African Journal of Biotechnology Vol. 7 (21), pp. 3836-3844, 5 November, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Genetic variation and population structure of willowy flounder *Tanakius kitaharai* collected from Aomori, Ibaraki and Niigata in Northern Japan

Yongshuang Xiao[1], Masakazu Takahashi[4], Takashi Yanagimoto[2], Yan Zhang[3], Tianxiang Gao[1]*, Mamoru Yabe[4] and Yasunori Sakurai[4]

[1]Key Laboratory of Mariculture, Ministry of Education of China, Ocean University of China, Qingdao, 266003, China.
[2]National Research Institute of Far Seas Fisheries, Fukuura, Kanazawa-ku, Yokohama, 236-8648, Japan.
[3]Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, 266071, China.
[4]Graduate School of Fisheries Sciences, Hokkaido University, Minato-cho, Hakodate, Hokkaido, 041-8611, Japan.

Accepted 3 July, 2008

The first hypervariable region (HVR-1) of the mitochondrial DNA control region was utilized for determination of genetic variation and population structure in willowy flounder (*Tanakius kitaharai*) collected from Aomori, Ibaraki and Niigata. A total of 35 haplotypes were detected among 66 individuals with a total of 30 variable sites out of 387 bp sequenced. Average sequence differences between populations (1.0 - 1.1%) were comparable to those within populations (0.9 - 1.2%), suggesting no genetic heterogeneity among samples. The pattern of distribution of genetic variability with high level of haplotype diversity (h = 0.94) and moderate nucleotide diversity (π = 1.0%) was also detected in the HVR-1 region of the mitochondrial DNA control region. AMOVA tests and the conventional population Φ_{st} comparisons revealed no significant genetic structure among the populations. Partitioning populations into coherent geographic groups divided willowy flounder samples (Φ_{ct} = -0.007, P > 0.05) into two major groups: a Sea of Japan group composed of Aomori and Niigata populations; a Pacific Ocean group composed of Ibaraki populations. The minimum spanning tree constructed with 35 haplotypes showed four low-divergent clades, corresponding to those defined in the NJ tree. However, these clades did not appear to have geographic structure. Altogether, the results indicate that willowy flounder is panmictic throughout the examined range in Aomori, Ibaraki and Niigata.

Key words: *Tanakius kitaharai*, mitochondrial DNA control region, genetic variability, genetic structure.

INTRODUCTION

Willowy flounder, *Tanakius kitaharai* (Jordan and Starks, 1904), belonged to the righteye flounder; family, Pleuronectidae, is a medium-sized flatfish, that inhabits the sandy bottom shallower than 400 m depths off southern Hokkaido southward of Japan, as well as the Yellow Sea, the Gulf of Po-Hai, East China Sea and around Taiwan (Sakamoto, 1984). The species is an important fisheries resource, especially for bottom trawlers in the North Pacific Ocean off the coast of Japan. With a rapid increase of total catches of willowy flounder, the species has been facing high fishing pressure (Narimatsu et al., 2007). And annual catches by bottom trawlers also have changed significantly over the last 30 years, from more than 200 t in the 1970s to less than 20 t in late 1980s (Narimatsu et al., 2006). Catches subsequently increased up to 240 t in 1998 and 1999, but decreased to approximately 100 t from 2001 to 2004. Catches of willowy flounder in Aomori and Ibaraki also decreased from 1996 to 2003 (Narimatsu et al., 2006). Therefore, the investigation of the genetic diversity and structure of local populations in Aomori, Ibaraki is necessary for stock management of the species. Although

*Corresponding author. E-mail: gaozhang@ouc.edu.cn.

Abbreviation: HVR-1, The first hypervariable region; **AMOVA**, Analysis of molecular variance.

Table 1. Sampling data of *Tanakius kitaharai* including sample abbreviation (ID), Locations, Sample size, Number of haplotypes and date of collection.

ID	Location	Date of collection	Sample size	No. of haplotypes	h	Л	S	k
Aom	Aomori	Jul. 2006	25	18	0.95	0.012	25	4.47
Iba	Ibaraki	Oct. 2005	13	11	0.97	0.011	13	4.24
Nii	Niigata	Jul. 2005	28	19	0.93	0.009	18	3.61
Total			66	35	0.94	0.010	30	3.93

Several diversity indices for HVR-1 region were also indicated.

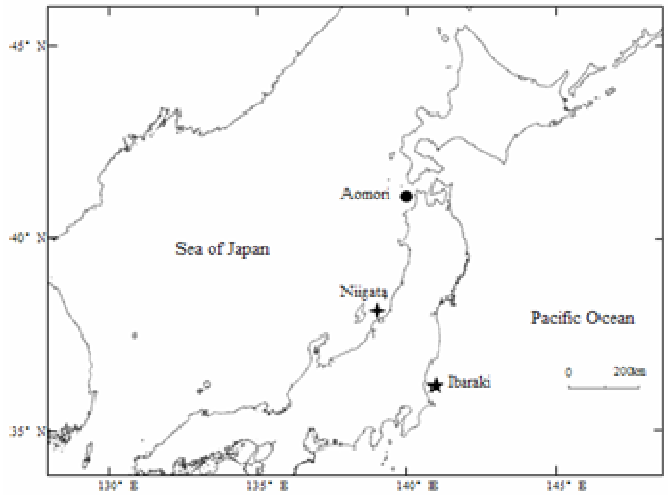

Figure 1. Map showing sample locations of *Tanakius kitaharai*, samples are marked by localities that correspond to Table 1.

a number of studies such as the early life stages with pelagic eggs and planktonic larval history, age, somatic growth, and reproductive mode for the species have been made, the basic biological information obtained so far is not enough to elucidate the population structure (Fujita, 1965; Yabuki, 1989; Minami, 1983; Hashimoto, 1995; Shimamura and Igarashi, 2000; Yagishita et al., 2005; Narimatsu et al., 2007). Also there is no information about the genetic diversity and population genetic structure researches of the wild fish.

Assessments of intraspecific genetic diversity and population genetic structure can provide important biology and evolutionary data and are invaluable for the successful conservation or management of exploited species (McMillen-Jackson and Bert, 2004). From a biological perspective, the assessment can imply whether the species is panmictic throughout its range. From a management perspective, an accurate definition of population structure is important for the management of commercial marine fishes (Utter, 1991). Failure to detect population units can lead to local overfishing and ultimately to severe declines (Waples, 1998).

Mitochondrial DNA (mtDNA) has been used in population studies because of its compactness, almost total maternal inheritance and fast evolutionary rate compared to nuclear DNA (Brown et al., 1982; Wilson et al., 1985). The highest rates of base substitutions and insertion/deletion events in mtDNA have been found in the first half of the mitochondrial DNA control region (adjacent to the proline transfer RNA gene), probably because of reduced functional constraints (Saccone et al., 1987; Prager et al., 1993; Lee et al., 1995). Therefore, population studies of fishes have focused on sequences in the control region (Fujii and Nishida, 1997; Stepien, 1999; Guarniero et al., 2002; Sekino et al., 2002). In the present study, to expand our understanding of the genetic diversity and population structure of willowy flounder off the coast of Japan, we also analyzed the DNA sequences from the first hypervariable region (HVR-1) of the mitochondrial DNA control region in willowy flounder collected from Aomori, Ibaraki and Niigata. The assessments of genetic diversity and population structure of willowy flounder will provide useful insight into management practices of the species.

MATERIALS AND METHODS

Sample collection

Sixty-six specimens were collected from Aomori, Ibaraki and Niigata in 2005 and 2006 (Figure 1, Table 1). All individuals were identified based on morphological features, and a piece of muscle tissue was obtained from each individual and preserved in 95% ethanol or frozen for DNA extraction. Two individuals of *Glyptocephalus stelleri* were also collected to serve as outgroup for phylogenetic analyses.

DNA extraction, amplification and sequencing

Genomic DNA was isolated from muscle of willowy flounder. Muscle tissues were digested with proteinase K in a buffer of 10 mM Tris-HCl (pH 7.5) with 125 mM NaCl, 50 mM EDTA, 8 M urea, and 1% SDS by at 50℃ temperature and purified by standard phenol-chloroform extraction. Fish primers L-15926 (5′-TCAAAGCTTACAGTCTTGTA-3′) and DL-R (5′-TGGTGGTTGCTCCCGCTTATG -3′) (Kocher et al., 1989), which target a portion of transfer RNA (tRNA)-pro and the central conserved region of the control region were used in a polymerase chain reaction (PCR) to amplify the first hypervariable region (HVR-1). The PCR amplification was carried out in a thermal cycler 9700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial 3 min denaturation at 94℃, and 39 alternating cycles of 1 min at 94℃ for denaturation, 1 min at 50℃ for annealing, and 1 min at 72℃ for extension, and a final extension

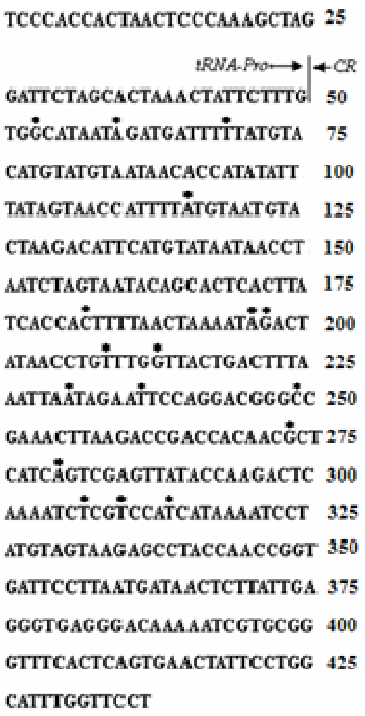

Figure 2. Nucleotide sequence from the 3' end of the tRNApro gene to the central part of the control region of the mtDNA for one individual of *Tanakius kitaharai*. Asterisks indicate parsimoniously informative variable sites in 35 haplotypes

at 72°C for 10 min. A total of 2 µL of each PCR product was used for 1% agarose gel electrophoresis for verifying the amplified fragment length with a standard size marker (TaKaRa, Dalian). PCR products were purified with Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai). Both strands were sequenced using the BigDye Terminator Cycle Sequencing Kit (ver.2.0, PE Biosystems, Foster City, California) and run on an ABI Prism 377 (Applied Biosystems) automatic sequencer according to the manufacturer's recommendations. The primers used for sequencing were the same as those for PCR amplification.

Sequence alignment and data analysis

All sequences were edited and aligned using Dnastar software (DNASTAR Inc., Madison, USA). Molecular diversity indices such as the number of haplotypes, polymorphic sites, transitions, and transversions were obtained using the program ARLEQUIN version 2.000 (Schneider et al., 2000). Haplotypes diversity (*h*), nucleotide diversity (π) and the mean number of pairwise sequences differences (*k*), and their corresponding variances were calculated following Nei (1987) as implemented in ARLEQUIN (Tajima, 1983; Nei, 1987). A mismatch distribution of pairwise nucleotide differences among HVR-1 region haplotypes was compared with the expectations of a sudden-expansion model (Rogers, 1992). The gamma distribution with shape parameter Γ for the rate heterogeneity among sites and nucleotide sequence evolution models were evaluated using likelihood-ratio tests implemented by the program Modeltest version 3.06 (Posada and Crandall, 1998). A hierarchical molecular variance analysis (AMOVA) and the conventional population Φ_{st} comparisons performed in ARLEQUIN were used to test for significant population genetic structure within willowy flounder (Excoffier et al., 1992). The haplotypes were permuted 1,000 times in AMOVA test and the significance of pairwise population Φ_{st} was tested by 10,000 permutations. The significance threshold of pairwise Φ_{st} was always adjusted by sequential Bonferroni correction (Rice, 1989). Genetic relationship among haplotypes of the species for control region data was reconstructed using the neighbor-joining method implemented in PAUP* (Saintou and Nei, 1987). Genetic distances were generated of phylogenetic reconstruction using the best fit model of Tamura and Nei corrected with the shape parameter of a gamma distribution (TrN + Γ, Γ = 0.85) substitution suggested by Modeltest (Tamura and Nei, 1993). The minimum spanning tree of haplotypes, drawn by hand, was based on the output (Excoffier, 1993).

RESULTS

Intraspecific sequence variation

A fragment of 480 base-pair (bp) in length was amplified after PCR and 437 bp sequenced unambiguously. Comparison with the sequence from the carp verified that the obtained sequence included part of the $tRNA^{pro}$ gene (50 bp) and the first hypervariable control region (HVR-1) (387 bp) (Chang et al., 1994) (Figure 2).

No intraspecific polymorphism was found in the 50 bp partial segment of $tRNA^{pro}$ gene. A total of 30 variable sites (17 parsimony informative sites) were checked within the HVR-1 fragment (Figures 2, 3), which defined 31 substitutions: 28 transitions and 3 transversions. Both transitions and transversions were also observed at the position 3. No indels were found in HVR-1 (Figure 3). High frequency of variable sites (> 50%) was detected at positions 143 - 187 and 253 - 267. The nucleotide composition of the fragment of control region was A□T-rich (A, 33%; T, 31%), and variations consisted predominantly of transition substations (Ti: Tv = 4.0), as is usual for this region in many flat-fishes (Fujii and Nishida, 1997; Stepien, 1999; Guarniero et al., 2002). A total of 35 haplotypes were defined by the 30 variable sites in the 66 individuals. Nine haplotypes were shared among two or more individuals, and 26 (74.3%) thus had unique haplotypes (Figure 3). Five of the nine shared haplotypes were represented in all sites. Shared haplotype h3 was the most common, occurring in 14 individuals and at all sites (Aomori, *n* = 5; Ibaraki, *n* = 2; Niigata, *n* = 7). Shared haplotype h2 was mainly occurring at Aomori population (*n* = 3), and shared haplotype h8 was mainly occurring at Niigata population (*n* = 3) (Figure 3).

Intrapopulation diversity indices are shown in Table 1. Overall, low level of intrapopulation genetic diversity was observed. Of all the populations examined, the haplotype diversity, nucleotide diversity and average number of pairwise nucleotide differences were 0.94, 1.0% and 3.93, respectively. The pairwise sequence difference of HVR-1 with an average within Ibaraki population (1.1%) was comparable with that within Aomori population (1.2%). The value within Niigata population (0.9%) was the lowest among the three populations. Pairwise sequence differences with an average between populations ranged from 1.0 to 1.1%. The mean numbers of pairwise sequences

Haplotype	Nucleotide position 11111 1111111111 1222222222 111611234 4455667888 9012355666 3059635923 6729232137 9563037047	Geographic population Aomori	Ibaraki	Niigata
Shared h12(2)	GAACACGCTA AGTTGGTAAT CCCGAATCTT	1		1
Shared h1 (3)	...T....C. ....T..	1	1	1
Shared h2 (4)	...T....C. ....C ....T..	3		1
Shared h3 (14)	...T....C. .....A.... ....T..	5	2	7
Shared h8 (4)	...T....C. ...C...... ....T..	1		3
Shared h9 (5)	...T....C. .....A.G.. T......T..	2	1	2
Shared h18(3)	...T....C. ...C...... T.........	1	1	1
Shared h20(3)	.G.T...... ...C...... ....T..		2	1
Shared h25(2)	...T....C. .....A.G.. ....T..		1	1
Aom h4 (1)	.... .T...A....	1		
Aom h5 (1)	A..T....C. .....A.... ....T..	1		
Aom h6 (1)	....G..... .... T.........	1		
Aom h7 (1)	A..T....C. ..C..A.G.. T......T..	1		
Aom h11(1)	.......T.. .T...A.G.. ....G.....	1		
Aom h13(1)	...T....C. ......C... ....TC.	1		
Aom h14(1)	...T....C. .....A.... ....TC.	1		
Aom h15(1)	........C. ....A...G. ....T..	1		
Aom h16(1)	...T....CG .....A.... ....TCC	1		
Aom h17(1)	T....A..C. ....TA.GCT..	1		
Aom h10(1)	...T....C. ...C......	1		
Iba h19(1)	...T....C. ....TC.		1	
Iba h21(1)	...T....C. G..C...... T.........		1	
Iba h22(1)	.... .T...A.... .T........		1	
Iba h23(1)	.... .T...A.... ....G..T..		1	
Iba h24(1)	.G.T....C. ...C...... ....T..		1	
Nii h26(1)	.... .T...A.G.. T...G.....			1
Nii h27(1)	...T....C. .....A.... T......T..			1
Nii h28(1)	....G.....			1
Nii h29(1)	...T....C. ...C...... ...A......			1
Nii h30(1)	...T....C. ...C...G.. T.........			1
Nii h31(1)	...T....C. G..C......			1
Nii h32(1)	...T....C. G..C...... ....T..			1
Nii h33(1)	T.G.....C. ....CT..			1
Nii h34(1)	...T....C. ...C...... T......T..			1
Nii h35(1)	...T..A.C. ...C...... T.........			1

Figure 3. Nucleotide variation and frequencies of the HVR-1 region haplotypes in *Tanakius kitaharai* samples. "." , identical base.

differences for Aomori (m = 4.44), Ibaraki (m = 4.35) and Niigata (m = 3.55) samples were also obtained from the mismatch analysis (Figure 4). There were more haplotypes than variable sites in the species, which indicated homoplasy, a common outcome in control region surveys (Liu et al., 2006a). A Γ value of 0.85 was calculated for willowy flounder, also suggesting strong homoplasy of substitution rates among nucleotide sites in its HVR-1.

Genetic structure analysis

The population structure was estimated from Aomori, Ibaraki and Niigata populations. All of the pairwise Φ_{st} values (Table 2) were low and statistically not significant. Moreover, it was remarkable that negative Φ_{st} values were observed in the pairwise comparisons among populations. And all the Φ_{st} values observed were not significantly different after application of the sequential Bonferroni correction, suggesting a pattern of overall mitochondrial DNA control region homogeneity of willowy flounder in Aomori, Ibaraki, and Niigata populations. In addition, we examined the population structure using 2 sets of hierarchical AMOVAs. First, we used entire data as one group to check the population structure. Second, we divided populations into two groups according to natural geographic boundary: a Sea of Japan group composed of Aomori and Niigata populations; a Pacific Ocean group

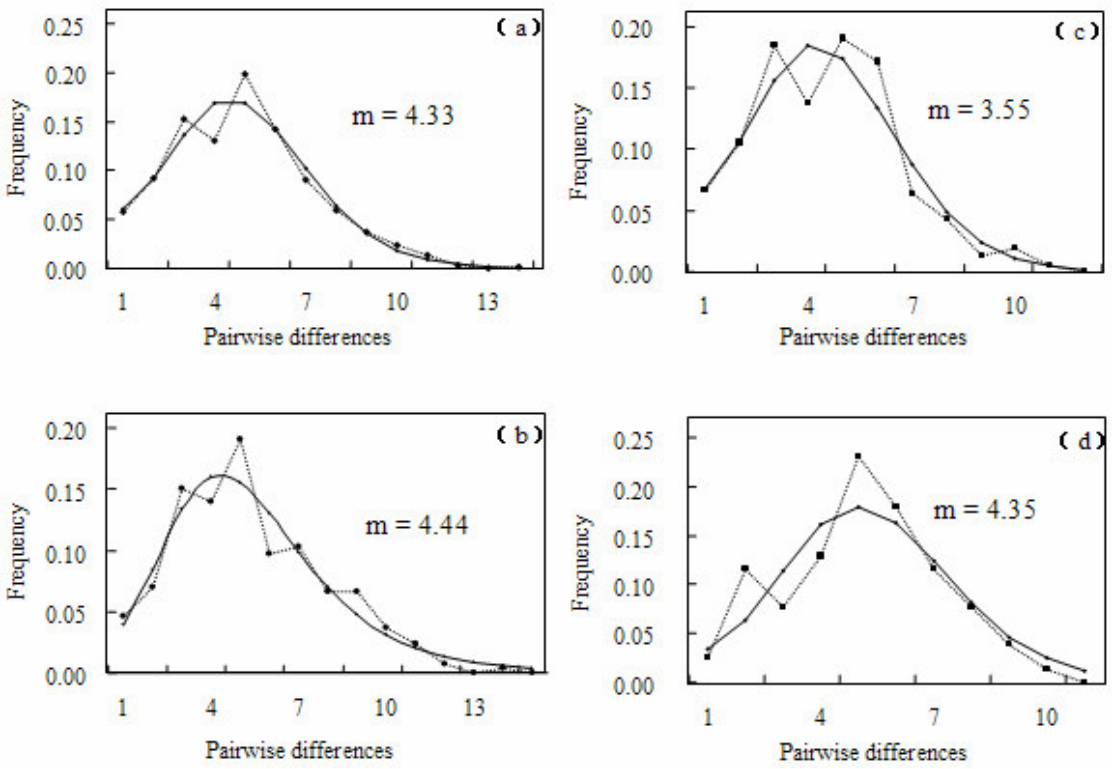

Figure 4. Distribution of pairwise differences (—, expected; ⋯⋯, observed) in the *Tanakius kitaharai* populations (*m*, observed mean of the Pairwise sequences differences). (a) Pooled populations; (b) Aomori population; (c) Niigata population; (d) Ibaraki population.

Table 2. AMOVA analysis and estimates of Φ_{st} values, and associated *P* values based on HVR-1 region sequence data.

Variance component	Variance	% total	Φ Statistics	P-value
A. Entire data set				
Among geographical populations	-0.013	-0.65		
Within geographical populations	2.040	100.65	Φ_{st}= -0.007	0.61
B. Geographical character data				
Among geographical groups	-0.014	-0.68	Φ_{ct} =-0.007	0.66
Among geographical populations within groups	-0.003	-0.13	Φ_{sc} = -0.001	0.85
Within geographical populations	2.040	100.81	Φ_{st} = -0.008	0.61
Aom vs. Iba			Φ_{st} = -0.006	0.50
Aom vs. Nii			Φ_{st} = 0.003	0.35
Iba vs. Nii			Φ_{st} = -0.028	0.85

composed of Ibaraki population (Table 2). The AMOVA, incorporating both sequence divergences and haplotype frequencies among populations, detected no significant structuring among populations within one group (Φ_{st} = -0.007, P > 0.05) according to the permutation tests. Population subdivision was also not significant using natural geographic boundary as the criterion (Φ_{ct} = -0.007, P > 0.05). Negative values were obtained for some comparisons. Negative values indicate great difference between two random individuals from the same population, rather than between two random individuals from different populations (Aris-Brosou and Excoffier, 1996) (Table 2).

Genetic relationships

The NJ tree constructed on the Tamura-Nei distances

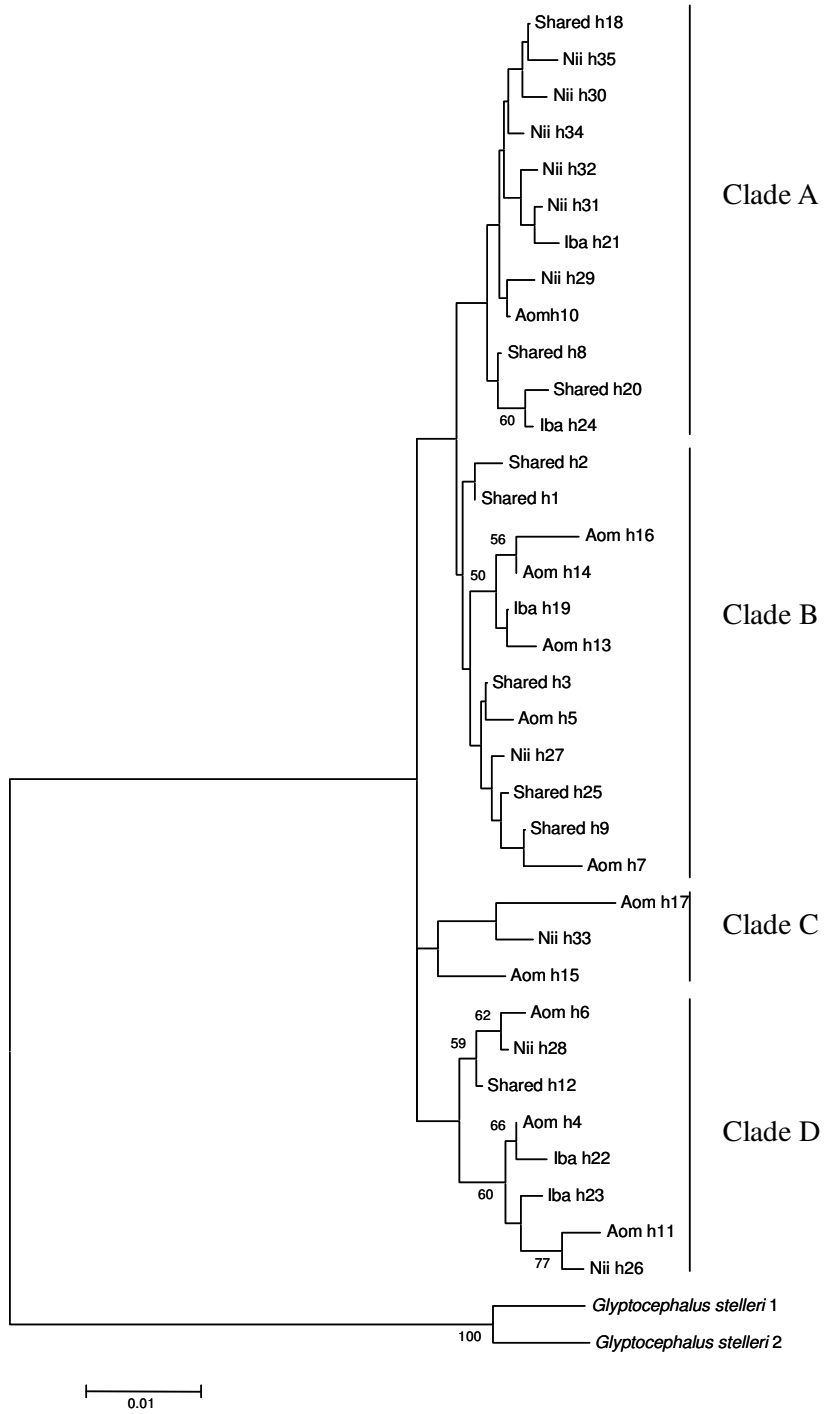

Figure 5. Neighbor-joining tree constructed using TrN distances with a gamma shape parameter of 0.85 for 35 HVR-1 region haplotypes of *Tanakius kitaharai*.

corrected with the shape parameter of a gamma distribution (α = 0.85) showed that the 35 haplotypes of willowy flounder were assigned into four closely related groups (Clade A, B, C, and D) (Figure 5). The clades A, B, C, and D included 12 (Aomori, *n* = 3; Ibaraki, *n* = 4; Niigata, *n* = 9), 12 (Aomori, *n* = 9; Ibaraki, *n* = 5; Niigata, *n* = 6), 3 (Aomori, *n* = 2; Niigata, *n* = 1), and 8 (Aomori, *n* = 4; Ibaraki, *n* = 2; Niigata, *n* = 3) haplotypes comprising 19 (Aomori, *n* = 3; Ibaraki, *n* = 5; Niigata, *n* = 11), 35 (Aomori, *n* = 16; Ibaraki, *n* = 6; Niigata, *n* = 13), 3 (Aomori, *n* = 2; Niigata, *n* = 1), and 9 (Aomori, *n* = 4; Ibaraki, *n* = 2; Niigata, *n* = 3) individuals, respectively. As expected from sequence

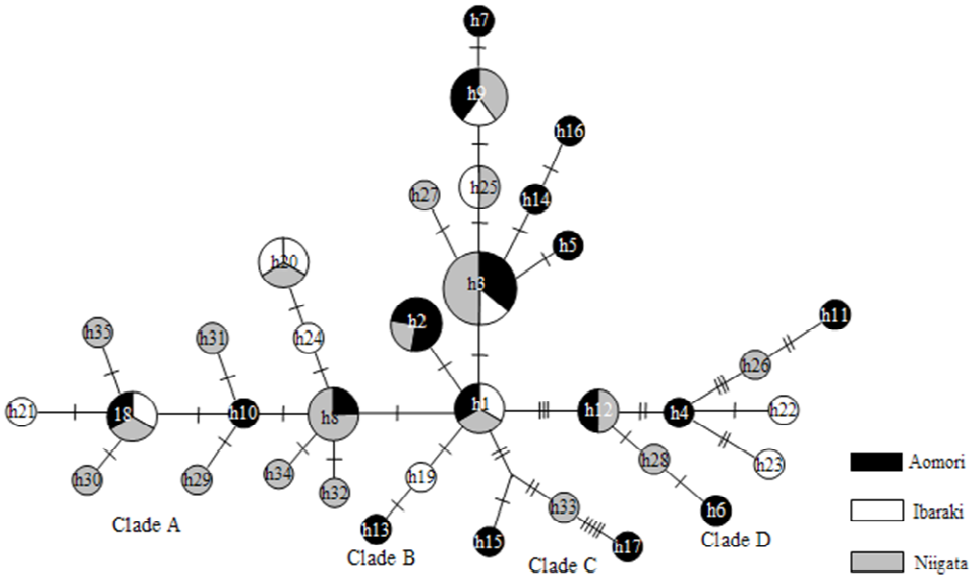

Figure 6. Unrooted minimum spanning trees showing genetic relationship among HVR-1 region haplotypes for *Tanakius kitaharai*.

variability and the NJ tree, minimum spanning tree appeared complex in willowy flounder (Figure 6). Minimum spanning tree of the species also identified four clades, corresponding to those defined in the NJ tree (Figures 5, 6). However, these clades did not appear to have geographic structure (Figure 6).

DISCUSSION

Variability of HVR-1 region in *T. kitaharai*

The mtDNA control region has been documented to be particularly sensitive in detecting genetic diversity and genetic structure of marine fishes and has been used for analysis of populations in various fishes. Low level of genetic diversity was observed in willowy flounder. The most notable finding in the present study was the pattern of distribution of genetic variability with high haplotype diversity (h = 0.94) and moderate nucleotide diversity (π = 1.0%) in HVR-1 region. Stepien (1999) also discerned the type of distribution of genetic variability (high h and moderate π) in mtDNA left-domain control region sequences of Dover sole populations.

There are two possible reasons for the pattern of distribution of genetic variability with high haplotype diversity (h) and moderate nucleotide diversity (π) in HVR-1 region of willowy flounder. Theoretically, the level of genetic variability held in a population tends to be positively correlated with the effective population size (Fujii and Nishida, 1997). High haplotype diversity (h) suggests large, stable, effective population sizes over time in the continental shelf fishes (Stepien, 1999). Thus, one possible reason is that willowy flounder might have continued having large population size and the high haplotype diversity (h) has been maintained in it. Indeed, the species is distributed widely off the coast of Japan. Over 200 t of the species was caught in a single year (Narimatsu et al., 2006). Alternatively, the fast evolution of the mtDNA control region may also be responsible for the high haplotype diversity (h). It has been postulated that the large number of haplotype, separated by only one or few mutations, may be produced by rapid expansion of the population, rapid population growth enhancing the retention of new mutations (Rogers and Harpending, 1992). Willowy flounder showed high levels of haplotype diversity but relatively low sequence divergence in the HVR-1 region, which means the species may have experienced population expansion. Slatkin and Hudson (1991) also believed that this type of distribution of genetic variability (high h and moderate-to-low π) might imply the population experienced expansion after the bottleneck. The mis- match analysis showing a smooth wave predicted for willowy flounder that had undergone a demographic expansion (Figure 4). Thus, the other possible reason is that the population expansion may be responsible for the moderate nucleotide diversity (π) in HVR-1 region of willowy flounder.

Population structure

The pairwise Φ_{st} statistics and AMOVA were used to test for the patterns with loss of genetic structure and check the hypothesis of the geographic isolation (the Sea of Japan and Pacific Ocean) for the species in Aomori, Ibaraki, and Niigata. No significant population structure and genetic differentiations corresponding to the Sea of

Japan and Pacific Ocean existed throughout the examined range of the species. Marine fishes generally show low levels of genetic differentiation among geographic region due to higher dispersal potential during planktonic egg, larval, or adult history stages coupled with an absence of physical barriers to movement between ocean basins or adjacent continental margins (Palumbi, 1994; Hewitt, 2000). According to our results, willowy flounder conforms to this pattern. Willowy flounder spawn pelagic eggs, larvae undergoing a planktonic life for more than one month before settling on the bottom (Fujita, 1965). Along the North-western coast of Japan, eggs and larvae could be transported by Tsushima Current, which flows from the Southwest to the Northeast. Therefore, Aomori population could get direct recruitment of larvae from the South-western areas. The Tsugaru Strait is the exclusive channel for eggs and larvae of willowy flounder between Aomori and Ibaraki. The Tsushima Current through the Tsugaru Strait collides with Oyashio Current off the eastern shore of Japan, and the eggs and larvae in the Tsushima Current may be forced to flow south following Oyashio Current, So Ibaraki population could get recruitment of larvae from the North-eastern areas. The Ibaraki population could also get direct recruitment of larvae from the South-western areas of Japan based on Kuroshio Current flowing from the Southwest to the Northeast. The phenomenon of migration (from northern waters to southern waters) for adults of willowy flounder appears based on the species distribution densities analysis (Narimatsu et al., 2006, 2007). Adults flounder in Ibaraki may also go up North against Oyashio Current or they may be transported to North along the coast where there is subtle flow from Kurosiho Current. The pelagic eggs and planktonic larval history for willowy flounder coupled with phenomenon of migration for adults flounder, might account for the metapopulations of the species maintained in a state of panmixia. The similar results can be found in *thalasoma bifasciatum* and *verasper variegates* (in Northern Japan) with little differentiation among populations (Shulman and Bermingham, 1995; Ortega-Villaizán Romo et al., 2006). Alternatively, a late historical isolation may also be responsible for such pattern of genetic structure for willowy flounder. The minimum spanning tree (MST) and the neighbor-joining (NJ) tree showed four low-divergent clades, but these clades did not also appear to have geographic structure.

In summary, the present mtDNA analysis of willowy flounder showed a pattern of distribution of genetic variability with high haplotype diversity (h) and moderate nucleotide diversity (π) in HVR-1 region, and no significant population genetic structure was checked among the geographic populations in Aomori, Ibaraki, and Niigata. In order to have further insight into the population genetic structure of willowy flounder in Japan, additional populations are needed throughout the range of the species and the historical demography of the species also should to be discerned.

ACKNOWLEDGMENTS

We are grateful to Professors Naritoshi Chow and Syuiti Abe for going through the paper. We also thank Mr. Nwafili Sylvanus and Ms. Mecklenburg for correcting the English. The study was supported by State 863 High-Technology R&D Project of China (No. 2006AA09Z418), the National Natural Science Foundation of China (No. 30471329) and The National Key Basic Research Program from the Ministry of Science and Technology, P.R. China (No. 2005CB422306).

REFERENCES

Aris-Brosou S, Excoffier L (1996). The impact of population expansion and mutation rate heterogeneity on DNA sequence polymorphism. Mol. Biol. Evol. 13: 494-504.

Brown WM, Prager EM, Wang A, Wilson AC (1982). Mitochondrial DNA sequences of primates: Tempo and mode of evolution. J. Mol. Evol. 18: 225-239.

Chang YS, Huang FL, Lo TB (1994). The complete nucleotide sequence and gene organization of carp (*Cyprinus carpio*) mitochondrial DNA gene. J. Mol. Evol. 38: 138-155.

Excoffier L, Smouse PE, Quattro JM (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics 131: 479-491.

Excoffier L (1993). MINSPNET: Minimum Spanning Network. Department of Anthropology, University of Geneva, Switzerland.

Fujita S (1965). Early development and rearing of two common flatfishes, *Eopsetta grigorjewi* (Herzenstein) and *Tanakius kitaharai* (Jordan et starks). Bull. Japan. Soc. Sci. Fish. 31: 258-262.

Fujii T, Nishida M (1997). High sequence variability in the mitochondrial DNA control region of the Japanese flounder *Paralichthys olivaceus*. Fish. Sci. 63: 906-910.

Guarniero I, Franzellitti S, Ungaro N, Tommasini S, Piccinetti C, Tinti F (2002). Control region haplotype variation in the central Mediterranean common sole indicates geographical isolation and population structuring in Italian stocks. J. Fish Biol. 60: 1459-1474.

Hashimoto R (1995). Studies on the age of *Tanakius kitaharai* (Jordan & Starks). Bull. Tohoku Reg. Fish. Res. Lab. 4: 156-164.

Hewitt GM (2000). The genetic legacy of the Quaternary ice ages. Nature 405: 907-913.

Kocher TD, Thomas WK, Meyer A, Edwards SV (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Pro. Natl. Acad. Sci. U. S. A. 86: 6196-6200.

Lee WJ, Conroy J, Howell WH, Kocher TD (1995). Structure and evolution of teleost mitochondrial control region. J. Mol. Evol. 41: 54-66.

Liu JX, Gao TX, Yokogawa KJ, Zhang YP (2006a). Differential population structuring and demographic history of two closely related fish species, Japanese se bass (*Lateolabrax japonicus*) and spotted sea bass (*Lateolabrax maculatus*) in Northwestern Pacific. Mol. Phylogenet. Evol. 39: 799-811.

Minami T (1983). The early life history of a flounder *Tanakius kitaharai.* Nippon Suisan Gakkaishi 49: 527-532.

McMillen-Jackson AL, Bert TM (2004). Genetic diversity in the mtDNA control region and population structure in the pink shrimp *Farfantepenaeus duorarum*. J. Crust. Biol. 24: 101-109.

Narimatsu Y, Yamanobe A, Takahashi M (2007). Reproductive cycle, age, and body at maturity and fecundity of female willowy flounder *Tanakius kitaharai*. Fish. Sci. 73: 55-62.

Narimatsu Y, Ito M, Hattori T, Fujiwara K (2006). Stock assessment of willowy flounder in the North Pacific Ocean off Japan in 2005. In: Stock Assessment of Fisheries Resource Around Japan. Fisheries Agency of Japan, pp 1292-1302.

Nei M (1987). Molecular Evolutionary Genetic. Columbia University Press, New York.

Ortega-Villaizán Romo M, Aritaki M, Suzuki S, Ikeda M, Asahida T, Taniguchi N (2006). Genetic population evaluation of two closely related flatfish species, the rare barfin flounder and spotted halibut, along the Japanese coast. Fish. Sci. 72: 556-567.
Prager EM, Sage RD, Gyllensten U, Thomas WK, Hübner R, Jones CS, Noble L, Searle JB, Wilson AC (1993). Mitochondrial DNA sequence diversity and the colonization of Scandinavia by house mice from East Holstein. Biol. J. Linn. Sci. 50: 85-122.
Posada D, Crandall KA (1998). Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817-818.
Palumbi SR (1994). Genetic divergence, reproductive isolation, and marine speciation. Annu. Rev. Ecol. Syst. 25: 547-572.
Rogers AR, Harpending H (1992). Population growth makes waves in the distribution of pairwise genetic difference. Mol. Biol. Evol. 9: 552-569.
Rice WR (1989). Analyzing tables of statistical tests. Evolution 43: 223-225.
Sakamoto K (1984). *Tanakius kitaharai* (Jordan et Starks). In: Masuda H, Amaoka K, Araga C, Uyeno T, Yoshino T (eds). The Fishes of the Japanese Archipelago. Tokai University Press, Tokyo, p. 339.
Shimamura S, Igarashi S (2000). Flatfish, *Tanakius kitaharai* (Jordan et Starks) caught in the coast of Fukushima region. Bull. Fukushima Pref. Fish. Exp. Start. 9: 29-52.
Saccone C, Attimonelli M, Sbiza E (1987). Structure elements highly preserved during the evolution of the D-loop-containing region in vertebrate mitochondrial DNA. J. Mol. Evol. 26: 205-211.
Stepien CA (1999). Phylogeographical structure of the Dover sole *Microstomus pacificus*: the larval retention hypothesis and genetic divergence along the deep continental slope of the northeastern Pacific Ocean. Mol. Ecol. 8: 923-939.
Sekino M, Hara M, Taniguchi N (2002). Loss of microsatellite and mitochondrial DNA variation in hatchery strains of Japanese flounder *Paralichthys olivaceus*. Aquaculture 213: 101-122.
Schneider S, Roessli D, Excoffier L (2000). ARLEQUIN, version 2,000: A Software of Population Genetic Data Analysis. University of Geneva, Geneva.
Saitou N, Nei M (1987). The neighbor-joining methods: a new method for reconstructing phylogenetic trees. Mol. Evol. 4: 406-425.
Slatkin M, Hudson RR (1991). Pairwise comparison of mitochondrial DNA sequences in stable and exponentially growing populations. Genetics 129: 555-562.
Shulman MJ, Bermingham E (1995). Early life histories, ocean currents, and population genetics of Caribbean reef fishes. Evolution 49: 897-910.
Tajima F (1983). Evolutionary relationship of DNA sequence in finite populations. Genetics 105: 437-467.
Tamura K, Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10: 512-526.
Utter FM (1991). Biochemical genetics and fishery management: an historical perspective. J. Fish Biol. 39: 1-20.
Waples RS (1998). Separating wheat from the chaff: patterns of genetic differentiation in high gene flow species. J. Hered. 89: 438-450.
Wilson AC, Cann RL, Carr SM, George M, Gyllensten UB, Helm-Bychowski KM, Higuchi RG, Palumbi SR, Prager EM, Sage RD, Stoneking M (1985). Mitochondrial DNA and two perspectives on evolutionary genetics. Biol. J. Linn. Sci. 26: 375-400.
Yabuki K (1989). Age determination of yanagimushigarei *Tanakius kitaharai* (Pleuronectidae) from otoliths in the Sea of Japan off Kyoto Prefecture. Nippon Suisan Gakkaishi 55: 1331-1338.
Yagishita N, Ohki S, Yamazaki A (2005). Age, growth and age composition of *Tanakius kitaharai* in western Wakasa Bay, Japan. Nippon Suisan Gakkaishi 71: 138-145.