi Rivers, was distantly related to another clade (42-43-44-45-46) comprising samples from the Kando, Gono and Sufu Rivers.

Ⅳ. Discussion

In this study, Nikkoiwana was distributed from the Omono to Hakuta Rivers, whereas Gogi was distributed from the Tenjin to Sufu Rivers. This observation is inconsistent with the findings of Imanishi (1967), Anonymous (1974) and Kimura (1989). The discrepancy may be attributable to the detailed investigation of the rivers in the present study and some ambiguity in the morphological criterion for Gogi.

Yamamoto et al. (2004) estimated that an ancestor of the white-spotted charr had extended the distribution range south- and westward during every glacial period via seaward migration. The last southward range expansion was estimated to have occurred at the end of the last glacial period (10-20 thousand years ago) (Sato, 1998). Haplotypes 1, 2 and 8 were widely distributed from the Tohoku or Hokuriku to San-in Region in this study. Thus, these haplotypes were likely to have repeatedly expanded their ranges via seaward migration. In contrast, haplotypes 29 and 31 were dominantly distributed only in the western San-in Region, suggesting the subsequent derivation of the ancestor in this region to seaward migration.

Three types of clades, comprising only Nikko-iwana, only Gogi and both subspecies, were identified in the dendrogram. Multiple subspecies-specific clades were observed for both Nikko-iwana and Gogi. Furthermore, a Nikko-iwana clade (haplotypes 2-3-6-11-14), distributed in the Tohoku to San-in Region, was rather distantly related to another clade (haplotypes 4-8-9-10-15-16), distributed in the Hokuriku to San-in Region. Similarly, 2 Gogi clades (haplotypes 30-31-34-36-41 distributed in the Hino and Iinashi Rivers, and haploytypes 42-43-44-45-46 distributed in the Kando, Gono and Sufu Rivers) were distantly related. These results suggest that both Gogi and Nikko-iwana have multiple origins, and may have tried to expand their ranges during different glacial periods.

On the other hand, the present results strongly suggest the possibility of co-existence of both subspecies from the Hakuta to the Tenjin River System in the San-in Region (70 km in straight distance). As many as 19 haplotypes were collected from the Hino River Basin, among which 11 and 10 haplotypes were recorded for Nikko-iwana and Gogi, respectively. The Nikko-iwana-specific haplotype 27 and the Gogi-specific haplotype 31 were the most dominant in the eastern and western regions, respectively. One explanation for this phenomenon is as follows. The ancestor of Gogi may have invaded the San-in Region by seaward migration during the earlier glacial periods, and Nikko-iwana ancestor may have invaded during the later glacial periods. Indeed, Gogi is estimated to have been derived the earliest among the 4 subspecies (Numachi, 1975). However, this is completely incompatible with the restricted distribution of Gogi within the Chugoku Region.

Another explanation is based on the supposed invasion of Gogi via the Korean Peninsula and subsequent eastward range expansion, similar to that of the cyprinid fishes (Mizuno, 1987) in the Pleistocene, taking advantage of highland marshes appearing during flood or stream capture events frequently occurring in peneplain-like topography of the western Chugoku Mountains (Obata, 1991). Thus, the large clear spots on the snout of the typical Gogi may be a product of adaptation to and selection in stream-like environments. In our previous study, a charr with short and wide spots on the snout was observed to favor rivers with low conductivity and riverbed gradient and high iron concentration (Kawai et al., 2000, 2004). The slightly lower P_{50} value of Gogi at pH 7.0 compared to that of Nikko-iwana, could also support the adaptation of Gogi to stream-like environments where fish is likely to be exposed to a lower oxygen concentration in the water (Kawai et al., 2003). Furthermore, the distribution of *Salvelinus* species has already been reported in Korean rivers (Sato, 1981; Jeon, 1987). However, further studies on the distribution of Gogi or relatives in the Korean Peninsula should be undertaken. Thus, generally the very steep Daisen Mountain mass, highly active in the Pleistocene (Kurasawa and Tsukumi, 2003), may have been a barrier to the non-seaward range expansion of Gogi. Indeed, haplotypes 24 and 29 were distributed in the Hino River and in the Iinashi and/or Hii Rivers facing to the Hino River across several very loose passes. Kikko et al. (2008) also suggested that white-spotted charr had dispersed into the northern inlet rivers of Lake

Biwa from adjacent inlet rivers of the Sea of Japan by watershed exchanges during the Pleistocene glacial periods. We have previously shown that some haplotypes are also shared by 3 facing rivers, the Sendai, Yoshii, and Chigusa Rivers, in the eastern Chugoku Region (Kawai et al., 2006). However, Gogi-like charr have also been reported to be distributed in the rivers of the Abukuma Peneplain (Sato, 1998), although the clear spots on the snout of the samples from the rivers were much longer (similar to those of the mackerel) than those of Gogi and easily distinguishable in our preliminary study.

Yet another explanation is the hypothesis of multiple origins of Gogi. Gogi may comprise a group of Nikko-iwana that expanded seaward and were selected for fitness in the stream-like environments of the western Chugoku Mountains resulting in clear, short, and wide spots on the snout, and one of the above-mentioned eastward-expanded populations. Indeed, at least 2 distantly related Gogi-specific clades were revealed in this study. Furthermore, 2 haplotypes (1 and 29) common to both subspecies were observed.

In this study, the research field was confined to the rivers flowing into the Sea of Japan due to the uncertain origins of the charr distributed in the rivers flowing into the region adjacent to the Pacific Ocean, particularly that adjacent to the Seto Inland Sea (Takeshita, 1988). Additionally, sampling was performed by fishing at the highest possible upper reaches for collection of native fish only; fish with remarkably worn fins or completely white berry, which are general features of cultured or introduced fish, were excluded. However, further exclusion of introduced fish, for instance by hearing at each sampling spot, should be performed for a more significant analysis.

【References】

Anonymous (1974): River fish. In: Kobeshinbun (ed.). *Hyogotanken Shizen-hen*, Kobeshinbunsha, Kobe, 339-343 (in Japanese).

Aoyama, J., Watanabe, S., Ishikawa, S., Nishida, M. and Tsukamoto, K. (2000): Are morphological characters distinctive enough to discriminate between two species of freshwater eels, *Anguilla selebesensis* and *A. interioris*. *Ichthyol. Res*., 47: 157-161.

Hosoya, K. (2000): Salmonidae. In: Nakabo T (ed.). *Nihonsan gyorui kensaku*, Tokai University Press, Tokyo, 256-261 (in Japanese).

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

Inamura, A. and 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)

Inoue, J. G., Miya, M., Tsukamoto, K. and Nishida, M. (2000): Complete mitochondrial DNA sequence of the Japaenese sardine *Sardinops melanostictus. Fish. Sci*., 66: 924-932.

Jeon, S. R. (1987): Freshwater fishes in Korea. In: Mizuno N, Goto A (eds.). *Japanese freshwater fish −its distribution, variation and speciation−*, Tokai University Press, Tokyo, 199-213 (in Japanese).

Kawai, K., Masuhara, A. and Imabayashi, H. (2000): Relationships between the spot patterns on the head of the Japanese char *Salvelinus leucomaenis*, distributed in the Chugoku Region, and water quality. *Fish. Sci*., 66: 901-907.

Kawai, K., Motozaki, S., Oikawa, S., Takeda, T. and Imabayashi, H. (2003): Comparison of affinity of total Hb to O_2 between 2 subspecies of Japanese char, Gogi, *Salvelinus leucomaenis imbrius* and Nikkoiwana, *S. l. pluvius*. *J. Grad. Sch. Biosp. Sci. Hiroshima Univ*., 42: 1-5.

Kawai, K., Masuhara, A. and Imabayashi, H. (2004): Relationships between the spot patterns on the head of the Japanese char, *Salvelinus leucomaenis*, in the Chugoku Region, and altitude or riverbed gradient. *Environ. Biol. Fish*., 70: 331-337.

Kawai, K., Katayama, T. and Imabayashi, H. (2006): Genetic relationships between chars distributed around the watershed borders in the eastern Chugoku Mountains, Japan, on the basis of RAPD analysis. *J. Grad. Sch. Biosp. Sci., Hiroshima Univ*., 45: 1-8.

Kikko, T., Kuwahara, M., Iguchi, K., Kurumi, S., Yamamoto, S., Kai, Y. and 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: Kawanabe H, Mizuno N (ed.). *Japanese freshwater fishes*,Yama To Keikoku-sha, Tokyo,128-131 (in Japanese)

Kurasawa, H., Tsukumi, M. (2003): Daisen Volcano. In: Physical Geography Encyclopedia New Edition Editorial Board (ed.). *Physical Geography Encyclopedia New Edition*, Heibonsha, Tokyo, 747 (in Japanese).

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

Mizuno, N. (1987): Establish of Japanese fresh water fish fauna. In: Mizuno N, Goto A (eds). *Japanese freshwater fish −its distribution, variation and speciation−*, Tokai University Press, Tokyo, 232-244 (in Japanese).

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

Obata, H. (1991): *Geographical features of the Chugoku Region*. Kokin Shoin, Tokyo (in Japanese).

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

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

Sato, S. (1998): *Setogiwa no Sakanatachi*, Tsuribitosha, Tokyo (in Japanese).

Swofford, D. L. (2000): *PAUP: Phylogenetic analysis using parsimony*. Version 4. Sinauer Associates Sunderland.

Takeshita, K. (1988): Distribution of the genus *Salvelinus* in the Chugoku Region. *Hiroshimadaigaku Gakkokyouiku Kenkyuka Shushironbunsho* 7: 123-126 (in Japanese).

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994): CLUSTAL W: improving the Sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucreic Acids Res*., 22: 4673-4680.

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

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