

## Molecular phylogeny of the white-spotted charr, *Salvelinus leucomaenis* from Japan and the position of the *S. l. japonicus* morphotype (nagaremon charr), demonstrating teleost diversification in an archipelago

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**ABSTRACT.** Four subspecies of white-spotted charr (*Salvelinus leucomaenis*) are known from the Japanese archipelago; *S. l. imbrius*, *S. l. japonicus*, *S. l. leucomaenis*, and *S. l. phivius*. All four subspecies are popular as fisheries resources as well as targets of recreational sports fishing. Japanese *S. leucomaenis* showcase a unique example of diversification of teleosts inhabiting mountainous streams in a limited geographic range such as an archipelago in the Far East, worthy of detailed study. The Japanese endemic subspecies *S. l. japonicus* are further divided into two morphotypes, a typical type and a morphotype known as the Nagaremon char. We extracted

complete mtDNA sequences of the four Japanese *S. leucomaenis* subspecies as well as that of the *S. l. japonicus* nagaremon morphotype. The mtDNA sequence of *S. l. leucomaenis* was 16,655 bp in length, whereas the typical type of *S. l. japonicus* had a sequence length of 16,654 bp and the nagaremon morphotype had 16,653 bp. There were one or two nucleotide deletions in the D-loop region of *S. l. japonicus* compared with the other *S. leucomaenis* subspecies. A phylogenetic tree showed that the typical morphotype of *S. l. japonicus* was the most basal subspecies, followed by *S. l. pluvius*, *S. l. imbrius* and the nagaremon morphotype, which formed a clade followed by *S. l. leucomaenis*. Comparison of whole mtDNA sequences among the four subspecies of *S. leucomaenis* revealed that the *S. l. japonicus* morphotype was most closely related to *S. l. imbrius*.

**Key words:** Mitochondrial DNA; *Salvelinus leucomaenis*; Nagaremon charr

## INTRODUCTION

The white-spotted charr, *Salvelinus leucomaenis* is widely distributed in eastern Asia. This salmonid is adapted to cold water habitats; in mid latitude regions its distribution is restricted to alpine streams at high altitudes (Nakano et al., 1996). However, at high latitudes, this species ranges from the headwaters to the mouth of rivers, often appearing in coastal waters, with some populations known to be anadromous. Ecological diversity of this group is considerable (Behnke, 1980; Fausch et al., 1994). In the Japanese Archipelago, the diversity of this group is high compared to other geographic regions. *S. leucomaenis* inhabiting Japan has been classified into four subspecies; Amemasu charr (*S. l. leucomaenis*), Yamato charr (*S. l. japonicus*), Nikko charr (*S. l. pluvius*) and Gogi charr (*S. l. imbrius*), based on morphological characters and zoogeographic patterns (Hosoya, 2013). All four subspecies are popular in Japan as food fish and also as target of recreational sports fishing such as fly fishing. *S.l.leucomaenis* and *S.l.pluvius* are cultured for human consumption in numerous mountainous regions of Japan. *S. l. japonicus* and *S.l.imbrius* are very difficult to culture and are traded at high prices. Except for *S. l. leucomaenis*, all three subspecies are endemic to Japan. The distribution of three subspecies, *S. l. imbrius*, *S. l. japonicus* and southern populations of *S. l. pluvius* are restricted to the high mountainous regions of Honshu Island and these three subspecies are landlocked (Nakano et al., 1996; Ymamoto et al., 2000; Shimoda et al., 2002). *S. l. imbrius* is endangered and classified as class II (VU) , and the southernmost population of *S. l. japonicus* is designated as a threatened local population (LP) by the Ministry of the Environment, Japan and conservation measures are taken (Red List 2019 of Ministry of the Environment). Populations of *S. l. leucomaenis* are distributed at the northern extent of the *S. leucomaenis* range, from Sakhalin Island and Kamchatka Peninsula, Russia, Hokkaido Island, to the northern region of Honshu Island, Japan. *S. l. leucomaenis* have white spots on the dorsal and ventral sides of the body and lack colored spots.

*S. l. japonicus* is distributed in the rivers of Honshu Island that drain into the Pacific Ocean. *S. l. japonicus* either lack or have very few white spots on the dorsal region and

yellow or orange spots are scattered on the ventral side of the body. A morphotype of *S.l.japonicus*, known as Nagaremon charr has a weak dark colored marble pattern on its ventral side (Figure 1), and is known from only a small number of rivers flowing into Lake Biwa (Takeda, 1975; Magnann et al., 2002; Kikko et al., 2007).

*S. l. pluvius* is distributed in the Pacific coast of Honshu east of Fuji River System in Yamanashi Prefecture and along the Sea of Japan east of the Hino River System in Tottori Prefecture.

The white spots on the dorsal region of *S. l. pluvius* are rather small, and do not cover the anterior half of the dorso-cephalic region. Yellow or orange spots are scattered on the ventral side of the body. Among the four subspecies, *S. l. imbrius* exhibit the most western distribution in Honshu, from Yoshii River water system in Okayama Prefecture and the Hii River water system in Tottori Prefecture. There are yellow or orange spots scattered on the ventral side of *S. l. imbrius*. Large white-spots are present on the dorsal region, which cover the dorso-cephalic region from the snout. The high degree of diversification of the four subspecies of *S. l. leucomaenis* in Japan provide a unique example of evolution of teleosts inhabiting mountainous streams. Molecular phylogenetics using only a limited region of mitochondrial DNA (mtDNA) and cytochrome b 557bp/1141bp have been conducted to examine evolutionary processes in the *S. leucomaenis* group (Yamamoto et al., 2004; Kikko et al., 2008). However, for a more detailed comparison, a complete mtDNA sequence is essential and should provide valuable information for conservation of this highly diverse and unique group of Salmonids. We previously identified the complete mtDNA sequences of two subspecies, *S. l. imbrius* and *S. l. pluvius*. In this study, the complete mtDNA sequences of *S. l. leucomaenis*, typical and morphotypes of *S. l. japonicus* were determined and compared with those of the four subspecies.

## MATERIAL AND METHODS

### Sample collection

Specimens of each subspecies of *S. leucomaenis* were collected by fly fishing or with an electric shocker. *S. l. leucomaenis* specimen was collected in Aomori Prefecture, northern Honshu in the upstream region of the Akaishi River system (40.759577° N, 140.157189° E), which flows out to Sea of Japan. Typical form of *S. l. japonicus* specimen (Figure 1) was collected in Nagano Prefecture, central Honshu, upstream of the Tenryu River system (34.646607° N, 137.801287° E) which flows out to the Pacific Ocean in Shizuoka Prefecture, central Honshu. The morphotype of this subspecies (Nagaremon charr) (Figure 1) was collected in Shiga Prefecture, central Honshu, from the upstream region of the Ane River system (35.387642° N, 136.215062° E), which flows to Lake Biwa and subsequently flows out to the Pacific Ocean via Osaka Bay. *S. l. pluvius* specimen was collected in Fukui Prefecture, north central Honshu upstream in the Kuzuryu River system (36.219987° N, 136.132300° E) flowing out to the Sea of Japan. *S. l. imbrius* specimen was collected from Shimane Prefecture, north western Honshu, upstream of the Takatsu River system (34.695647° N, 131.819614° E) which flows out to Sea of Japan.

All the specimens from Japan are landlocked forms. Specimens of all four subspecies obtained are of natural origin and were caught from parts of each river system

where no known artificial restocking of *S. leucomaenis* took place in the past. All samples were preserved frozen until DNA extraction.



**Figure 1.** Morphotype of *Salvelinus leucomaenis japonicus* (nagaremon charr). This morphotype of *S. l. japonicus* has a weak dark colored marble pattern on its ventral side and is called nagaremon iwana in Japanese. This specimen was 18 cm in length. This species grows to an average length of 14.6 to 21.9 cm (Kikko et al., 2007).

### Sequencing of mtDNA

The mtDNA was extracted by phenol/chloroform methods, and four primer sets for its amplification. The primer set for amplification of mtDNA was designed using forward primer 1: 5'-CAA CTG ATA TCC GCC AGG GAA C-3' and reverse primer 1: 5'-GGT TGA GAA AAA TCA TCG TGT G-3', forward primer 2: 5'-ACT TCC ACT ACC TGC CAC ACT AG-3' and reverse primer 2: 5'-CCG TGC CAT GAG TTC GTG-3', forward primer 3: 5'-CAT TGT CCG CAG TAC TAG CCA C-3' and reverse primer 3: 5'-CAG TGG CTC CTC AGA AGG-3', forward primer 4: 5'-GAC TTG ACC TTA CAC TCC CAC-3' and reverse primer 4: 5'-GGG GCT GAA CTT CTA TTC ATT TCC-3'. After PCR with KAPA HiFi HS DNA polymerase (NIPPON Genetics), the PCR products were purified using FastGene Gel/PCR Extraction Kit (NIPPON Genetics) and direct sequenced using a BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems) with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

### Phylogenetic analysis

The phylogenetics of two types of *S. l. japonicus* (a typical specimen and a morphotype) were compared with the other three subspecies of *S. leucomaenis*. Multiple mtDNA sequence comparisons were performed to evaluate the levels of similarity and difference between nucleotides using MEGA software (version 7).

A phylogenetic tree was obtained using the maximum likelihood method with 1000 bootstrap simulations using the MEGA software (Hall, 2013).

### RESULTS

The whole mtDNA sequence of *S. l. leucomaenis* was 16,655 bp in length. The sequence was submitted to GenBank (Access No. LC516603). The typical *S. l. japonicus* specimen had a mtDNA sequence length of 16,654 bp (submitted to GenBank, Access No. LC513886), and the morphotype (Nagaremon charr) had a sequence length of 16,653 bp (submitted to GenBank, Access No. LC516602). The subspecies *S. l. imbrius* (LC424298)

and *S. l. pluvius* (LC424299) also had mtDNA sequence lengths of 16,655 bp (Arai et al., 2019).

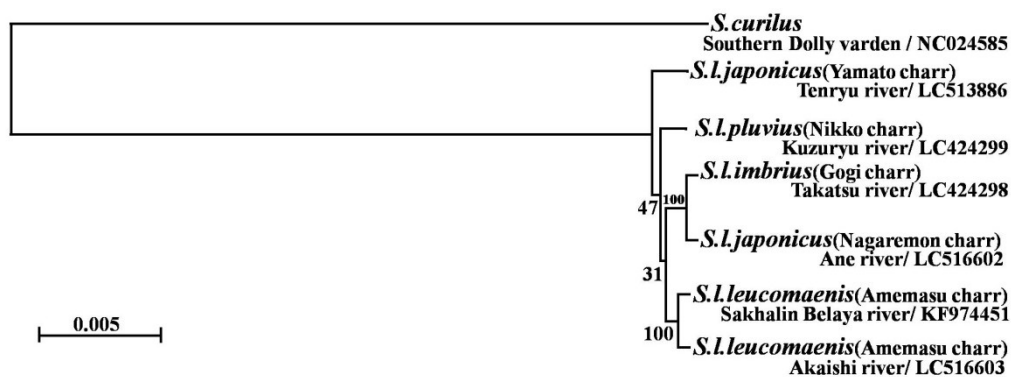
There were 120 regions of polymorphisms with a frequency of 0.72%. Missense mutations occurred at 10 nucleotides (Table 1). Most were transition mutations; however, transversion mutations occurred at eight nucleotides (6.7%). The most frequent polymorphism was in the ND5 region, and among the 16 nucleotide mutations, missense mutations occurred at four sites. In the CYTB region, there were 13 polymorphisms and all of them were silent mutations. Only three polymorphisms were observed in the ATP8, COX3, ND3 and ND4L regions. A deletion of one base was observed in the typical *S. l. japonicus* specimen and deletions of two bases were observed in the D-loop region of the morphotype (Table 1).

**Table 1.** Missense mutations of mitochondrial DNA in *Salvelinus leucomaenis*.

	Accession no.	length (bp)	D-loop 930-931	980	ND1 4625	ND2 6012	ATP8 8965	9013	ND4L 11527	ND5 12970	13023	13776	14275	ND6 14879-14881
<i>S.l.leucomaenis</i> (Amemasu charr)	KF974451	16655	AA	C	A(Met)	G(Val)	G(Ara)	<b>A(Ile)</b>	C(Thr)	G(Ser)	C(Leu)	A(Thr)	C(Thr)	ACC(Gly)
<i>S.l.leucomaenis</i> (Amemasu charr)	LC516603	16655	GA	C	A(Met)	G(Val)	G(Ara)	G(Val)	C(Thr)	G(Ser)	C(Leu)	<b>G(Ara)</b>	C(Thr)	ACC(Gly)
<i>S.l.japonicus</i> (Yamato charr)	LC513886	16654	GA	-	A(Met)	G(Val)	<b>A(Thr)</b>	G(Val)	C(Thr)	<b>A(Asn)</b>	C(Leu)	A(Thr)	C(Thr)	GCC(Gly)
<i>S.l.japonicus</i> (Nagaremon charr)	LC516602	16653	-	C	A(Met)	G(Val)	G(Ara)	G(Val)	C(Thr)	G(Ser)	<b>A(Met)</b>	A(Thr)	C(Thr)	GCC(Gly)
<i>S.l.pluvius</i> (Nikko charr)	LC424299	16655	GA	C	<b>G(Val)</b>	<b>A(Ile)</b>	G(Ara)	G(Val)	<b>T(Ile)</b>	G(Ser)	C(Leu)	A(Thr)	C(Thr)	GCC(Gly)
<i>S.l.imbrius</i> (Gogi charr)	LC424298	16655	GA	C	A(Met)	G(Val)	G(Ara)	G(Val)	C(Thr)	G(Ser)	C(Leu)	A(Thr)	<b>A(Lys)</b>	<b>GCT(Ser)</b>

## DISCUSSION

In the maximum likelihood tree (Figure 2), the typical type of *S. l. japonicus* branched off first, showing basal tendencies among Japanese *Salvelinus* spp., followed by *S. l. pluvius* (Nikko charr). *S. l. imbrius* (Gogi charr) and *S. l. japonicus* (Nagaremon charr) formed a clade and *S. l. leucomaenis* (Amemasu charr) formed a sister clade.



**Figure 2.** Maximum-likelihood tree based on whole mitochondrial DNA sequences of four subspecies of white-spotted charr (*Salvelinus leucomaenis imbrius*, *S. l. japonicus*, *S. l. leucomaenis*, and *S. l. pluvius*) in Japan. The bootstrap value based on 1,000 replicates is shown at each node.

This molecular tree indicates that the nagaremon type of *S. l. japonicus* was most closely related to *S. l. imbrius*, both of which has the western most distribution of *S. leucomaenis* subspecies found in Japan. This tree provides an interesting phylogenetic hypothesis of *S. leucomaenis* diversification in Japan. *S. l. japonicus* was the first to inhabit

the Japanese archipelago and extended its range throughout Honshu. Geological event such as glaciation and subsequent warming caused reproductive isolation resulting in diversification of *S. l. pluvius* in north eastern Honshu (Yamamoto et al., 2004; Kikko et al., 2008). *S. l. imbrius* and nagaremon morphotype of *S. l. japonicus* diversified in western Honshu with a very limited distribution. *S. l. leucomaenis* diversified in north eastern Honshu and Hokkaido. Similar studies conducted on *Salvelinus* of the Eurasian continent place *S. leucomaenis* as the basal *Salvelinus* (Balakirev et al., 2016; Oleinik et al., 2019a, 2019b), supporting the molecular phylogeny obtained in our study. Further research using specimens from numerous river systems throughout Japan will be necessary in order to completely understand the evolutionary process of *S. leucomaenis* of Japan as well as the currently elusive taxonomic status of nagaremon morphotype.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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