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Abstract

In an attempt to develop a useful molecular marker for the genetic stock identification of chum salmon (*Oncorhynchus keta*) in the ocean, we examined the nucleotide sequences of about 500 bp variable portion from the 5' end of mitochondrial (mt) DNA control region in more than 500 individuals from 12 populations that were captured in 11 rivers: six in Hokkaido and five in Honshu, Japan. A comparison of the sequences showed 10 variable sites, defining a total of 12 haplotypes in the examined individuals. All the 12 haplotypes occurred in seven Hokkaido populations, whereas only six haplotypes were found in five Honshu populations. Among these haplotypes, two were common in all the Hokkaido and Honshu populations. The AMOVA analysis inferred a genetic differentiation among three geographic regions: Hokkaido, Pacific Ocean coast in Honshu, and Japan Sea coast in Honshu. The haplotype diversity was higher in the populations of Hokkaido than those of Honshu, indicating a greater genetic variation in Hokkaido than Honshu populations. The estimates of pairwise population F_{ST} suggested that the regional differentiation is mostly ascribed to the divergence between populations in Hokkaido and Pacific coast in Honshu. The observed nucleotide sequence variation in the mtDNA control region may be useful for the stock identification of chum salmon. Further mtDNA analysis should be conducted for other chum salmon populations in Russia, USA, and Canada under the auspices of the North Pacific Anadromous Fish Commission.

Introduction

Stock identification of salmon has been attempted by using tagging, scale characteristics, parasite tagging, thermal otolith marking, and/or protein genetic (allozyme) characters (Ishida et al.., 1989; Winans et al.., 1994; Willmot et al.., 1998; Urawa et al.., 1998). Although variability of allozymes is beneficial for genetic stock identification (GSI) of chum salmon (*Oncorhynchus keta*), allozyme analysis requires careful collection and handling of tissues (Park et al., 1993). Furthermore, resolution of allozymes remains mostly at the regional- to continental-levels (Wilmot et al.., 1998).

Recently developed molecular techniques are a powerful replacement which compensates deficits of the use of allozymes with an increase in accuracy and resolution. Because of the higher sequence variability than single copy nuclear genes (Brown et al., 1979) and clonal haploid inheritance, analysis of mitochondrial (mt) DNA has become a method of choice in phylogenetic and population genetic studies (Moritz et al.., 1987). In fact, mtDNA studies, mostly based on the analysis of restriction fragment length polymorphisms (RFLPs), has been conducted so far in many fish species including salmon (Meyer, 1993). Low levels of mtDNA sequence variation shown by RFLP analysis were reported in chum salmon (Park

et al.., 1993) and other species of *Oncorhynchus* (Wilson et al.., 1987), providing the estimates of genetic divergence similar to those obtained from allozyme analyses (Seeb and Crane, 1999a).

The control region is considered to be the most variable portion of mtDNA, showing two to five times higher rate of nucleotide substitution than the protein-coding regions (Moritz et al., 1987; Meyer, 1993). However, a low variation in the sequence of the mtDNA control region sequence of chum salmon was reported after RFLP (Cronin et al., 1993) and sequence analyses (Park et al., 1993), probably because the samples in these studies were small or collected from small populations of restricted area.

In the present study, we collected more than 500 chum salmon specimens from 11 rivers in the northern Japan to examine the variation of nucleotide sequence of mtDNA control region. Then, genetic variability within or among populations was estimated based on the sequence variation, in order to evaluate the potential of mtDNA sequence analysis in chum salmon GSI.

Materials and Methods

Samples

Liver or blood samples were collected from 537 individuals of 12 populations which returned to their natal river in the latter half of breeding season from 1996 to 1999 (Table 1). The homing migration of northern populations is earlier than southern populations, and the peak runs in Hokkaido occur about one month earlier than those in Honshu in Japan (Salo, 1991). Early run may include descendants of populations which were transferred from rivers of other areas, particularly, in Honshu. Therefore, the samples from fish returned in the latter half of breeding season were used in the present study. Mostly late run fish were obtained from six rivers in Hokkaido. A possible genetic difference, if any, between early and late run fish were examined for those collected in the Tokoro River. Late run fish were obtained from five rivers in Honshu, i.e. two in Iwate and one in Miyagi Prefecture in the Pacific Ocean coast, and one each in Akita and Yamagata Prefectures in the Japan Sea coast (Table 1). The samples from the Otsuchi River were collected at random from juveniles of late run fish in the hatchery, which were obtained by the artificial fertilization of gametes from arbitrarily selected multiple parents and kept in large aquarium. The collected samples were stored at -80°C until DNA extraction.

DNA extraction

DNA was isolated from the stored specimens following the routine phenol-chloroform method (Sambrook et al.., 1989). About 50 ml of whole blood or liver homogenate were added to 500 ml sodium tris EDTA buffer (0.1 M NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH8.0) containing 500 mg/ml proteinase K and 0.5% SDS, and incubated at 37°C overnight. DNA was extracted with a mixture of phenol (250 ml) and 24:1 chloroform:isoamylalcohol (250 ml) three times and then twice with 500 ml of 24:1 chloroform:isoamylalcohol alone. DNA in aqueous phase was recovered by ethanol precipitation, dried in air, and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

PCR amplification

The control region of mtDNA was amplified by PCR in a 100 ml of reaction mixture containing 25-100 ng of template DNA, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, and 1.5 mM MgCl2, 250 nM each dNTPs, 250 nM of forward and reverse primers, 0.001% gelatin, and 1.25 U of Taq DNA polymerase (TaKaRa, Tokyo). PCR primers (Fig. 1) were designed based on the reported sequences of rainbow trout *O. mykiss* mtDNA (Zardoya et al., 1995) and chum salmon control region (Shedlock et al., 1992). The tRNAglu is located at 5' to cytochrome b gene, but the others are in tRNA site flanking the control region. The condition of PCR amplification using a Gene Amp PCR System 9700 was as follows: preheating at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec, and elongation at 72°C for 1 min, and completed with final extensions at 72°C for 7 min. The PCR products were purified by the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) after examining the size and quality with 1.5% agarose-gel electrophoresis and ethidium bromide staining.

Nucleotide sequence analysis

The mtDNA fragment amplified with tRNAglu and tRNAphe were cloned into vector pCR2.1-TOPO with the TOPO TA cloning™ system (Invitrogen, Carlsbad, CA). After cloning, the insert was analyzed for the sequence using a Hitachi SQ-5500L DNA sequencer (Hitachi, Tokyo). The nested fragment amplified with tRNAthr-2 and tRNAphe-2 was used as a template for direct sequence analysis. Sequence reaction was preformed using a Thermo SequenaseTM pre-mixed cycle sequencing kit (Vistra

Systems, Sunnyvale, CA) according to the manufacturer's instruction. The forward and reverse sequence primers labeled with texas-red included M13 reverse and T7 primers for cloned products and four primers for direct sequence analyses, which were designed based on own sequence data of the mtDNA control region (Fig. 1).

Data analysis

The sequence data were aligned by GENETIX-WIN version 4.0.1 (Software Development Co., Ltd, Japan) to find out nucleotide variations among the sequences of control region, from which the control region haplotypes were defined. Phylogenetic relationship among the haplotypes was estimated by the neighbor-joining method (Saitou and Nei, 1987), based on genetic distance estimated by Kimura's two-parameter method (Kimura, 1980). A parsimony network which connects the observed haplotypes was drawn after Bandelt (1994).

The heterogeneity of the haplotype frequencies within and between geographic regions was evaluated using the contingency χ^2 test (Roff and Bentzen, 1989), with 1,7000 Monte Carlo simulations generated by the CHIRXC program (Zaykin and Pudovkin, 1993). Some intra- and interpopulation parameters were calculated using Arequin version 1.1 program package for the mtDNA sequence data (Schneider et al, 1997). Haplotype diversity, being equivalent to expected heterozygosity for diploid data, was calculated for each population according to Nei (1973a). In order to assess the extent of genetic differentiation at the different level of geographic hierarchy, the overall molecular variance was partitioned into component corresponding to the population divergence within and among regions using the analysis of molecular variance model (AMOVA; Excoffier et al, 1992). For AMOVA, populations were grouped geographically referring to the tree obtained by the above neighbor-joining algorism, in which the topology was tested by a bootstrap analysis with 10,000 pseudoreplicate trees. Significance of the variance components and Φ_{ST} values was tested with permutation method.

Pairwise F_{ST} values were calculated to estimate the genetic distance between populations, according to Slatkin (1995). The amount of gene flow (Nm) between populations was estimated using the approximation $F_{ST} = 1/(2M+1)$, where M = Nm for haploid data. The estimation of Nm, however, was not conducted if its validity was not supported by the neutrality test after Tajima (1989) and by the regional equilibrium test after Hutchison and Templeton (1999).

Results

Nucleotide sequence of mtDNA control region and its variation

About 2300 bp fragment was successfully amplified with a primer pair of tRNAglu and tRNAphe from DNA samples of two individuals, and cloned for sequencing by fluorochrome-labeled M13 reverse, tRNApro-3, T7, and Okdl-H1 primers. The base homology analysis with reported rainbow trout mtDNA sequence (Zardoya et al., 1995; EMBL/GenBank accession number L29771) revealed that the insert contained a 1002 bp control region sequence. It was flanked by a 3' part of cytochrome b, tRNAThr, and $tRNA^{Pro}$ at the 5' side and with a 5' part of $tRNA^{Phe}$ at the 3' side (DDBJ/EMBL/GenBank accession number AB039956), as shown in Fig. 1, in agreement with the previous findings in salmon (Shedlock et al., 1992; Zardoya et al., 1995). The observed sequence of the control region was identical with the previously reported chum salmon sequence (Shedlock et al., 1992), except for a few substitutions and indels probably reflecting an interindividual variation.

Subsequently, about 1200 bp fragment was amplified with a primer pair of tRNAthr-2 and tRNAphe-2 on DNA samples from 30 individuals from the Chitose and the Otsuchi populations. The direct sequence analysis on the fragment containing the entire control region and flanking tRNA sites was performed by fluorochrome-labeled tRNAthr-3, Okdl-L1, Okdl-H1, Okdl-H2, and tRNAphe-2. Multiple alignment of the obtained sequences revealed a cluster of variable sites (six of seven sites) within 500 bp from 5'-end of the chum salmon control region. Estimation of the 481 bp sequence in the 5' variable portion disclosed 10 variable sites in a total of 537 individuals from 12 populations, defining 12 haplotypes designated as OKDL-1 to OKDL-12, as shown in Fig. 2a. The observed variation included base substitutions and indels with an excess of transversion substitutions (six of eight substitutions). The entire chum salmon control regions sequences have been registered in the DDBJ/EMBL/GenBank with accession numbers AB039890 to AB039901. Nucleotide variation in the 3' downstream to the variable portion occurred only in the OKDL-4 haplotype (data not shown).

A parsimony network which connects the 12 control region haplotypes is presented in Fig. 2b. The observed haplotypes of chum salmon could be grouped into three clades based on the nucleotide variation,

i.e., OKDL-1 to -5 in clade 1, OKDL-9 to -12 in clade 2, and OKDL-6 to -8 in clade 3. The T to C transition at nucleotide 30 separated the clade 2 from the clade 1, and a deletion at nucleotide 386 and the C to A substitution at nucleotide 395 discriminates clade 3 from clades 1 and 2, respectively (Fig. 2a). The T to C transition at nucletotide 231 in OKDL-3, -8, and -12 was homoplasmic. The neighbor-joining consensus tree resulted from the bootstrap analysis of the 481 bp sequence and rooted with the corresponding region of the rainbow trout mtDNA showed haplotype clustering identical to that observed in the network, but with less than 50% of bootstrap support for the nodes (data not shown).

Genetic differentiation among chum salmon populations

Table 2 shows the distribution of 12 haplotypes among 12 populations of chum salmon. All the 12 haplotypes occurred in seven Hokkaido populations, whereas only six haplotypes were found in five Honshu populations. All the phylogenetic clades were found in Kawabukuro, Gakko, and Koizumi populations, whereas clade 3 was not found in Tsugaruishi and Otsuchi populations (Table 2). The OKDL-1 and -9 were common in the 12 populations. The OKDL-7 haplotype was seen in all Hokkaido and three Honshu populations. Early and late run populations from Tokoro river in Hokkaido showed essentially similar haplotype distribution, but differed in haplotype frequencies. The Hokkaido populations showed a higher level of haplotype diversity than Honshu populations (Table 2).

The contingency χ^2 test revealed a highly significant heterogeneity in the haplotype frequencies for the entire set of populations ($p<0.001$), all Hokkaido ($p<0.001$), and all Honshu populations ($p<0.005$). Such significant heterogeneity was also observed for the set of populations from Hokkaido and Pacific coast or Japan Sea coast in Honshu ($p<0.001$), although no significant heterogeneity was shown for the populations within the two regions of Honshu ($p>0.3$). These findings suggest a greater genetic difference among populations between regions than within regions.

Using the NJ method, three population clusters, Pacific coast in Honshu, all Hokkaido and the Kawabukuro, and the Gakko, were obtained on the consensus tree, with more than 90% of bootstrap support for the clusters of Pacific coast in Honshu and the Gakko, respectively (Fig. 3). Early and late runs from the Tokoro River were separated from each other, but with less than 50% of the nodal value, and the heterogeneity in their haplotype frequencies was not significant by the χ^2 test ($p>0.5$). Taking together these observations and the results of the above contingency χ^2 tests, grouping of three geographic regions, i.e. Pacific coast in Honshu, Hokkaido, and Japan Sea coast in Honshu (including both the Kawabukuro and Gakko) was considered to be most appropriate for AMOVA. The AMOVA analysis indicated that the magnitude of variance among three regional groups was moderate but significant (11.32%, $P<0.001$). However, the variance among populations within regions was very low (<1%, $P>0.1$). This fact suggests that the populations within regions are genetically indistinguishable. The most of the variation occurred within populations (87.70%, $P<0.0001$). The results of AMOVA imply that the three regional groups of populations are genetically differentiated from each other.

In fact, an increased level of genetic isolation among populations between regions was suggested for Hokkaido, except for the Yurappu, and Pacific coast in Honshu by high F_{ST} values (0.103 to 0.328) compared with other pairwise estimates, as shown in Table 3. These results imply a low level of gene flow among populations between Hokkaido and Pacific coast in Honshu. Tajima's test (1989) supported the neutrality for the mtDNA variations in all populations ($p<0.01$, $D= -2.092$ to 2.840 or $p<0.05$, $D= -1.734$ to 2.130), except for the one in the Nishibetsu population ($p>0.1$, $D= 3.7568$). In addition, the relationship between F_{ST} and geographic distance in the seven Hokkaido populations inferred a lack of regional equilibrium, i.e. absence of isolation by distance (Fig. 4). From these observations, estimation of Nm was not conducted in the present study. Correlation between genetic and geographic distance was not tested for other regions, because the number of populations was small and pairwise F_{ST} values between populations were mostly not significant.

Discussion

The sequence variation detected in the control region of Japanese chum salmon was similar to or even higher than the diversity in other *Oncorhynchus* species including *O. mykiss, O. kisutch* and *O. tshawytscha* (Nielsen et al., 1994). Thus, the number of observed haplotypes is apparently greater than those obtained from previous RFLP analysis on chum salmon mtDNA (Cronin et al., 1993; Park et al., 1993; Bickham et al., 1995; Seeb and Crane, 1999b). These findings indicate an increased potential of mtDNA sequence analysis to estimate the genetic structure of chum salmon populations.

The 12 haplotypes were phylogenetically classified into three clades (Fig. 2). Because of their

prevalence in the examined populations, the haplotype OKDL-1, -9 and -7 are thought to be ancestral one within the clade 1, 2 and 3, respectively, and hence parental to the other haplotyopes less frequent in each clade. However, the frequency of OKDL-7 was low in Honshu populations and this haplotype was not detected in the Tsugaruishi and Otsuchi (Table 2). The decreased number of haplotypes in these two populations suggest that they might have gone through the decrease of population size, which resulted in extinction of the lineage with the OKDL-7 and other haplotypes.

AMOVA results were corroborated by the contingency χ^2 test for the heterogeneity in the haplotype frequencies among populations. Genetic differentiation among the three regional groups of Japanese chum salmon, as inferred from the AMOVA, has also been suggested by the previous allozyme analysis (Kijima and Fujio, 1979). Little genetic differentiation among populations within regions may partly be associated with a level of straying rate (about 14%) in spawning migration of chum salmon (Salo, 1991), which could cause a gene exchange between river populations. The estimates of haplotype diversity and pairwise population F_{ST} strongly suggest that the regional differentiation is mostly ascribed to the divergence between populations in Hokkaido and the Pacific coast in Honshu. Probable low gene flow between Hokkaido and the Pacific coast in Honshu may be contributed by the differences in the route of spawning migration (Kijima and Fujio, 1982; Okazaki, 1986) and run timing (Salo, 1991). In fact, previous allozyme study suggested that separation by at least 600 km is required for genetic differentiation to occur (Kijima and Fujio, 1982). This estimation well fits the minimum distance between the populations of Hokkaido, except for the Yurappu, and the Pacific coast in Honshu.

The estimated genetic structure of Japanese chum salmon populations may reflect either the effect of historical factors such as the glacial advancement and resulting shift of the species range to southward, or contemporary forces such as human-mediated extensive transplantation of stocks, or both. In fact, the Hokkaido and Honshu populations have undergone extensive hatchery operations and transplantation of eggs and fry from one river population to another in history of raising commercial salmon production (Kijima and Fujio, 1982). Such an extinsive transplantation of stocks from Hokkaido might be a cause of the Kawakuburo being positioned within a cluster of Hokkaido populations (Fig. 3). Although a certain level of the geographic structure was suggested for Hokkaido populations by a previous allozyme study (Wilmot et al., 1998), no such structure was apparent in the present NJ dendrogram (Fig. 3). The reason for this discrepancy is not known at present. Absence of isolation by distance in Hokkaido population (Fig. 4) might be a consequence of an artificial transplantation of stocks among rivers. The lack of such regional equilibrium also suggests little gene flow and mostly divergence by drift in the formation of Hokkaido populations (Hutchison and Templeton, 1999). The regional equilibrium test should also be performed on Honshu using a larger number of populations. In any case, mtDNA analysis on foreign populations such as Russia, USA, and Canada will be helpful to understand how population structures in Japanese chum salmon were established.

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Table 1. Sampling location, date of collection, and number of chum salmon samples used for mtDNA analysis.

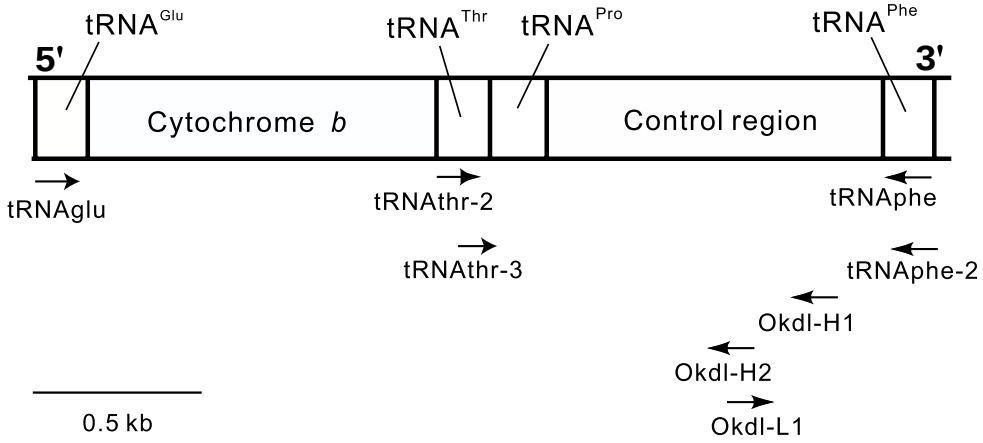
Sampling location	Date of collection	No. of samples
Hokkaido island		
Chitose river	14 Oct. 1996	51
Tokushibetsu river	23 Sep. 1997	51
Tokoro river	20 Nov. 1998	44
Tokoro river	13 Oct. 1999	49
Nishibetsu river	25 Sep. 1997	41
Tokachi river	17 Oct. 1996	46
Yurappu river	17 Nov. 1998	40
Honshu island		
Tsugaruishi river, Iwate Pref.	10 Dec. 1997	44
Otsuchi river, Iwate Pref.	8 Apr. 1999	49
Koizumi river, Miyagi Pref.	21 Nov. 1996	47
Kawabukuro river, Akita Pref.	18 Nov. 1997	30
Gakko river, Yamagata Pref.	10 Dec. 1996	45
Total		537

Table 2. Distribution of mtDNA control region haplotypes and haplotype diversity within the 12 populations of Japanese chum salmon.

	Number of individuals with haplotype												
	1	2	3	4	5	6	7	8	9	10	11	12	Haplotype diversity
Hokkaido island													
Chitose	22	1	0	0	0	0	14	2	10	0	0	2	0.71±0.04
Tokushibetsu	30	0	0	0	0	0	13	0	8	0	0	0	0.58±0.05
Tokoro	26	0	0	1	0	0	8	0	8	0	0	1	0.60±0.07
Tokoro-early	21	0	0	0	0	1	16	0	9	0	0	2	0.69±0.04
Nishibetsu	12	0	0	0	0	0	18	0	11	0	0	0	0.67±0.03
Tokachi	18	0	2	0	4	0	12	0	8	1	1	0	0.75±0.04
Yurappu	24	0	0	0	0	0	6	0	10	0	0	0	0.57±0.06
Honshu island													
Tsugaruishi	25	0	0	0	0	0	0	0	19	0	0	0	0.50±0.03
Otsuchi	26	0	2	0	0	0	0	0	21	0	0	0	0.54±0.03
Koizumi	24	0	1	0	0	0	1	0	21	0	0	0	0.55±0.03
Kawabukuro	19	0	0	0	0	0	5	0	5	0	0	1	0.56±0.09
Gakko	26	0	0	0	0	0	4	1	14	0	0	0	0.57±0.05
Total	273	1	5	1	4	1	97	3	144	1	1	6	0.64±0.01

Table 3. Pairwise estimates of F_{st} based on mtDNA sequence data.

			Hokkaido Island					Honshu Island			
Population	CHT	TKS	TKR	TKR-E	NIS	TOK	YRP	TSU	OTS	KIZ	KBK
Hokkaido Island											
Chitose											
Tokushibetsu	-0.007										
Tokoro	0.009	-0.007									
Tokoro-early	-0.017	-0.003	0.026								
Nishibetsu	0.013	0.045	0.093	-0.005							
Tokachi	-0.012	-0.012	-0.005	-0.006	0.033						
Yurappu	0.026	0.009	-0.019	0.048	0.121	0.008					
Honshu Island											
Tsugaruishi	0.193	0.199	0.125	0.235	0.328	0.163	0.090				
Otsuchi	0.190	0.198	0.122	0.234	0.328	0.162	0.088	-0.019			
Koizumi	0.167	0.175	0.103	0.208	0.296	0.141	0.070	-0.018	-0.018		
Kawabukuro	0.009	-0.007	-0.028	0.029	0.099	-0.006	-0.026	0.125	0.119	0.100	
Gakko	0.061	0.051	0.003	0.091	0.173	0.039	-0.014	0.037	0.035	0.023	-0.006



Forward (5'-3')	
tRNAglu	AAC CAC CGT TGT TAT TCA ACT A
tRNAthr-2	TCT TGT AAT CCG GAA GTC GGA
tRNAthr-3	GGT TAA AAC CCT CCC TAG TG
Okdl-L1	AGC TTG CAT ATA TAC AAG TGC A

Reverse (5'-3')	
tRNAphe	CA(G/T) CTT CAG TG(T/C) TAT GCT TT
tRNAphe-2	AAC AGC TTC AGT GTT ATG CT
Okdl-H1	ATG GGT TCT CTG GAA TTC AA
Okdl-H2	TGG GTA ACG AGC AAT AAG AT

Fig. 1. Schematic diagram of mtDNA control region and its flanking regions of chum salmon. Horizontal arrows show the positions of primers used for PCR amplification and nucleotide sequencing.

A

	Variable site from 5' end of control region									
Haplotype	30	57	96	108	154	194	231	386	395	471
OKDL- 1	T	A	-	A	C	A	T	G	C	A
OKDL- 2	.	.	.	.	.	T	.	.	.	.
OKDL- 3	.	.	.	.	.	.	C	.	.	.
OKDL- 4	.	.	.	.	.	.	.	.	.	C
OKDL- 5	.	.	A	.	.	.	.	.	.	.
OKDL- 6	.	.	.	.	.	.	.	-	A	.
OKDL- 7	.	.	.	.	G	.	.	-	A	.
OKDL- 8	.	.	.	.	.	.	C	-	A	.
OKDL- 9	C	.	.	.	.	.	.	.	.	.
OKDL-10	C	T	.	.	.	.	.	.	.	.
OKDL-11	C	.	.	T	.	.	.	.	.	.
OKDL-12	C	.	.	.	.	.	C	.	.	.

Hyphen and dot represent the deletion and the same nucleotide as in the OKDL-1, respectively.

B

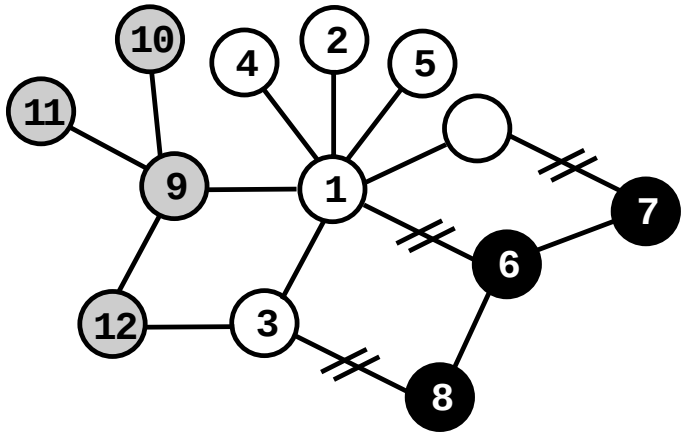


Fig. 2. Variable nucleotide sites from the 5' end of mtDNA control region defining 12 haplotypes and a parsimony network connecting the observed haplotypes

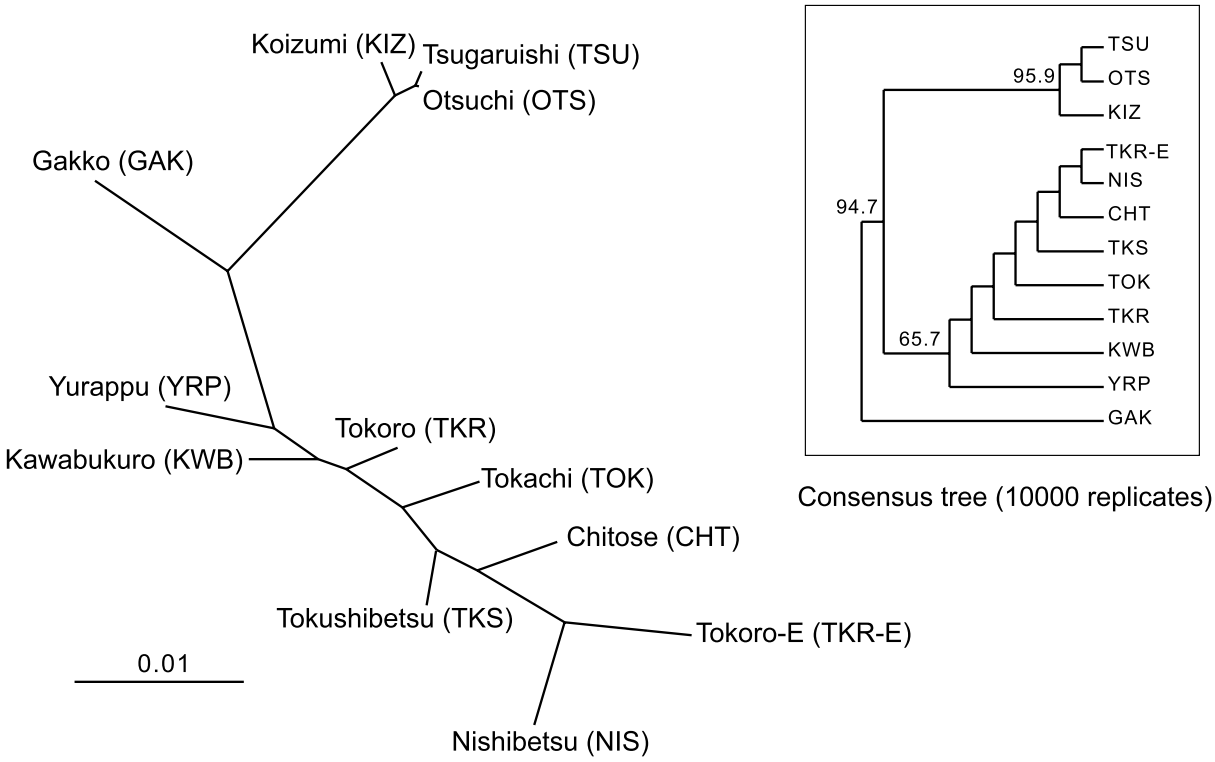


Fig. 3. Neighbor-Joining tree and phenogram (inset) of chum salmon populations

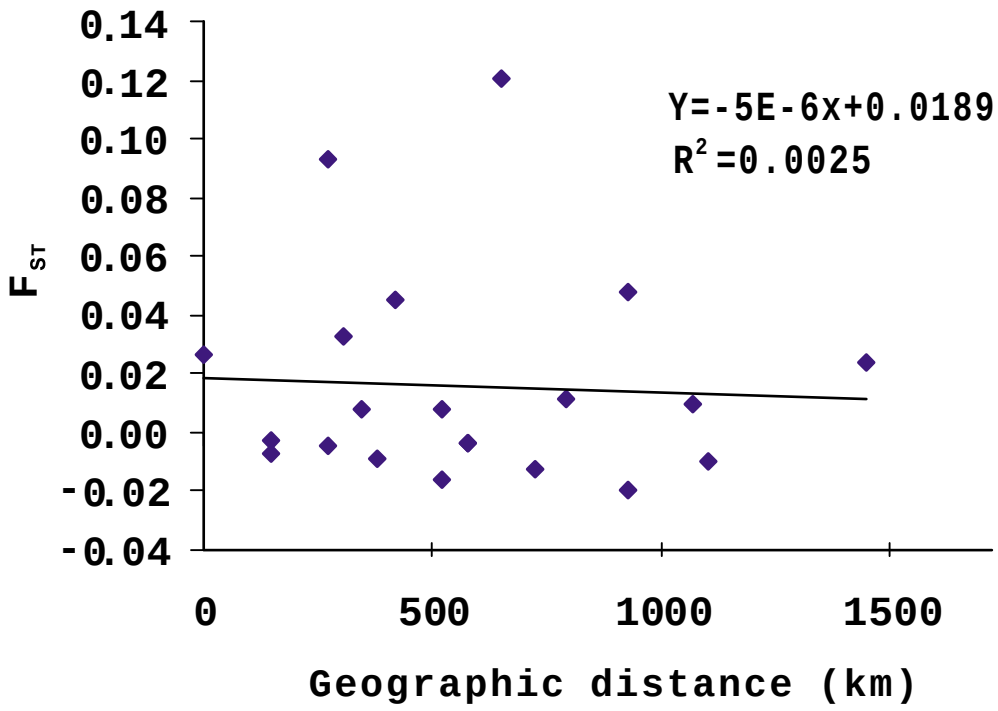


Fig. 4. Absence of isolation by distance on Hokkaido