

## **Genetic characterization of ayu *Plecoglossus altivelis altivelis* occurred in Nakaumi Lagoon**

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**Abstract:** Ayu *Plecoglossus altivelis altivelis* is a common fish that is composed by two major forms, amphidromous and landlocked forms, based on its annual life history. Although recent decline in the reserves of amphidromous stocks in rivers has prompted frequent transplantations from landlocked stocks, a low survival rate of larvae of the landlocked form occurs during periods of high water temperature in seawater. In brackish water environment, however, non-native landlocked form may survive and show sympatric distribution with mother populations. This study was conducted to verify the genetic status of ayu occurred in Nakaumi Lagoon, which is the second largest brackish lake in Japan. Nucleotide sequence polymorphism of the first 300 bp of the mitochondrial DNA control region was analyzed for 30 individuals including each 15 specimens collected from 2 locales of Nakaumi Lagoon in May 2009, along with each 15 reference specimens of the amphidromous and landlocked stocks. Comparatively high levels of nucleotide substitution were observed with 35 polymorphic sites from Nakaumi population and 40 sites from the amphidromous stock, as contrasted with 24 sites from the landlocked stock. Based on nucleotide sequences at 5 hypervariable sites, 2 haplotypes occurred in only the amphidromous stock were obtained at a high frequency in Nakaumi population. These results suggest that the amphidromous form forms the majority of Nakaumi population.

**Key words:** *Plecoglossus altivelis altivelis*, ayu, genetic structure, Nakaumi Lagoon, brackish lake

### **Introduction**

Ayu *Plecoglossus ativelis altivelis*, being the basis of major commercial freshwater fishing in Japan, includes amphidromous and landlocked forms that are different in their life history. The former

normally ranges over the Japanese Archipelago, and the latter is native to Lake Biwa, and they are well-known to differ in some behavioral, physiological, morphological, and reproductive characteristics. For example, the landlocked form tends to dominate optimal feeding territories in rivers, and its more aggressive behavior depending on water temperature excludes the other amphidromous fish from these areas (Kawanabe 1976; Shibuya et al. 1995). The landlocked form has more scales (Nishida 1986; Nishida and Sawashi 1987) and spawns a greater number of smaller eggs earlier in the season (Azuma 1973; Nishida 1988; Iguchi 1993). Furthermore, it is noteworthy that there is a low survival rate of larvae of the landlocked form in seawater (Iguchi and Yamaguchi 1994), particularly during periods of high water temperature (Tabata and Azuma 1986), which makes them maladaptive to reproduce in amphidromous life history. Decline in the reserves of amphidromous stocks has prompted frequent transplantations from non-native origins with the primary aim of stock enhancement, and transplantation fish have been largely supplied from the Lake Biwa population that is the landlocked form genetically differentiated from the amphidromous form (Nishida 1985; Seki et al. 1988; Iguchi et al. 1997). The consequence of transplantation on the reproductive success of native stock is not well known, despite possible negative ecological effects and genetic disturbances (Iguchi 1997).

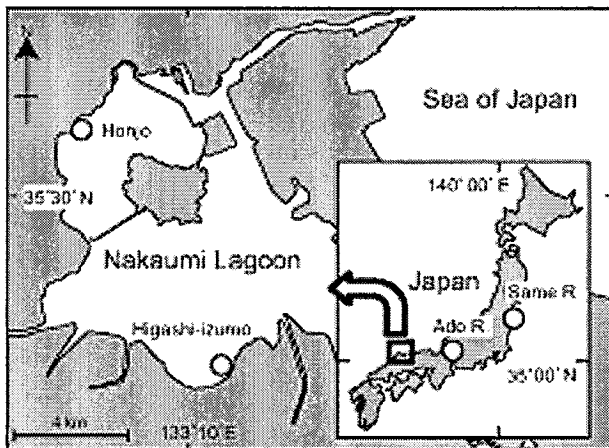


Fig. 1. Maps showing field sampling locales of ayu at Honjo and Higashi-izumo in Nakaumi Lagoon and sites of Same River (Same R.) and Ado River (Ado R.).

Nakaumi Lagoon is the second largest brackish lake with an area of 87 km<sup>2</sup> and located along the southwest coast of the Sea of Japan within Shimane and Tottori Prefectures of Japan (Fig. 1). It is representative of the highly eutrophicated and strongly enclosed coastal lagoon

systems in Japan. If larva and juvenile of landlocked ayu could utilize this brackish

water environment for their nursery ground, transplanted non-native landlocked individuals may survive and show sympatric distribution with the native individuals in Nakaumi Lagoon. In addition, the successful in reproduction not only of themselves but also with the native ones may cause

distinct changes in the genetic variability from the mother population. In this study, mitochondrial DNA sequence analysis was conducted for individuals in Nakaumi Lagoon in order to verify their current genetic status. Iguchi et al. (1997, 1999) reported that the first portion of the mitochondrial DNA control region of ayu was highly polymorphic and variable between the amphidromous and landlocked forms. We thus determined nucleotide sequences of the first 300 bp of this region for a better resolution of the genetic variability of Nakaumi population.

## **Materials and methods**

### **Sample Collection**

Fishes were collected by small set-net catch at Honjo and Higashi-izumo locales in Nakaumi Lagoon in May 2009 (Fig.1), and then 15 individuals were chosen randomly from each of two catches. All specimens were stored in a laboratory freezer at  $-20^{\circ}\text{C}$  until DNA preparation.

### **DNA Preparation**

Total genomic DNA was prepared from small scraps (~20 mg) of dorsal muscle tissue by the modified urea-SDS-Proteinase K method (Aranishi 2005a, 2005b). Samples were incubated in the extraction buffer (10 mM Tris-HCl, pH 7.5, 20 mM EDTA, pH 8.0, 1 % SDS, 4 M urea) containing 25  $\mu\text{g}$  Proteinase K at  $55^{\circ}\text{C}$ , and then 5 M NaCl was added and mixed. DNA was isolated with phenol-chloroform-isoamyl alcohol and subsequent chloroform-isoamyl alcohol followed by precipitation with ethanol. DNA pellet was washed with ethanol, dried, and resuspended in 10T0.1E (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, pH 8.0).

### **PCR Amplification**

Approximately 470 bp mitochondrial DNA fragment involving the first half of the control region was amplified using the universal primers; 5'-TTAAAGCATCGGTCTTGTA-3' and 5'-CCTGAAGTAGGAACCAGATG-3' (Shields and Kocher 1991). PCR amplification was performed in GoTaq Green PCR Master Mix (Promega) containing 2 mM  $\text{MgCl}_2$ , 0.5  $\mu\text{M}$  each primer, and template DNA according to the protocol consisted of an initial denaturation at  $94^{\circ}\text{C}$  for 2 min, followed by 30 cycles of 10 sec at  $94^{\circ}\text{C}$ , 10 sec at  $55^{\circ}\text{C}$  and 20 sec at  $72^{\circ}\text{C}$ , and a final

extension at 72 °C for 5 min in a Techgene thermal cycler (Techne). PCR products were analyzed using a DNA-1000 Reagent Kit (Shimadzu) containing a SYBR Gold Nucleic Acid Gel Stain (Invitrogen) in a MCE-202 MultiNA microchip electrophoresis system (Shimadzu).

## Sequence Analysis

Nucleotide sequencing of double strands of PCR products was accomplished using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in an automated 3730xl DNA Analyzer (Applied Biosystems). Multiple sequence alignments were performed using the program MEGA 4.0 (Tamura et al. 2007), together with each 15 reference sequences of the amphidromous stock in Same River and landlocked stock in Ado River (Iguchi et al. 1997), which were reanalyzed in this study. Nucleotide diversity was calculated from the frequency of haplotypes within a population using the program Arlequin 3.0 (Excoffier et al. 2005).

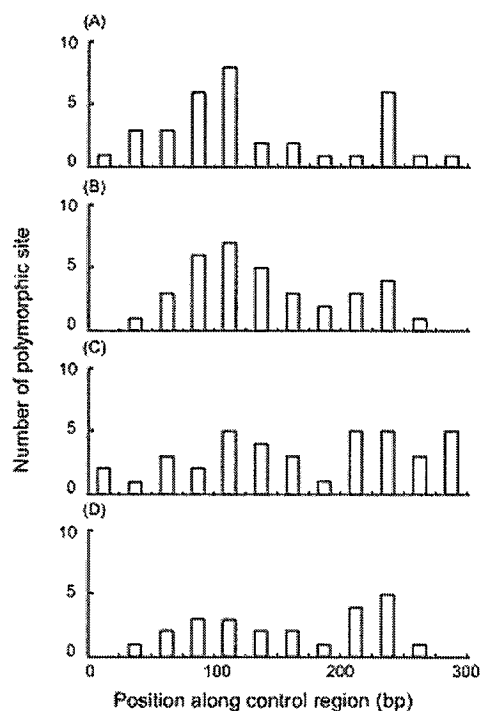


Fig. 2. Distribution of polymorphic sites along the first 300 bp of the mitochondrial DNA control region of ayu collected at Honjo (A) and Higashi-izumo (B) in Nakaumi Lagoon, in comparison with the amphidromous form (C) and landlocked form (D). Each bar represents a 25 bp segment.

## Results and Discussion

Nucleotide sequence polymorphism of the first 300 bp of the mitochondrial DNA control region was analyzed for each 15 individuals of ayu collected at Honjo and Higashi-izumo in Nakaumi Lagoon. Among a total of 30 individuals, a high level of nucleotide substitution with 43 polymorphic sites was detected at the relative frequency of nucleotide substitution to be 14.3 % (Fig. 2). All substitutions were due to a single nucleotide alteration, and neither insertion nor deletion events occurred.

Comparatively high levels of nucleotide substitution were observed with 35 polymorphic sites/locale from Nakaumi population, in contrast to 40 and 24 sites from the amphidromous and landlocked stocks, respectively (Table 1). Of 35

**Table 1.** Summary for genetic characterization using the first 300 bp of the mitochondrial DNA control region of ayu collected at Honjo and Higashi-izumo in Nakaumi Lagoon, in comparison with the amphidromous and landlocked forms

Locality	Number of haplotype		Number of polymorphic site			Nucleotide diversity
	<i>n</i>	<i>k</i>	variable site	transition	transversion	$\pi \pm SD$
Honjo	15	14	35	30	5	2.56±1.42
Higashi-izumo	15	15	35	32	5	2.96±1.62
Amphidromous	15	15	40	28	12	3.37±1.83
Landlocked	15	14	24	21	3	2.13±1.20

polymorphic sites of Nakaumi population, approximately 30 transition and 5 transversion sites were determined regardless of sampling locales, and the transition/transversion ratio was calculated to be 6.0. It is noteworthy that the transition/transversion ratio of the amphidromous stock (28/12 = 2.33) was much lower than that of the landlocked stock (21/3 = 7.0). Nucleotide diversity varied from 2.13 % of the landlocked stock to 3.37 % of the amphidromous stock, and that of Nakaumi population ranging 2.56 % at Honjo and 2.96 % at Higashi-izumo lay between the landlocked and amphidromous stocks.

**Table 2.** Haplotype groups assorted by nucleotide substitution at 5 hypervariable sites along the first 300 bp of the mitochondrial DNA control region of ayu collected at Honjo and Higashi-izumo in Nakaumi Lagoon, in comparison with the amphidromous and landlocked forms

Portion/Substitution					Distribution			
109	160	237	241	243	Honjo	Higashi-izumo	Amphidromous	Landlocked
C	G	C	A	G	0	5	6	0
C	A	C	A	G	6	2	2	0
A	G	C	A	G	0	2	3	1
A	A	C	A	G	5	3	2	3
A	A	A	G	A	1	0	2	4
G	A	C	A	G	0	0	0	1
A	A	C	A	A	0	1	0	6
C	A	C	A	A	2	1	0	0
C	G	C	A	A	1	0	0	0
C	G	C	G	G	0	1	0	0

Iguchi et al. (1997) reported that 5 nucleotides such as positions 109, 160, 237, 241, and 243 along the mitochondrial DNA control region of ayu were significantly hypervariable. Based on nucleotide sequences at these 5 hypervariable sites, a total of 10 haplotypes were obtained from 60 individuals comprising 30 Nakaumi individuals and each 15 individuals of the amphidromous and

landlocked stocks (Table 2). A high frequency of 2 haplotypes, which occurred only in the amphidromous stock, and a very low frequency of other 2 haplotypes, which occurred only in the landlocked stock, were observed in Nakaumi population. In addition, 3 haplotypes of Nakaumi population occurred in neither the amphidromous nor landlocked stocks, and one of these haplotypes (CACAA) have so far been recorded as the wild stock of Shinano River that drains into the Sea of Japan (Iguchi et al. 1997).

The genetic characterization of Nakaumi population using the polymorphic portion of the mitochondrial DNA control region allowed us to demonstrate comparatively high levels of nucleotide substitution and a high frequency of unique haplotypes of the amphidromous stock. These results suggest that the amphidromous form of ayu could form the major part of Nakaumi population, and transplanted landlocked individuals might not survive in brackish water environment of Nakaumi Lagoon. Seki et al. (1988, 1994) inferred limited reproductive contribution of released landlocked individuals to native amphidromous stock. Otherwise, Iwata et al. (2007) recently reported that drifting larvae reproduced by landlocked females were observed in the early spawning season. Therefore, further emphasis on the investigation of the genetic structures of larva and/or juvenile stages is needed to elucidate the impact of transplanted individuals to the mother population in Nakaumi Lagoon.

### **Acknowledgement**

We thank T. Sasaki, Shimane Prefectural Fisheries Technology Center, for assistance of field sampling of ayu in Nakaumi Lagoon. This study was supported in part by grants from the Ministry of Land, Infrastructure, Transport and Tourism of Japan and Shimane University.

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