Contrasting genetic population structures between congeneric flounder species, *Hippoglossoides dubius* and *H. pinetorum*

SHIGEAKI KOJIMA¹,²*, KEI SAKUMA² & TAKASHI YANAGIMOTO³

¹ Graduate School of Frontier Sciences, The University of Tokyo, Chiba 277–8563, Japan
² Atmosphere and Ocean Research Institute, The University of Tokyo, Chiba 277–8564, Japan
³ National Research Institute of Fisheries Science, Fisheries Research Agency, Kanagawa 236–8648, Japan

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**Abstract:** The Sea of Japan is a semi-closed marginal sea where genetic break and/or speciation have been reported for some deep-sea taxa. Population structure was compared between the congeneric deep-sea flounder species *Hippoglossoides dubius* and *H. pinetorum* inhabiting the Sea of Japan and neighboring seas. Based on nucleotide sequences of mitochondrial control regions, the *H. dubius* population was concluded to be genetically homogeneous, while 2 genetically distinct groups were recognized among *H. pinetorum*. Although the rare group of *H. pinetorum* populates the Sea of Japan, the Yellow Sea, the Okhotsk Sea, and the northwestern Pacific, frequency was the highest in the Sea of Japan. The unique phylogeographic pattern of *H. pinetorum* may be attributable to secondary contact with the Sea of Japan lineage, which was isolated from other sea areas during the last glacial period. A difference in bathymetrical distribution between the two species might explain their contrasting genetic population structures.

**Key words:** glacial periods, *Hippoglossoides*, mitochondrial control region, Phylogeography, Sea of Japan

**Introduction**

The Sea of Japan is one of the marginal seas of the northwestern Pacific Ocean characterized by a unique history of periodic environmental changes during the Quaternary (1.6–1.8 million years before present). The Sea of Japan is semi-closed and only connected to neighboring sea areas (namely the Okhotsk Sea, the East China Sea, and the northwestern Pacific Ocean) by shallow and narrow straits. The deep parts of this sea area have repeatedly suffered severe decreases of dissolved oxygen during glacial periods. During the last glacial maximum (LGM, 30,000–19,000 years ago), deep areas of the Sea of Japan were thought to have been completely anoxic due to a massive influx of freshwater from the Asian Continent and strong stratification (Oba et al. 1991, Tada et al. 1999). As a result, the deep-sea fauna was thought to have become extinct and was replaced by the present fauna, established by recolonization from adjacent habitats after the LGM (Oba et al. 1991). However, recent phylogeographical studies of deep-sea demersal fish (Kodama et al. 2008, Kodama & Kojima 2009, Kojima et al. 2011) and snails (Iguchi et al. 2007) suggest that some species survived in the oxic habitats which remained in restricted parts of the Sea of Japan during the LGM (Gorbarenko et al. 2004, Itaki et al. 2004). The existence of a few endemic deep-sea species in the Sea of Japan, such as the deep-sea whelks *Buccinum tsubai* and *B. striatissimum* (Amano 2004), the eelpouts *Lycodes japonicus* and *L. teraoi* (Nakabo 2013), and the snailfish *Careproctus notosaikaiensis* (Kai et al. 2011b), suggests that long-term geographical isolation of these Sea of Japan populations has continued. Kai et al. (2011a, 2011c) analyzed the molecular phylogeny of the *Careproctus rastrinus* species complex, which contains the third dominant deep-sea demersal fish of the Sea of Japan, the cactus-skin snailfish *C. trachysoma*, and concluded that fish in the Sea of Japan have undergone genetic deviation and speciation from those of the Pacific Ocean. Environmental changes in the Sea of Japan may have produced unique phylogeographic distributions, which are shared by some deep-sea species distributed in the Sea of Japan.

Based on nucleotide sequences of the mitochondrial
control region, a large genetic deviation between populations in the Sea of Japan and in neighboring seas was shown for the Japan-sea eelpout Bothrocara hollandi, which is the dominant deep-sea demersal fish in the Sea of Japan (Kodama et al. 2008, Kojima et al. 2011). Extremely low dispersal ability without ontogenetic vertical migration of this species is thought to have resulted in long-term geographical isolation of the Sea of Japan from neighboring sea areas (Kodama et al. 2008). This pattern corresponds to phylogeographic category I of Avise (2000). In contrast, Adachi et al. (2009) reported no such deviation for the darkfin sculpin Malacocottus zonurus, which is the second most common deep-sea demersal fish of the Sea of Japan. Ontogenetic vertical migration was suggested to have enabled this species to recolonize the Sea of Japan after the LGM and to maintain high gene flow between sea areas (Adachi et al. 2009). Similar results have been reported for the willowy flounder Tanakius kitaharai (Xiao et al. 2008) and the blackfin flounder Glyptocephalus stelleri (Xiao et al. 2010). Their patterns correspond to phylogeographic category IV in Avise (2000).

The flathead flounder Hippoglossoides dubius and the pointhead flounder H. pinetorum are also dominant demersal fish species in deep parts of the Sea of Japan (Okiyama 2004). They are commercially important flatfish inhabiting the Sea of Japan, the Okhotsk Sea, and the northwestern Pacific Ocean. Hippoglossoides pinetorum is also found in the Yellow Sea and the Bohei Sea (Yamada et al. 2007). In the present study, the phylogeography of these congeneric flounder species was analyzed and a third phylogeographic pattern in demersal fish inhabiting deep areas of the Sea of Japan was discovered, which corresponds to phylogeographic category II of Avise (2000).

**Materials and Methods**

Fifty-three specimens of Hippoglossoides dubius were collected from the Sea of Japan and 46 from the northwest Pacific Ocean. For H. pinetorum 20 specimens were collected from the Sea of Japan (off Ohda) and the northwest Pacific Ocean (off Miyako; Table 1 and Fig. 1). All the specimens were kept frozen (−30°C) until use. Total DNA was extracted from a small piece of muscle with a Puregene DNA purification kit (Gentra Systems, Minneapolis, MN, USA) or a DNeasy Tissue Extraction Kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s instructions. Also, total DNA samples from 70 specimens of H. pinetorum (Table 1), which were extracted by Yanagimoto et al. (2007) and kept frozen (−30°C), were used.

Mitochondrial DNA fragments, including the 5′ region of the control region were amplified by PCR. Primers Pro-L (5′-CTACCTCCAACTCCCAAAGC-3′) (Palumbi et al. 1991) and either 1612SAR-H (5′-ATAGTGGGGTATCATC-3′) (Palumbi et al. 1991) or KareiCR-1R (5′-AAAGAGAACCCCTTACCCGC-3′) were used for H. dubius. Primers ZThr-L (5′-AGAGCGCCGCTTTCGTA-3′) (Kodama et al. 2008) and either 1612SAR-H or KareiCR-1R were used for H. pinetorum. The primer KareiCR-1R was synthesized on the basis of determined sequences. The steps used to perform PCR were as follows: incubation at 95°C for 120 s, followed by 30 to 40 cycles at 95°C for 40 s, 60°C (H. dubius) or 57°C (H. pinetorum) for 60 s, and 72°C for 90 s. To degrade remaining primers and nucleotides, 5 μl of the PCR products were mixed with 1 μl of ExoSAP-IT (United States Biochemical, Cleveland, OH, USA) and incubated at 37°C for 15 min and 80°C for 15 min. Each purified PCR product was used in cycle sequence reactions with either of the same primers as for PCR, using the BigDye Terminator Cycle Sequencing Kit, version 3.0 (Applied Biosystems, Foster City, CA, USA). The sequences were determined bi-directionally using an ABI 3130 automated DNA sequencer (Applied Biosystems). The nucleotide sequences reported in the

<table>
<thead>
<tr>
<th>No.</th>
<th>Sea area</th>
<th>Site</th>
<th>H. dubius</th>
<th>H. pinetorum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sea of Japan</td>
<td>Off Rumoi</td>
<td>33</td>
<td>3*</td>
</tr>
<tr>
<td>2</td>
<td>Off Ohda</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Okhotsk Sea</td>
<td>Off Abashiri</td>
<td>29*</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NW Pacific</td>
<td>Off Kushiro</td>
<td>31</td>
<td>30*</td>
</tr>
<tr>
<td>5</td>
<td>Off Hachinohe</td>
<td>1</td>
<td>8*</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Off Miyako</td>
<td>14</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Yellow Sea</td>
<td></td>
<td>27**</td>
<td></td>
</tr>
</tbody>
</table>

*Total DNA was extracted by Yanagimoto et al. (2007)
**Sequence data were referred from Yanagimoto et al. (2007)
present study have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB859317–859483. Sequences for *H. pinetorum* from the Yellow Sea are from Yanagimoto et al. (2007).

The sequences were aligned using Clustal W (Thompson et al. 1994, Jeaemougin et al. 1998) in MEGA ver. 5.0 (Tamura et al. 2007), using the default settings. The alignments were also checked visually. Transitions, transversions, and insertions/deletions were equally weighted. Phylogenetic networks were constructed by the median-joining method, using Network ver. 4.5.1.6 (Bandelt et al. 1999) based on differences in nucleotide sequences containing indels.

Based on nucleotide sequences containing indels, the genetic diversity of specimens was estimated based on both gene (haplotype) diversity (*h*), which is the probability that 2 randomly chosen sequences are different (Nei 1987), and nucleotide diversity (*π*), which is the probability that 2 randomly chosen homologous sites are different (Tajima 1983, Nei 1987), using Arlequin ver. 3.5.1.2 (Excoffier et al. 2010). The unbiased fixation index, *F*<sub>ST</sub> (Weir & Cockerham 1984), was estimated and its significance was tested using a nonparametric permutation approach (10,000 permutations) performed with Arlequin. The significance of population structure was tested using analysis of molecular variance (AMOVA) via a permutational approach (Excoffier et al. 1992) with Arlequin. The demographic population history was analyzed based on the mismatch distributions of pairwise differences between all individual pairs. Probable population expansion was examined by performing mismatch distribution analysis (Rogers & Harpending 1992) using Arlequin.

### Results

#### *Hippoglossoides dubius*

There were 66 different kinds of sequences (haplotypes) identified from 99 specimens. Out of 475 sites, 43 were variable; 5 sites contained indels, and transition/transversion bias was 1.978. Each of three dominant haplotypes was obtained from more than 6 specimens from both the Sea of Japan and the Pacific Ocean. Each of the remaining haplotypes was obtained from less than 4 specimens from a single sea area. Figure 2a shows the median-joining network based on 43 variable sites among the haplotypes, which can be classified as the same phylogeographic pattern as *Malacocottus zonurus*, *Tanakius kitaharai*, and *Glyptocephalus stelleri* (phylogeographic category IV; Avise 2000).

The genetic diversity of local *H. dubius* populations is summarized in Table 2. The test performed to examine the unbiased *F*<sub>ST</sub> between local populations where more than 10 specimens were available, showed no significant genetic difference (*P*>0.05). Mismatch distribution analysis showed that all individuals (mismatch observed mean=3.965, *r*=3.887, *θ*<sub>0</sub>=0.019, *θ*<sub>1</sub>=99999, SSD=0.0009, *P*=0.450; Fig. 3a), the local populations of the Sea of Japan (mismatch observed mean=3.686, *r*=3.748, *θ*<sub>0</sub>=0.000, *θ*<sub>1</sub>=99999, SSD=0.0016, *P*=0.390), and the Pacific Ocean (mismatch observed mean=4.315, *r*=3.451, *θ*<sub>0</sub>=0.724, *θ*<sub>1</sub>=99999, SSD=0.0016, *P*=0.480) have all experienced an expansion in population size.

![Fig. 2. Median-joining network of haplotypes of (a) *H. dubius* and (b) *H. pinetorum*. The area of the circles is proportional to the frequency of occurrence of the haplotypes. The black, gray, hatched, and white sectors indicate the relative frequency of specimens from the Sea of Japan, the Okhotsk Sea, the Yellow Sea, and the northwestern Pacific, respectively. I and II denote the first and second clusters, respectively, of *H. pinetorum.*](image-url)
On the basis of nucleotide sequences in the 3′ part of the mitochondrial control region (429–430 base pairs), 101 haplotypes were identified from 137 specimens. Of 430 sites, 74 were variable; a single site contained insels, and transition/transversion bias was 4.431. On the median-joining network (Fig. 2b), haplotypes formed 2 distinct groups, resembling that of *Bothrocara hollandi* (Kodama & Kojima 2009, Kojima et al. 2011). However, significant differences were the presence of specimens from all 4 sea areas in both the groups. Therefore, a third phylogeographic pattern is proposed, corresponding to phylogeographic category II of Avise (2000). Such a pattern has not been reported in demersal fish populations inhabiting deep regions in the Sea of Japan. The second cluster (II in Fig. 2b) consists of fewer specimens (N=17) than the first cluster (N=110). The frequency of specimens of the second cluster was higher in the Sea of Japan (21.7%) than in the Okhotsk Sea (10.3%), the Yellow Sea (11.1%), or the northwestern Pacific (10.3%).

The genetic diversity of local *H. pinetorum* populations is summarized in Table 2. The test performed to examine the unbiased $F_{ST}$ between populations where more than 10 specimens were available showed a significant genetic difference between local populations off Ohda and off Kushiro ($P<0.05$). No significant genetic structure was apparent among local populations within the Sea of Japan, Okhotsk Sea, the Yellow Sea, or the Pacific Ocean (AMOVA; $P>0.05$) nor among the four sea areas (AMOVA; $P>0.05$).

Mismatch distribution analysis showed that the Sea of Japan population (mismatch observed mean=6.664, $r=3.438$, $\theta_{e}=4.217$, $\theta_{l}=99999$, SSD=0.0043, $P=0.720$), the Yellow Sea population (mismatch observed mean=6.385, $r=5.092$, $\theta_{e}=1.225$, $\theta_{l}=99999$, SSD=0.0079, $P=0.190$), and the Pacific Ocean population (mismatch observed mean=5.396, $r=3.037$, $\theta_{e}=2.000$, $\theta_{l}=99999$, SSD=0.0031, $P=0.440$) have experienced an expansion in population size while all individuals together and the Okhotsk Sea population have not ($P<0.05$). In contrast to *H. dubius*, mismatches among all individuals of *H. pinetorum* showed a bimodal distribution (Fig. 3), which supports the existence of 2 distinct groups.

**Discussion**

The congeneric species *Hippoglossoides dubius* and *H. pinetorum* show contrasting phylogeographical patterns
(Fig. 2). Because eggs and larvae of both species inhabit the surface layer (Miyamoto et al. 1993, Tominaga et al. 2000), it can be considered that *H. dubius* and *H. pinetorum* would have become extinct in most regions of the Sea of Japan during the LGM when the surface was covered by fresh water flowing from the Asian Continent (Oba et al. 1991, Tada et al. 1999, Itaki et al. 2004, Yokoyama et al. 2007). Adults of both species can inhabit shallower bottom habitats than the Tsugaru Straits, which connect the Sea of Japan and the northwestern Pacific (Nakabo 2013). The present Sea of Japan populations of both species are thought to be the result of recolonization, at least in part, from other regions after the glacial period. Avise’s (2000) category IV genetic population structure of *H. dubius* can be clearly explained by this scenario.

A constant inflow of fresh seawater through the Tsushima Straits and the resultant oxic condition in the bottom layer are suggested to have occurred in the most western part of the Sea of Japan throughout the last glacial period (Gorbarenko & Southon 2000). It is considered that both species could have survived there during the last glacial period. Adults of *H. dubius* can inhabit shallower bottom habitats (up to 40 m) than *H. pinetorum* (100 m; Yamada et al. 2007, Nakabo 2013). It seems likely that only *H. dubius* could have migrated between the Sea of Japan and the East China Sea, which was inhabitable due to lower temperatures during glacial periods than at present. If so, only the Sea of Japan population of *H. pinetorum* should have been isolated and deviated genetically during the last glacial period. A small cluster of *H. pinetorum* (II in Fig. 2b) may have originated from the Sea of Japan population that had been isolated from other populations during the last glacial period as indicated by a higher frequency of this cluster in the Sea of Japan than other sea areas. Higher nucleotide diversity of *H. pinetorum* compared to *H. dubius* (Table 2) is attributable to genetic deviation between the 2 lineages.

No genetic divergence has been reported for the willowy flounder *Tanakius kitaharai* (Xiao et al. 2008) and the blackfin flounder *Glyptocephalus stelleri* (Xiao et al. 2010). They inhabit deep areas in the Sea of Japan. The shallowest habitat of *G. stelleri* is around 50 m (Yamada et al. 2007, Nakabo 2013). While Nakabo (2013) reported the depth of the shallowest habitat of *T. kitaharai* to be 100 m, Yamada et al. (2007) reported it from around 40m. The similar phylogeographic pattern suggests that *H. dubius*, *G. stelleri*, and *T. kitaharai* share a common population history.

The existence of genetically distinct groups has also been reported for some shallow-water marine fish species in the northwestern Pacific: *Chelon haematocheilus* (Liu et al. 2007), *Ammodytes personatus* (Han et al. 2012), and *Chaenogobius annularis* (Hirase et al. 2012). This demonstrates that isolation in the deep-sea basin is not the unique cause of genetic breaks among marine fish species in this area. Thus, the phylogeographic patterns of deep-sea demersal fish might not be formed only by larval vertical migration and bathymetric distribution of adults. Differences in life history and adaptive ability to environmental changes as well as accidental events might have affected the pattern, which suggests a requirement for more detailed ecological research on deep-sea species.

The Sea of Japan is an interesting region for phylogeographic research because of the periodic environmental changes that occurred during the Quaternary. The present results show that the population structures of deep-sea organisms inhabiting the Sea of Japan is more diverse than expected. In order to reveal how such environmental changes formed the unique fauna of this region, it is necessary to compare population structures among various marine species. This will be of use in predicting the effects of changes in the marine environment caused by human activities, such as global warming and ocean acidification.

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**References**


