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Low Genetic Diversity and Shallow Population Structure of the Japanese Halfbeak *Hyporhamphus sajori* Revealed from Mitochondrial DNA in the Northeast Asia

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ABSTRACT This study was conducted to know the genetic diversity and population structure of Japanese halfbeak (*Hyporhamphus sajori*) in the Northeast Asia, using mitochondrial DNA control region. In the present study, a total of 70 individuals were collected from three locations of China (Liaoning), Korea (Tongyeong) and Japan (Wakasa Bay), and 47 individuals sequences from three locations of Japan (Wakasa Bay, Toyama Bay and Mikawa Bay) were downloaded from genbank. A total of 7 haplotypes were identified with 7 polymorphic sites from 358 bp length sequences. Haplotype and nucleotide diversity were very low and ranged from 0 to 0.295±0.156 and 0 to 0.0009±0.0011, respectively. Ancestral haplotype was shared by 94% individuals. An extremely low haplotype and nucleotide diversity, and starlike minimum spanning tree indicated that the species have undergone a recent population expansion after bottleneck. Pairwise F_{ST} values were low and there was no significant differences among populations suggesting a gene flow among the populations. Dispersal of the eggs with the aid of drifting seaweed and currents might be the major responsible factor for the genetic homogeneity.

Key words: *Hyporhamphus sajori*, mtDNA control region, genetic diversity, population structure, egg dispersal

INTRODUCTION

Knowledge of the genetic structure of fish populations is important for the sustainable management of fish stock to reduce the risk of depletion, as well as for the recovery of fisheries resources and monitoring of populations (Roldan *et al*., 2000; Laikre *et al*., 2005). Many marine fish populations show high genetic connectivity with little or no differentiation over a large geographical scale (Hauser and Carvalho, 2008); however, studies have also shown clearly structured fish populations (e.g., Gwak and Nakayama, 2011; Kitanishi *et al*., 2013). Historical processes associated with climatic oscillations are one of the most important factors of the current distribution of species (Yan *et al*., 2015). On the contrary, genetic patterns in marine species are greatly influenced by contemporary factors such as the dispersal ability of larvae and adults, ocean currents, adaptations to a confined area, temperature of the sea, spatial isolation, and reproductive behavior (Palumbi, 1994).

The Japanese halfbeak *Hyporhamphus sajori* (Temminck and Schlegel, 1846), belonging to the family Hemiramphidae, is a pelagic fish inhabiting the coastal waters of China, Korea, and Japan (Kim *et al*., 2005). This species lives in shallow water and is not capable of long-distance migration; the females usually mature at 2 years while the males mature at 1∼2 years, with spawning usually occurring from April to July (Kim *et al*., 2004; Kim *et al*., 2005). After spawning, the eggs attach to drifting seaweed (Kim *et al*., 2004), which may help in long-distance dispersal. *H. sajori* has a high nutritional content and is of commercial importance in Northeast Asia (Tsuji and Sadakata, 2000);

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the meat is delicious and also possesses some medicinal value (Tang, 1987). Despite their commercial importance, the total population size of *H. sajori* has recently decreased (Tsuji and Sadakata, 2000). Therefore, a sustainable management plan for this species is needed urgently.

Studying the population genetics of *H. sajori* is important to obtain fundamental data for conservation and development of a fisheries management strategy. Yu *et al.* (2016) studied the genetic population structure of *H. sajori* but found no structured population in Korea and Japan. In this study, we investigated the genetic variation of *H. sajori* over a broad geographical area in Northeast Asia. We studied a mitochondrial DNA (mtDNA) control region, which is a useful genetic marker because of its hypervariable sites and high mutation rate (Aquadro and Greenberg, 1983; Nesbø *et al.*, 1998), to understand the genetic population structure of *H. sajori* in China, Korea, and Japan.

MATERIALS AND METHODS

1. Sample collection

A total of 70 specimens of *H. sajori* were collected from 3 locations of China, Korea and Japan in 2014 and 2015 (Table 1, Fig. 1). After collection, specimens were transported to the laboratory in a frozen state. A piece of dorsal muscle tissue was collected from each individual and preserved in 99% ethanol for DNA extraction. In addition 47 mtDNA control region sequences data of *H. sajori* from Takahana Bay, Toyama Bay and Mikawa Bay (Japan) were downloaded from the genbank and added with the new sequences for analysis. The sequences of the Takahana Bay were added with the Wakasa Bay sequences as the two sites are located at the same place (Table 1).

2. DNA extraction, amplification and sequencing

Genomic DNA was extracted from muscle tissue of *H. sajori*. Muscle tissues were digested with Proteinase K and Wizard genomic DNA purification kit (Promega, USA). Polymerase chain reactions (PCR) were used to amplify the mtDNA control region using the universal primer pair L-15924 (5′-AGCTCAGCGCCAGAGCGCCGGTCTTGTAAA-3′) (Kocher *et al.*, 1989) and H-16498 (5′-CCTGAAGTAGGAACCAGATG-3′) (Meyer *et al.*, 1990).

Table 1. Sampling locations, date of collection and sample number of *H. sajori* used in this study

Country	Sampling location	*n*	Collection date
China	Liaoning (HSCH)	30	2014 September
Korea	Tongyeong (HSTY)	30	2015 August
Japan	Wakasa Bay (HSWA)	30	2014 May and downloaded data from GenBank*
	Toyama Bay (HSTO)	14	Downloaded from GenBank**
	Mikawa Bay (HSMI)	13	Downloaded from GenBank***

n: number of samples; *: GenBank accession number - KX427013 to KX427014; **: GenBank accession number - KX427002 and KX427004; ***: GenBank accession number - KX427015 to KX427017

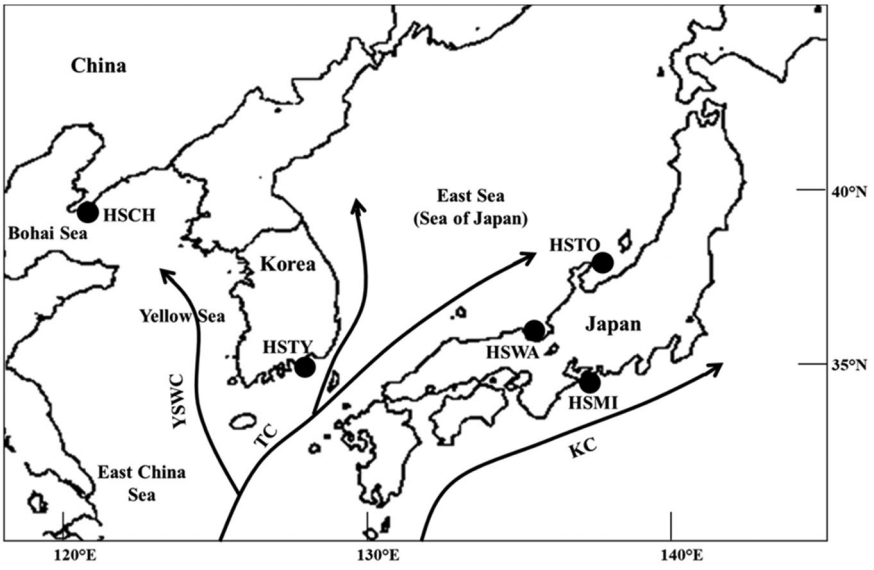

Fig. 1. Sampling locations for *H. sajori*. HSCH: Liaoning province, HSTY: Tongyeong, HSWA: Wakasa Bay, HSTO: Toyama Bay, HSMI: Mikawa Bay. Black line with arrow represents the direction of different Sea currents. KC: Kuroshio Current, TC: Tsushima Current, YSWC: Yellow Sea Warm Current.

PCR was carried out with a reaction mixture containing template DNA 0.6 μL, 10 × Ex Taq DNA polymerase buffer 1.5 μL (Takara, Otsu, Japan), 5 μM primers 1.5 μL, 2.5 mM deoxyribonucleoside triphosphate (dNTP) 1.5 μL, Ex Taq DNA polymerase 0.1 μL (Takara) and then total volume was made to 15 μL by adding sterilized water. Initial denaturation was for 5 min at 94°C followed by 30 cycles with denaturation for 30 s at 94°C, annealing for 30 s at 50°C, extension for 30 s at 72°C and final extension for 7 min at 72°C. Then resultant PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide and observed on a UV-light source. PCR products were purified with a mixture of 0.5 μL ExoSAP-IT (United States Biochemical Corporation, USA) and 1.5 μL of sterilized water for 30 min at 37°C and another 15 min at 80°C. Purified mtDNA products were sequenced on an ABI 3730XL DNA Analyzer (Applied Biosystems Inc., USA) using an ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems Inc., USA).

3. Sequence alignment and data analysis

The mtDNA control region sequences were checked and aligned with ClustalW (Thompson *et al*., 1994). Molecular diversity indices of each population including transition and transversion sites, numbers of haplotype, haplotype diversity (h) and nucleotide diversity (π) were calculate by the software programs ARLEQUIN version 3.5.1.2 (Excoffier and Lischer, 2010). Pairwise F_{ST} was calculated by ARLEQUIN and significance was tested by 1000 permutations for each pairwise comparison. The phylogenetic relationships among haplotypes were reconstructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) using Tamura 3 parameter and gamma distribution with invariant sites in MEGA 5.05 (Tamura *et al*., 2011). The robustness in the nodes of the phylogenetic tree was evaluated by 1000 bootstrap replicates (Felsenstein, 1985). A sequence of the mtDNA control region of the species *Hyporhamphus intermedius* was used as an out group. The minimum spanning tree was created using Arlequin and HapStar software program (Teacher and Griffiths, 2011).

RESULTS

1. Genetic diversity

In the present study 440 bp of *H. sajori* mtDNA control region was amplified and sequenced successfully from 70 individuals collected from three locations of China, Korea, and Japan. After adoption with the sequences from the genbank 358 bp long sequences were obtained. Among 117 sequences, a total of 7 haplotypes were identified with 7 polymorphic sites. Haplotype diversity and nucleotide diversity were extremely low in all the populations with highest were observed in Wakasa Bay ($h = 0.295 \pm 0.156$, $\pi = 0.0009 \pm 0.0011$) whereas lowest were observed in Toyama Bay and Mikawa Bay ($h = 0, \pi = 0$). The numbers of transitions, transversions, substitutions, haplotypes together with haplotype diversity and nucleotide diversity are presented in Table 2. One haplotype is widely distributed and occurred in 110 individuals which is about 94% of the total number. The number of rare haplotypes occurred in one individual only was 4 among which Liaoning, Tongyeong and Wakasa Bay belong to 2, 1 and 1, respectively. In addition, a separate analysis was conducted with 440 bp long sequence obtained from 70 individuals of Liaoning (30), Tongyeong (30) and Wakasa Bay (10). In that case haplotype and nucleotide diversity were lowest in Wakasa Bay whereas other populations also exhibit low haplotype and nucleotide diversity (data not shown).

2. Population structure, phylogenetic relationship and demographic history

Pairwise F_{ST} values indicated that there was no significant difference between the populations from China, Japan and Korea. All of the pairwise F_{ST} values among five locality were very low and in most cases negative. The highest pairwise difference observed between Liaoning

Table 2. Molecular diversity results for the mtDNA control region sequences of *H. sajori* from five locations of China, Korea and Japan

Location	n	Substitutions (ti + tv)	N	h (SD)	π (SD)
HSCH	30	3 (2 + 1)	3	0.131 (0.082)	0.0006 (0.0007)
HSTY	30	3 (2 + 1)	4	0.251 (0.102)	0.0007 (0.0009)
HSWA	30	2 (2 + 0)	3	0.295 (0.156)	0.0009 (0.0011)
HSTO	14	0	1	0	0
HSMI	13	0	1	0	0

n: number of samples, Substitutions: transitions (ti) + transversions (tv), N: number of haplotypes, h: haplotype diversity, π: nucleotide diversity, SD: standard deviation

Table 3. Pairwise F_{ST} (below diagonal) and associated P values (above diagonal) among populations of *H. sajori*

Location	HSCH	HSTY	HSWA	HSTO	HSMI
HSCH	–	0.522	0.462	0.999	0.999
HSTY	0.009		0.541	0.999	0.999
HSWA	0.010	− 0.007		0.999	0.999
HSTO	− 0.332	− 0.316	− 0.328		0.999
HSMI	− 0.140	− 0.128	− 0.1304	0.000	

Significance level $P < 0.05$

Fig. 2. Neighbor-joining (NJ) tree showing the relationships between 7 haplotypes of mtDNA control region sequences. Numbers at branches indicate bootstrap probabilities in 1,000 bootstrap replications. Bar indicates genetic distance. HI: *Hyporhamphus intermedius* was used as outgroup.

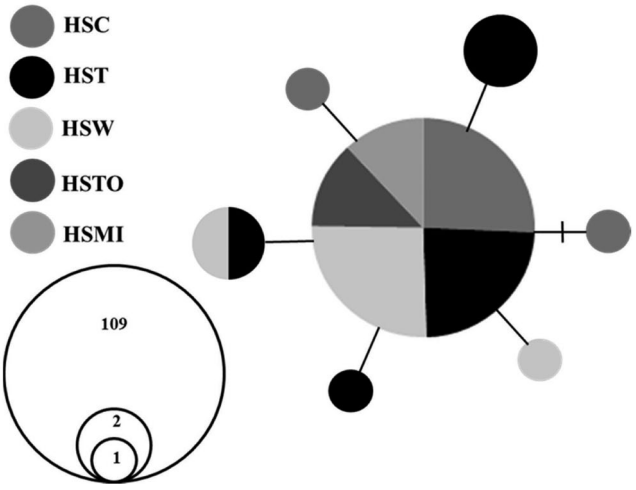

Fig. 3. Minimum spanning network based on 7 haplotypes of *H. sajori* mtDNA control region. Circle sizes represent the frequencies of each haplotype. Each line connecting haplotypes represents one mutational step.

and Wakasa Bay ($F_{ST}=0.010$, $P=0.462$) (Table 3). No significant pairwise F_{ST} was observed between any pair of comparison. The neighbor-joining tree of *H. sajori* with 7 haplotypes was shallow and there were no significant genealogical branches of samples corresponding to sampling locality (Fig. 2). Haplotypes from individuals of a population were scattered and well mixed with other populations. In order to depict the phylogenetic and geographical relationship, a minimum spanning tree of the observed 7 haplotypes was also constructed (Fig. 3). The obtained network of *H. sajori* was somewhat starlike topology from which 6 haplotypes radiating from the central haplotype. The most common haplotype is distributed in all five populations which were shared by the 94% of the total sample suggesting that this haplotype is the ancestral haplotype. All individuals from Toyama and Mikawa Bay shared the common haplotype. An analysis with 440 bp long sequences of 70 individuals also exhibit no significant difference among populations (data not shown).

DISCUSSION

1. Genetic diversity

The haplotype and nucleotide diversity of *H. sajori* were extremely low, ranging from 0 (Toyama and Mikawa Bays) to 0.295 ± 0.156 (Wakasa Bay) and 0 (Toyama and Mikawa Bays) to 0.0009 ± 0.0011 (Wakasa Bay), respectively. In Toyama and Mikawa Bays of Japan, the haplotype and nucleotide diversity were estimated as 0. Yu *et al*. (2016) also observed a low haplotype and nucleotide diversity in eight locations in Korea and Japan, but in Toyama and Mikawa Bays, they reported results different from the zero observed in our study, which is due to the similarity of some haplotypes in the mtDNA control region used in this study. A single haplotype was shared by the majority of individuals of all populations in both studies. This pattern of low genetic diversity is not usual in marine fishes but has been reported in species such as *Gadus macrocephalus* ($h=0.410$, $\pi=0.0010$, Gwak and Nakayama, 2011), *Pleurogrammus azonus* ($h=0.033$, $\pi=0.00007$, Canino *et al*., 2010), and *Merluccius paradoxus* ($h=0.53$, $\pi=0.0014$, von der Heyden *et al*., 2010). The number of rare haplotypes is extremely low. In this study, we found 2, 1, and 1 rare haplotypes in Liaoning, Tongyeong, and Wakasa Bays, respectively. Additional rare haplotypes, as well as haplotypes unique to one population, may be detected in other populations if we increase the number of sampled individuals. With detailed studies, population-specific haplotypes could be used as genetic markers in identifying *H. sajori* individuals from different geographical locations (Slatkin, 1985). The adaptation of a fish population to a changing environment depends on its genetic diversity level. A high genetic diversity within a population will allow more individuals and offspring to adapt to the new environment, thereby ensuring that the population survives for further generations (Sun and Tang, 2018). A number of factors influence genetic diversity such as environmental heterogeneity, life-history characteristics, and population dynamics (Avise, 2000). Grant and Bowen (1998) categorized marine fishes into four classes depending on a combination of haplotype and nucleotide diversity values. According to their theory, *H. sajori* falls under the first cat-

egory with low haplotype and nucleotide diversity ($h<0.5$, $\pi<0.005$), which may be due to a recent population expansion after a bottleneck or the founder effect. Low population numbers during colonization, might survived with single or few mtDNA lineages which cause extremely low genetic diversity.

2. Population structure and historical insight

Pairwise F_{ST} comparison using 358 bp sequences of five *H. sajori* populations from China, Korea, and Japan showed no significant differences, indicating frequent gene flow among the populations. Both the neighbor-joining tree and the minimum spanning tree revealed absence of geographical association, which also strongly suggests a high rate of gene flow. A single haplotype was found to be shared by the majority of specimens from all locations. The results of analysis using 440 bp sequences for three populations was also congruent with panmictic populations. Many marine pelagic fish species show low levels of genetic differentiation among geographical regions because of high dispersal of the planktonic egg and larvae by the ocean current (Palumbi, 1994; Selkoe and Toonen, 2011). Although *H. sajori* is not capable of long-distance migration, this species possesses a special reproductive behavior that may influence the genetic homogeneity over a large geographical range. The eggs of *H. sajori* attach to drifting seaweed and hatch approximately 15 days after fertilization (Kim *et al*., 1984). The drifting seaweed may facilitate long-distance dispersal of the eggs by the Tsushima and Kuroshio Warm Currents to different locations of the Korean Peninsula and Japanese Archipelago. The Yellow Sea Warm Current, which is a branch of the Tsushima Current, runs through the Yellow Sea carrying warm, saline water to the Bohai Sea (Xu *et al*., 2009), influencing the west coast of Korea, and may play an important role in transporting drifting seaweed. Therefore, the ocean current patterns in Northeast Asia may play an important role in gene flow through the passive transport of the eggs. Similar genetic homogeneity over large geographical ranges owing to larval transport by ocean currents has also been reported in other fish species in this area such as *Sebastes schlegelii* (Zhang *et al*., 2016) and *Branchiostegus japonicus* (Nohara *et al*., 2010).

The minimum spanning tree has a star-like topology where some haplotypes radiate from a central haplotype, which is a signature of population expansion (Slatkin and Hudson, 1991). Therefore, the minimum spanning tree indicates that the population has expanded from a single lineage. An extremely low haplotype and nucleotide diversity of the present study also support the theory of population expansion by Grant and Bowen (1998). Therefore, *H. sajori* is likely to recover from a small effective population size or population bottleneck. During the Pleistocene glaciations including the Last Glacial Maximum (LGM), the sea level is thought to have been approximately 120~140 m below the present sea level. Marginal seas of the Northwestern Pacific such as the beds of the Yellow Sea and most of the East China Sea were exposed, and the East Sea was almost separated by the closing of the Tsushima Strait (Park, 1994; Xu and Oda, 1999; Domitsu and Oda, 2006). The resultant halt in the Tsushima Current, as well as inflow of the cold Oyashio Current to the East Sea through the Tsugaru Strait, not only reduced the salinity and created an anaerobic to weakly aerobic condition but also lowered the temperature by 10°C in the East Sea (Gorbarenko and Southon, 2000). This may have created unfavorable conditions for many organisms and resulted in a drastic reduction of shallow water species such as *H. sajori* in the East Sea. Despite its susceptibility to the marine environmental changes, the sharing of a single lineage suggests that *H. sajori* could have survived in small numbers either in the East China Sea or the East Sea during the glacial period. Therefore, the present populations could have been created recently by migration from refugial populations after the LGM, which might be responsible for the low genetic diversity and lack of genetic structure that we found in this study. Some species around China, Korea, and Japan, such as *Branchiostegus japonicus* (Nohara *et al*., 2010), *Gadus macrocephalus* (Gwak and Nakayama, 2011), *Oplegnathus fasciatus* (Park *et al*., 2018), and *Pleurogrammus monopterygius* (Canino *et al*., 2010), also show population expansion. Therefore, *H. sajori* might also be undergoing general population expansion owing to vigorous environmental changes, similar to that in other marine fish species.

Assessment of genetic diversity and population structure is a vital tool for the management of fish populations. Based on mtDNA analysis, *H. sajori* exhibits high levels of genetic connectivity with low levels of differentiation among the populations of China, Korea, and Japan. The results of this study suggested that *H. sajori* may have faced a drastic population reduction in the past owing to environmental fluctuation, which is evidenced by its low mtDNA diversity. However, contemporary factors, such as dispersal of the eggs with the help of seaweed and ocean current, may also be a major factor for the genetic homogeneity of this species. This study may be beneficial for the conservation and sustainable management of this species. Additional insight into the genetic differentiation of *H. sajori* will require further sampling from locations in

China and the use of highly sensitive DNA markers such as microsatellite DNA.

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Mitochondrial DNA를 이용한 동북아시아 학꽁치 *Hyporhamphus sajori*의 유전적 다양성과 집단 구조

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요 약 : 학꽁치(*Hyporhamphus sajori*)의 유전적 다양성과 집단구조를 조사하기 위해 동북아시아에서 시료를 채집하여 mitochondrial DNA control region (mtDNA CR)을 분석하였다. 시료는 중국(Liaoning), 한국(통영), 일본(Wakasa Bay) 3곳에서 총 70개체를 채집했고, 일본 3곳(Wakasa Bay, Toyama Bay and Mikawa Bay)에서 분석된 47개체의 mtDNA CR 염기서열을 Genbank에서 다운로드했다. 분석결과 총 358 bp가 나타났고, 7개의 변이와 함께 haplotype이 7개 확인되었다. Haplotype diversity과 nucleotide diversity는 각각 0~0.295±0.156 및 0~0.0009±0.0011이고, main haplotype을 94%의 개체가 공유했다. 매우 낮은 haplotype diversity와 nucleotide diversity 그리고 starlike minimum spanning tree는 집단이 최근에 병목현상을 거친 후, 팽창되었음을 나타낸다. 집단 간에 Pairwise F_{ST} 값은 낮고 유의하지 않은 것으로 나타났고, 이것은 집단 간 gene flow가 있음을 시사한다. 학꽁치의 genetic homogenity는 부유조와 해류가 주요 원인으로 생각된다.

찾아보기 낱말 : 학꽁치, mtDNA, 유전적 다양성, 집단구조, 난 분산