Population structure of the sailfin sandfish, *Arctoscopus japonicus* (Trichodontidae), in the Sea of Japan

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Abstract We analyzed patterns of genetic diversity in the sailfin sandfish (*Arctoscopus japonicus*), focusing on population subdivisions within the Sea of Japan. We observed 270 specimens from nine sampling sites in 1999–2000, i.e., seven sites in the Sea of Japan and two sites from the Pacific coast of Hokkaido. An additional site (30 specimens) was sampled from eastern Korea in the spawning season of 2004 for comparison. Forty haplotypes, compiled into three haplogroups (A–C), were detected based on the comparison of a 400-bp sequence of the anterior part of the mitochondrial control region. In accordance with previous hypotheses from morphological and molecular analyses, genetic discontinuity between the Sea of Japan and the Pacific coast of Hokkaido was conspicuous. Within the Sea of Japan, eight sampling sites were not genetically uniform, and most of the variations among sites were detected between eastern Korea [the "eastern Korea" (EK) population: distributed from the Korean Peninsula to Mishima, Yamaguchi Prefecture] and the other sites along the coast of Japan [the "western Japan" (WJ) population: from Oki Islands to western Hokkaido] ($\Phi_{CT} = 0.096$, $P = 0.0183$). The WJ population, having lower genetic variability, showed significant departure from neutrality, indicating influences through a recent population expansion. The period of the expansion can be estimated to have begun on the order of 10^4 years ago. We consider that the present Japan Sea populations have been formed through the invasion of a small ancestral stock to the Sea of Japan and its population expansion during the last glacial period or later. On the other hand, we failed to detect distinct evidence of a population expansion in the EK population. Haplogroup C, detected in a high frequency in this population, was estimated to have mixed with haplogroup A after rapid differentiations of the latter. Therefore, the EK population, strongly influenced by such a mixture, might possess haplogroup C in a higher frequency and a different haplotype composition from the WJ population.

Key words *Arctoscopus japonicus* · Sea of Japan · Recent population expansion · Population structure

Marine pelagic and demersal fishes are likely to facilitate extensive gene flow and poor intraspecific genetic structuring, mainly because of the development of ocean current systems and/or the lack of physical barriers (Ward et al., 1994; Palumbi, 1994). Molecular approaches, however, have recently revealed valuable information to upset such a long-held view (Miya and Nishida, 1997; Rocha-Olivares et al., 1999; Hutchinson et al., 2001; Knutsen et al., 2003). In the Sea of Japan, Kojima et al. (2001) examined the cryptic population structure in the deep-sea eelpout *Bothrocara hollandi* (Jordan and Hubbs, 1925).

The sailfin sandfish, *Arctoscopus japonicus* (Steindachner, 1881), is widely distributed from the east coast of the Korean Peninsula, the Sea of Japan off Honshu, around Hokkaido, to extensive areas of the Sea of Okhotsk (Okiyama, 1970, 1990; Chereshnev and Nazarkin, 2002; Mecklenburg, 2003). This species is one of the important fishery resources in Japan, and its remarkable phylogenetic position has been studied recently through molecular (Smith and Wheeler, 2004) and morphological research (Imamura et al., 2005). Although this species ordinarily inhabits sandy bottom areas in the Sea of Japan, in early but severe winters it appears very near shore within seaweed beds for spawning (Ochiai and Tanaka, 1986). Large spawning areas are known in Akita Prefecture (northern part of Honshu) and the east coast of the Korean Peninsula, and also several spawning areas are found around Hokkaido; *A. japonicus* has been said to show a distinct annual migration for its reproduction (Okiyama, 1970, 1990; Kobayashi and Kaga, 1981; Ochiai and Tanaka, 1986).

If each population of the sandfish has a spawning ground of its own, there seems to be a distinct population structure in the Sea of Japan, although it is a rather restricted sea area. Okiyama (1970) recognized three populations from morphometric and mark–recapture analyses in the Sea of Japan (Fig. 1A); i.e., regions of western Hokkaido (WH), northern Honshu (NM: Honshu = mainland of Japan), and eastern coast of Korea (EK). Okiyama (1970) also noticed that populations for the Pacific coast of Hokkaido (called the southern Hokkaido population: SH) were a "highly sepa-

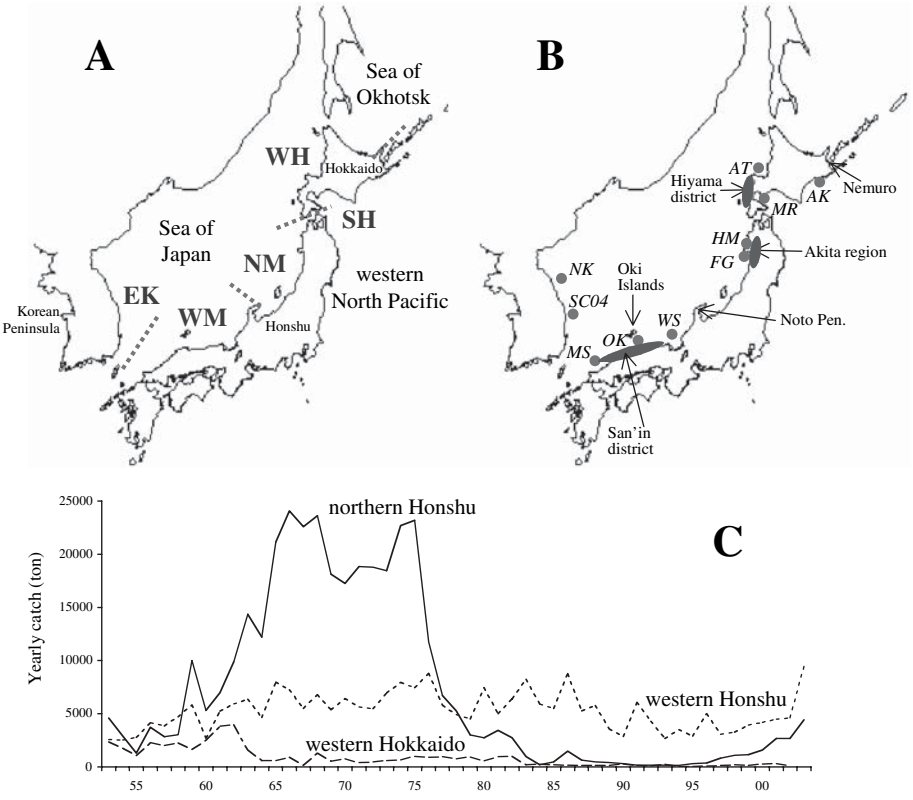

Fig. 1. Schematic of the hypothesized populations of *Arctoscopus japonicus* of Okiyama (1970) (**A**), the location of the ten sampling sites used in this analysis, with some regional names of Japan for explanation (**B**), and annual catches of three areas within the Japan Sea during 1952–2004 (**C**) [from The Annual Report of Fishery and Aquaculture Products in Japan (The Ministry of Agriculture, Forestry and Fisheries of Japan)]. For abbreviations of sample sites and populations, see Table 1. The locality of *NK* is ambiguous, but it must be a certain area of North Korea

rated lineage" from the three Japan Sea populations. Sandfish in western Japan (west of Noto Peninsula), called the western Honshu (WM) population, were explained as a mixture of the NM and EK populations; Okiyama noted that components of this population would change annually depending on the stock level of the NM and EK populations.

Fujino and Amita (1984), from their study of isozymes, agreed with Okiyama's (1970) SH and EK populations, and also recognized a third population, called the "population of the western coast of Honshu," inhabiting along western Hokkaido and Honshu. The western limit of the third group was then considered to be off Hyogo Prefecture (near the eastern end of the San'in district of Fig. 1B), and the eastern limit of EK was seen near the Oki Islands. Yanagimoto (2004) analyzed the control and 12S–16S rRNA regions of mitochondrial DNA with the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Yanagimoto concluded that sandfish around Japan were separated into two groups; one was the SH of Okiyama (1970) and another was a rather large population, seen from Korea along the Japan Sea coast of Honshu and Hokkaido, to the coast of the Sea of Okhotsk through the Soya Strait, and further to the Nemuro area. Other kinds of experiments to determine the population structure have recently been carried out using statistical methods (Watanabe et al., 2004) and parasites (Yanagimoto and Konishi, 2004).

In the present study, we examined nucleotide differences of mitochondrial DNA control region (mt-CR) using specimens collected in 1999 and 2000, and investigated the former hypotheses, focusing on their population structure within the Sea of Japan. As a result, we found sandfish in the Sea of Japan are not uniform genetically, which thus denies the single-population hypothesis in the Sea of Japan.

Materials and Methods

Sampling design.—Before all the manipulations, to find a hypervariable domain in the mt-CR region of *Arctoscopus japonicus*, we first observed the whole mt-CR region as assay no. 1 using 21 specimens collected from Muroran (*Muroran*, 5 specimens), Atsuta (*Atsuta,* 5), Akita region (5), and San'in district (6) (Table 1). Then, we observed 300 adult specimens (assay no. 2: Table 1) collected from ten sampling sites covering its distributional area in Japan and the Korean Peninsula (Fig. 1B) during the end of 1999 and July 2000 (except "*Samcheok04*"). Collections were made in one to three fishing areas in each of the five populations assessed by Okiyama (1970), i.e., SH (2 sites), WH (1), NM (2), WM (west of Noto Peninsula; 2), and EK (3: including the "*Mishima*" site) (names of all the sampling sites are given in italics in this article). Thirty specimens were collected in each sampling site. Of the Korean sites, the *North Korea* was caught from the sea off North Korea in 1999 (imported material to Akita Prefecture), and the *Samcheok04* was collected from Samcheok, Kanwon-do, South Korea, in November 2004, which was used as a representative of the EK population.

DNA extraction, PCR, and sequencing.—Muscle samples were stored individually at –20°C in 2-ml tubes containing

Table 1. Collections of sailfin sandfish (*Arctoscopus japonicus*) samples for the present study

Sample site	Abbreviation	Locality	Date collected	Assay no. 1		Assay no. 2	Collector
				n	Accession number	*n*	
Southern Hokkaido	SH						
Akkeshi	*AK*	Akkeshi Bay	2000.5.2			30	S. Takeyama
Muroran	*MR*	Kojohama, Shiraoi	1999.11.30[a]	5	AB243691–AB243694	30	K. Maeda
Western Hokkaido	WH						
Atsuta	*AT*	Ishikari Bay	1999.11.16[a]	5	AB243695–AB243699	30	K. Maeda
Northern Honshu	NM						
Hachimori (Akita)	*HM*	Off Hachimori	1999.12.15[a]	1		30	H.S.
Funagawa (Akita)	*FG*	Off Funagawa	1999.12.28[a]	2	AB243700	30	H.S.
Kitaura (Akita)	—	Off Kitaura	1999.12.15[a]	2			H.S.
Western Honshu	WM						
Wakasa	*WS*	Wakasa Bay	2000.7.18			30	S.M.S.
Oki	*OK*	Near Oki Islands	2000.3.9			30	R.K.
San'in district	—	Offshore of Tottori Prefecture	2000.7	6	AP003090, AB243701–AB243703		S.M.S.
Eastern coast of Korea	EK						
Mishima	*MS*	Off Mishima, Yamaguchi	2000.3.8			30	RK
North Korea	*NK*	Sea of North Korea	1999 autumn			30	—
Samcheok04	*SC04*	Samcheok, Korea	2004.11.28[a]			30	Y.J. Kang
Total				21		300	

Assay no. 1, sequencing analysis of complete mt-CR using 21 specimens

Assay no. 2, population genetics using 300 specimens (see Table 2 for their haplotypes and accession numbers)

[a] Sampled in spawning season

99.5% ethanol. All DNA was extracted using DNeasy Tissue Kit (Qiagen) following the manufacturer's instructions. Polymerase chain reaction (PCR) was performed with Z-taq (TaKaRa) on an ABI 9700 Cycler under the following conditions: 25–27 cycles with denaturation at 98°C for 5 s, annealing at 55°–57°C for 8 s, and extension at 72°C for 20 s (reaction volume, 12 µl). PCR products were purified using ExoSAP-IT (Amersham Biosciences), sequenced in both directions with the BigDye termination reaction chemistry kit, and analyzed with an ABI 377 sequencer. Sequences were aligned with AutoAssembler 2.1 (ABI) and DNASIS 3.7 (Hitachi).

The first assay was proceeded using primers L-Pro.Arja (5′-TAA CTC CCA CCC CTA ACC CCC AAA G-3′) and H-Phe.Arja (5′-AGG ACC AAG CCT TTG TGC CTT CG-3′). For the assay no. 2 (population genetic analysis), the following two primers were used: L-CR02.Arja (5′-CCA TTT ATT AAT GAT AAA GTA G-3′) and H-CR01.Arja (5′-TGC CTC CAA AAA TWC ACC TT-3′). All the primers were original, based on the complete mitochondrial DNA sequences of *Arctoscopus japonicus* (Shirai, Miya, and Nishida, unpublished data: AP003090).

Population genetic analysis.—Identical nucleotide sequences were assigned haplotypes, and frequencies were recorded for each sampling site. Mitochondrial DNA variation within samples and regions was summarized with haplotype (h) and nucleotide diversity (π) statistics following the formulae of Nei (1987). A statistical parsimony network was constructed for all ten samples. For construction of the network we used the computer program TCS 1.21 (Clement et al., 2000), which implements the statistical parsimony algorithm described by Templeton et al. (1992). Levels of genetic diversity within and among the sampling sites of the sandfish were tested by a hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992) using the Φ statistics, which includes information on mitochondrial haplotype frequency (Wier and Cockerham, 1984) and genetic distances (pairwise difference). Partitioning of genetic variability among sampling sites was performed by pooling populations into alternative structures by maximizing Φ_{CT} values. The significance of pairwise Φ_{ST} was tested by multiple permutation (10 000 times) of the original data set; P values were adjusted with sequential Bonferroni correction (Rice, 1989). All AMOVA tests were implemented in Arlequin 2.0 (Schneider et al., 2000) and SAMOVA 1.0 (Dupanloup et al., 2002).

A mismatch analysis was performed with Arlequin to compare the demographic history of the samples and to test for recent population expansion. We computed the moment estimator of time to the expansion (τ) and the mutation parameters before (θ_0) and after (θ_1) the expansion using a parametric bootstrap approach (10 000 simulations) (Rogers, 1995; Schneider and Excoffier, 1999). Tajima's (1989) D statistic and Fu's (Fu, 1997) F_S test for selective neutrality were calculated to assess evidence for population expansion, under which significant negative values are ex-

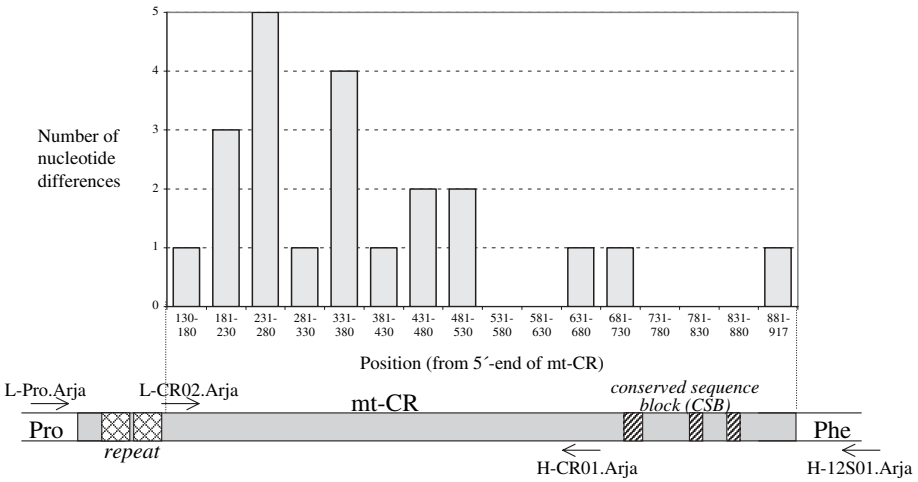

Fig. 2. Brief map of the mitochondrial control region of *Arctoscopus japonicus* with the position of the four primers. Chart shows numbers of nucleotide differences found in 21 individuals from *Muroran*, *Atsuta*, Akita region, and San'in district

pected; estimation and testing were done by bootstrap resampling (10 000 replicates) using Arlequin.

Results

Deciding a DNA region for comparison. We first sequenced the whole of the mt-CR using 21 specimens to select an appropriate region for the present observation: 14 unique haplotypes were detected, deposited in DDBJ/EMBL/GenBank (accession numbers: AB243691–AB243703, AP003090). Total size of the mt-CR was 870 bp in 1 specimen and 916–917 bp in the others (Fig. 2). In the latter 20 specimens, two repeating units (45 bp) were found in the 38th–82nd and 85th–129th sites of the mt-CR with or without a few differences between them (the position was counted from the 5′-end of the mt-CR). The short sequence of the former specimen was caused by the loss of the first or second repeat (45 bp) and their intermediate nucleotides between the repeats (2 of 3 bp). Subsequent sequences (data not shown) indicated that the presence or absence of the 47–48 bp sites might be caused by a kind of heteroplasmy: two (or more) kinds of copies, with or without a repeating region, were sometimes shared in a single specimen. Except for such an unstable region, many substitutions were found in the 130th–730th region (Fig. 2); the following region to the 3′-end was rather well preserved including estimated CSBs (conserved sequence blocks), and there was only one mutation among the 21 specimens examined. For assay no. 2, we selected a region of 181st–580th sequences (400 bp), including a large part of nucleotide variations in the mt-CR and no insertions/deletions. A PCR primer set, L-CR02.Arja/H-CR01.Arja, was newly designed.

Haplotypes. Sequences from the 300 individuals from the ten sampling sites comprised 40 different haplotypes defined by 25 polymorphic sites including 28 substitutions (19 transitions and 9 transversions) (Tables 2, 3). Of these, 20 haplotypes were singletons and 4 were found in several specimens of one sampling site. The other 16 haplotypes were shared among (at least) two different sampling sites; of these, 3 were restricted in southern Hokkaido, and 9 were in the Sea of Japan. Nine of 40 haplotypes were found in the specimens of assay no. 1, and the others were deposited as AB243704–AB243734 (Table 3).

The haplotype network is given in Fig. 3. Forty haplotypes were subdivided into three haplogroups: A, B, and C. Haplogroup A is composed of 28 haplotypes, which differ from each other by a few mutations. Haplotype a01, the most abundant haplotype within the Sea of Japan (also see Fig. 4), was a center of radiation in a02–a13; other starlike topologies were found stemming from a14 and a15. Haplogroups B (translated to A at 60th site from 5′-end of aligned fragments) and C (C at 246th and T at 266th sites) were separated from haplogroup A at least by four and six nucleotides, respectively. Although haplogroup B was composed of closely related haplotypes (b01–b07), haplogroup C was rather heterogeneous, not showing a starlike relationship.

As in Table 3 and Fig. 4, haplogroup A appeared in high frequency at all sites within the Sea of Japan (83%–100%) and at somewhat lower frequency in *Akkeshi* (47%) and *Muroran* (73%) (both are in southern Hokkaido). Many haplotypes of this group (a01–a22) were detected in the Sea of Japan; of these, the frequency of haplotype a01 was 80% or more in two Akita sites (*Hachimori* and *Funagawa*), 70% in *Wakasa* and *Oki*, and rather low (about 50%) in *Atsuta*, *Mishima*, *North Korea*, and *Samcheok04*. Haplotypes a23–a28 were limited in *Akkeshi* and/or *Muroran*; only 4 haplotypes (a01, a02, a13, and a14) appeared in the Sea of Japan and the Pacific Ocean. Haplogroup A includes many rare haplotypes; 16 of 28 haplotypes were singletons. Haplogroup B was restricted in two sampling sites of southern Hokkaido in high frequency (53% in *Akkeshi* and 27% in *Muroran*). Haplogroup C was detected in only the Sea of Japan: high in *North Korea* (17%), *Samcheok04* (17%), and *Mishima* (13%), low in *Atsuta* and *Funagawa* (3%), and not observed in the other sites.

Number of haplotypes was 10 to 12 in southern Hokkaido and three sites west of *Mishima*, and fewer in the sites along west coast of Japan (see Table 2). Number of substitutions was especially low in *Hachimori*, *Wakasa*, and *Oki*.

Table 2. Mitochondrial DNA control region (mt-CR) sequence diversity in ten sampling sites

Sample site	No. of haplotypes	Substitutions [ti + tv]	*h*	*k*	π
Akkeshi	10	12 [7 + 5]	0.862 (0.040)	4.768 (2.397)	0.012 (0.007)
Muroran	12	13 [7 + 6]	0.860 (0.047)	4.034 (2.072)	0.010 (0.006)
Atsuta	9	17 [12 + 5]	0.667 (0.090)	2.007 (1.163)	0.005 (0.003)
Hachimori	5	6 [5 + 1]	0.308 (0.108)	0.524 (0.451)	0.001 (0.001)
Funagawa	6	13 [10 + 3]	0.363 (0.111)	1.053 (0.719)	0.003 (0.002)
Wakasa	8	6 [4 + 2]	0.513 (0.110)	0.061 (0.674)	0.002 (0.002)
Oki	9	7 [5 + 2]	0.515 (0.111)	1.026 (0.706)	0.003 (0.002)
Mishima	10	14 [10 + 4]	0.736 (0.080)	3.184 (1.693)	0.008 (0.005)
North Korea	12	17 [13 + 4]	0.779 (0.077)	3.763 (1.951)	0.009 (0.005)
Samcheok04	12	18 [13 + 5]	0.683 (0.096)	3.818 (1.976)	0.010 (0.005)
Total	40	28 [19 + 9]			

Standard deviations are in parentheses
h, haplotype diversity; *k*, mean pairwise differences; π, nucleotide diversity

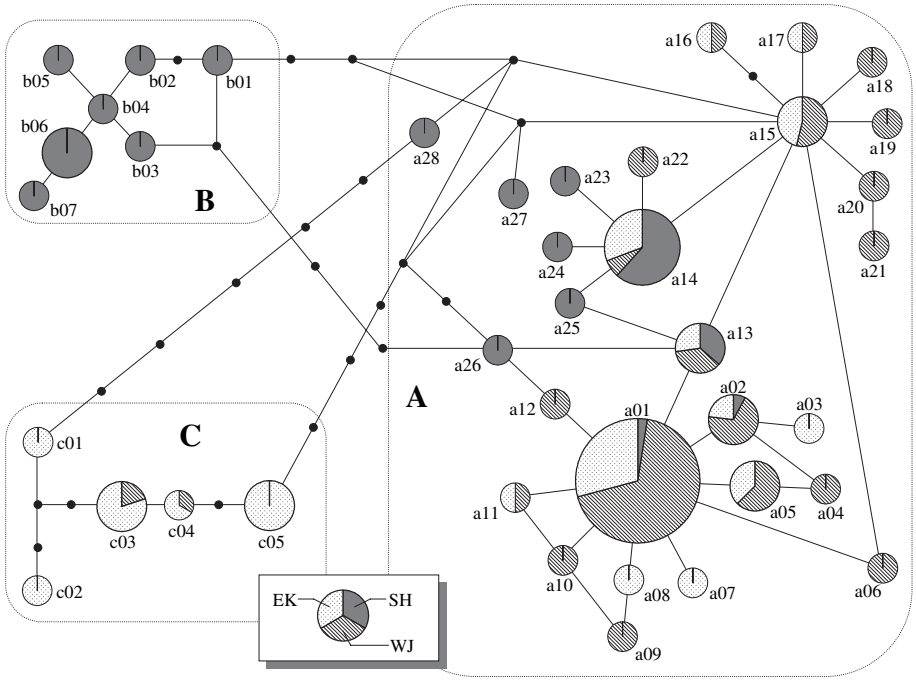

Fig. 3. Haplotype network [composed of 3 (*A*–*C*) haplogroups] for *Arctoscopus japonicus* generated with TCS 1.21 (Clement et al., 2000; 95% connection limit). *Each circle* represents a single haplotype; the *size of the circles* is proportional to the number of individuals with that haplotype. *Pie slices* represent the fraction of three populations recognized in the present study (*SH*, southern Hokkaido; *WJ*, west coast of Japan; *EK*, eastern Korea). *Small closed circles* indicate a missing intermediate

Mitochondrial DNA diversity. Haplotype diversity (*h*) was high in southern Hokkaido (0.86 in both sites), and rather low within the Sea of Japan (see Table 2). In the latter, it was higher in *Atsuta* (0.67) and three western sites near the Korean Peninsula (0.68–0.78) and particularly low in the two Akita sites (0.31, 0.36). Mean pairwise difference (*k*) was high in southern Hokkaido (4.0, 4.8) and three western sites (3.2–3.8) and low in the sites along the Japanese Archipelago north of *Oki*. Nucleotide diversity (π) was low overall (0.001–0.012) and followed the same pattern as the mean pairwise differences; rather high in southern Hokkaido (0.010, 0.012) and three western sites (0.008–0.010), and low in *Atsuta* (0.005), the two Akita sites (0.001, 0.003), *Wakasa* (0.002), and *Oki* (0.003).

Population subdivision. An AMOVA was performed in more than one step (Table 4). First, the variance among all sampling sites was large with a fixation index of 0.204 (Φ_{ST}). Second, when ten sites were divided into two areas, southern Hokkaido and the Sea of Japan, the Φ_{CT} value was maximized (0.357, $P < 0.0001$) and 35.7% of the mt-CR genetic variability was distributed between areas. In the next step, examining within the Sea of Japan showed that differences among sites was rather low but distinct ($\Phi_{ST} = 0.041$, $P = 0.0015$). When two gene pools were assumed within the Sea of Japan (west of *Mishima* and east of *Oki*), the Φ_{CT} value was maximized (0.096, $P = 0.0183$) and the genetic variability was 9.6% between the areas. The secondary maximized Φ_{CT} value was detected when *Atsuta* + west of

Table 3. Variable sites of the 40 mt-CR haplotypes and number of individuals (with frequency of haplogroups: %) for each haplotype by sampling areas

Haplotypes	Position of differences[a]																												*n*	frequency (%)	Sampling sites[b] and number of samples										Accession no.
														1	1	1	1	1	1	2	2	2	2	2	3	3			*AK*	*MR*	*AT*	*HM*	*FG*	*WS*	*OK*	*MS*	*NK*	*SC04*	
	2	3	4	4	5	5	5	6	6	6	7	8	0	0	0	6	6	9	9	0	4	5	6	7	0	4			30	30	30	30	30	30	30	30	30	30	
	7	6	0	6	0	8	9	0	1	9	1	9	1	4	2	5	5	7	1	6	2	6	6	4	6															
Haplogroup A																																								
a01	G	G	T	C	A	C	T	T	C	G	T	C	A	G	T	A	T	A	A	T	T	C	T	G	G		158	52.67	3	1	17	25	24	21	21	15	14	17	AB243701
a02	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		13	4.33	1		4		2	1	2	1	1	1	AB243704
a03	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.		1	0.33									1		AB243705
a04	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.		1	0.33						1					AB243706
a05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.		8	2.67			1	2	1	1		1	1	1	AB243707
a06	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.		1	0.33							1				AB243708
a07	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		2	0.67									2		AB243709
a08	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1	0.33								1			AB243710
a09	.	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1	0.33				1							AB243711
a10	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1	0.33				1							AB243712
a11	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		2	0.67					1					1	AB243713
a12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.		1	0.33							1				AB243714
a13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.		11	3.67		4	1			2	1	1	1	1	AB243715
a14	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.		26	8.67	6	10				1	1	4	2	2	AB243691
a15	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.		13	4.33			3		1	2	1	3	2	1	AB243716
a16	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	G	G	.	.	.	.	.	.		2	0.67			1							1	AB243695
a17	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	C	.	.	.	.	.	.	.	.	.		2	0.67							1		1		AB243717
a18	.	.	.	.	.	T	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.		1	0.33						1					AB243718
a19	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.		1	0.33				1							AB243719
a20	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.		1	0.33			1								AB243720
a21	.	.	.	T	.	.	.	.	.	A	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.		1	0.33							1				AB243721
a22	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.		1	0.33			1								AB243698
a23	A	.	A	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.		1	0.33		1									AB243722
a24	.	.	A	.	.	.	.	.	.	.	G	.	.	A	.	C	.	.	.	.	.	.	.	.	.		2	0.67		2									AB243723
a25	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.		5	1.67	2	3									AB243724
a26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.		1	0.33	1										AB243725
a27	.	.	.	.	.	.	.	.	A	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	A		1	0.33		1									AB243726
a28	.	.	.	.	.	.	.	.	.	.	.	.	.	A	C	C	.	T	.	.	.	.	.	.	.		1	0.33	1										AB243727

Haplogroup B														
b01	. . . . . . A A . . . . . A C C . . . . A . .	3	1.00	2	1									AB243692
b02	. . . . T C A A . . . . . A C C . . . . A . .	2	0.67	2										AB243728
b03	. . . . . C A A . . . . . . C C . . . . A . .	1	0.33		1									AB243729
b04	. . . . T C A A . . . . . . C C . . . . A . .	3	1.00	3										AB243730
b05	. . A . . T C A A . . . . . . C C . . . . A .	1	0.33		1									AB243731
b06	. . . . T C A A . . . . . . C C . . . . A A .	13	4.33	9	4									AB243693
b07	. C . . T C A A . . . . . . C C . . . . A A .	1	0.33		1									AB243694
				0	0	1	0	1	0	0	4	5	5	
Haplogroup C														
c01	. . . . T A . . C A C C . T . C . T . A . .	2	0.67								1	1		AB243732
c02	C . . . . T A . . C A C C . T . C C T C A . .	1	0.33										1	AB243733
c03	. . . . T A . . C A . C . T . C . T C A A .	5	1.67			1					1	2	1	AB243734
c04	. . . . T A . . . A . C . T . C . T C A A .	3	1.00					1					2	AB243700
c05	. . . . A . C . T . C . T C A A .	5	1.67								2	2	1	AB243703

[a]The position of differences is from 5′-end of aligned fragments
[b]Abbreviations of sampling sites are shown in Table 1

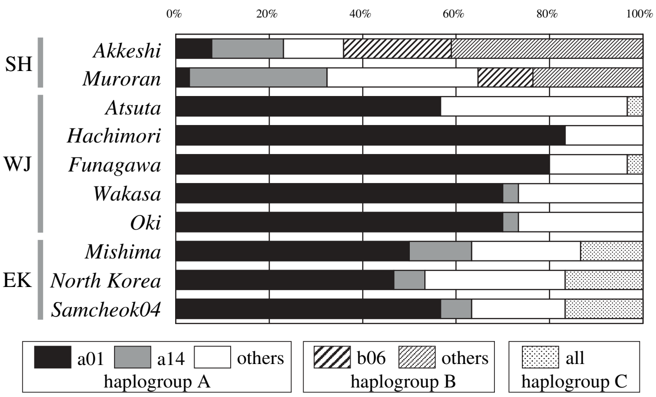

Fig. 4. Frequencies of three haplogroups (with several individual haplotypes) in ten sampling sites. Each sample is composed of 30 specimens. *SH*, southern Hokkaido; *WJ*, west coast of Japan; *EK*, eastern Korea

Mishima are separated from other four sites (0.079, $P < 0.0001$). When three gene pools were considered, the separation into north of *Oki*, *Mishima*, and *North Korea* + *Samcheok04* was selected ($\Phi_{CT} = 0.084$, $P = 0.0058$), but several other separations showed similar Φ_{CT} values.

The pairwise Φ_{ST} values are shown in Table 5. Two sites of southern Hokkaido were significantly different from all the Japan Sea sites (pairwise $\Phi_{ST} = 0.181$–0.482, $P < 0.001$) but not from each other (pairwise $\Phi_{ST} = 0.087$, $P = 0.029$). In the Sea of Japan, Φ_{ST} population pairwise comparisons exhibited no clear differentiation. Of the 28 possible comparisons, only 2 showed significant statistical values (*Hachimori* vs. *Mishima*; *Hachimori* vs. *North Korea*). Pairwise comparisons between sites of west of *Mishima* vs. north of *Oki* (except *Atsuta*) showed rather large Φ_{ST} values (pairwise $\Phi_{ST} = 0.063$–0.144, $P = 0.000$–0.044).

Historical demography. The mismatch distribution showed a distinct or indistinct bimodal curve in each of the three populations recognized here (Fig. 5). The first (left-hand) mode suggests characteristic of a recent exponentially growing population and starlike phylogeny around haplotypes e.g., a01, a15, or b04 (see Fig. 3). The second (right-hand) mode means larger pairwise differences between haplotypes of different haplogroups. In five sites north of *Oki* (Fig. 5B), a skewed exponential distribution fits the theoretically expected curve under the sudden expansion model (Schneider and Excoffier, 1999); this population is also characterized by parameters of lower τ and same values of θ_0 and θ_1 (Table 6), suggesting the possibility of a recent population expansion. Although sudden demographic expansion could not be denied, two sites of southern Hokkaido (Fig. 5A) and three sites west of *Mishima* (Fig. 5C) had higher frequencies of the second mode, characterized by larger τ and differences between θ_0 and θ_1, suggesting more stable demographic history during a recent period.

Neutrality tests were performed in each of the three populations (Table 6). Significant departures from the equilibrium were found in five sites north of *Oki* (*Atsuta* to *Oki*: $D = -1.894$, $F_S = -10.614$; $P < 0.01$), suggesting a recent

Table 4. Multistep hierarchical analyses of mt-CR diversity in samples of *Arctoscopus japonicus* collected from the ten sampling sites

Analysis	Source of variation	*df*	Φ Statistics	Percentage of variation
All sites	Among sites	9	0.2039 ($P < 0.0001$)	20.39
	Within sites	290		79.61
Two gene pools (southern Hokkaido/Japan Sea)[a]	Among sites	1	0.3566 ($P < 0.0001$)	35.66
	Within areas	8	0.0470 ($P = 0.0017$)	3.02
	Within sites	290	0.3868 ($P < 0.0001$)	61.32
Japan Sea only	Among sites	7	0.0414 ($P = 0.0015$)	4.14
	Within sites	232		95.86
Two gene pools (east of *Oki*/west of *Mishima*)[a]	Among sites	1	0.0957 ($P = 0.0183$)	9.57
	Within areas	6	–0.0129 ($P = 0.8374$)	–1.17
	Within sites	232	0.0840 ($P = 0.0017$)	91.60
Three gene pools (east of *Oki*/*Mishima*/ *Samcheok04* and *North Korea*)[a]	Among sites	2	0.0843 ($P = 0.0058$)	8.43
	Within areas	5	–0.0122 ($P = 0.6322$)	–1.11
	Within sites	232	0.0732 ($P = 0.0017$)	92.68
(*Atsuta*/Akita to *Oki*/west of *Mishima*)	Among sites	2	0.0837 ($P < 0.0001$)	8.37
	Within areas	5	–0.0180 ($P = 0.9441$)	–1.65
	Within sites	232	0.0837 ($P = 0.0017$)	93.28

Significance was tested by nonparametric permutation tests (10 000 permutations)
[a] By maximizing Φ_{CT}

Table 5. Pairwise Φ_{ST} (below diagonal) and associated *P* values (above diagonal) for ten sample sites of *Arctoscopus japonicus*

	AK	*MR*	*AT*	*HM*	*FG*	*WS*	*OK*	*MS*	*NK*	*SC04*
Akkeshi		0.029	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Muroran	0.087		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Atsuta	0.368*	0.279*		0.008	0.174	0.523	0.747	0.271	0.233	0.214
Hachimori (Akita)	0.482*	0.435*	0.068		0.749	0.086	0.049	0.000	0.001	0.002
Funagawa (Akita)	0.446*	0.391*	0.019	–0.001		0.406	0.479	0.015	0.022	0.044
Wakasa	0.423*	0.347*	–0.007	0.034	0.002		0.932	0.023	0.021	0.020
Oki	0.431*	0.355*	–0.013	0.041	–0.002	–0.02		0.024	0.019	0.032
Mishima	0.290*	0.181*	0.009	0.144*	0.090	0.066	0.063		0.948	0.871
North Korea	0.282*	0.195*	0.013	0.131*	0.080	0.071	0.065	–0.027		0.994
Samcheok04	0.285*	0.203*	0.015	0.121	0.070	0.070	0.065	–0.023	–0.029	

* Significant at $P < 0.001$ (after Bonferroni correction)

population expansion. In the Pacific coast of Hokkaido and west of *Mishima*, the neutrality was not rejected.

Discussion

The present analyses demonstrated details of polymorphism of the mt-CR sequences of sandfish around Japan for the first time. Different compositions of haplotypes and results of AMOVA and mismatch distribution analyses supported a distinct genetic discontinuity for this species between populations of the Sea of Japan and the Pacific coast of Hokkaido (Okiyama, 1970; Fujino and Amita, 1984; Yanagimoto, 2004). In the following sections, we discuss the population structure within the Sea of Japan.

Population structure within the Sea of Japan. In opposition to Okiyama (1970) and Fujino and Amita (1984), Yanagimoto (2004) suggested panmictic population structuring for this species within the Sea of Japan (including the Sea of Okhotsk) because of its "low genetic differences." However, our AMOVA test revealed the Japan Sea populations have low but distinct genetic differences of their own and supported the two-gene pool model that separates three sites west of *Mishima* from five sites north of *Oki*. These two subgroups showed a heterogeneous composition of haplotypes from each other as seen in the results of nucleotide diversity, neutrality tests, and mismatch distribution. Here we concluded that such evidence showed significant levels of genetic structuring within the Japan Sea population.

Three sites west of *Mishima* included haplogroup C in a higher frequency and showed higher genetic diversities within the Sea of Japan. One of them is *Samcheok04*, composed of spawning adults at the eastern side of the Korean

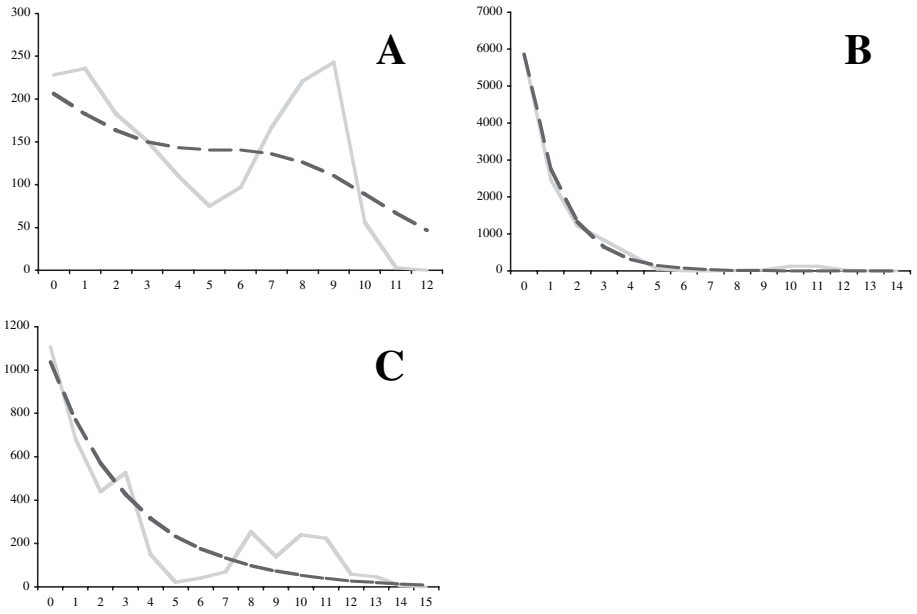

Fig. 5. Observed (*solid line*) and simulated distribution (*dotted line*) of pairwise sequence divergences under the sudden expansion model in each population recognized here: **A** southern Hokkaido (SH); **B** west coast of Japan (WJ); **C** east coast of Korea (EK). *x*-axis, genetic differences; *y*-axis, frequency

Table 6. Results of the mismatch analysis and neutrality tests

Population (abbreviation)	τ	obs. mean	θ_0	θ_1	Test of goodness of fit		Neutrality tests	
					SSD (*P*)	Ragged (*P*)	Tajima's *D* (*P*)	Fu's *Fs* (*P*)
Southern Hokkaido (SH)	8.812	4.615	0.006	7.565	0.014 (0.586)	0.017 (0.867)	1.295 (0.918)	–2.335 (0.218)
West coast of Japan (WJ)	1.438	1.124	0.914	0.914	0.002 (0.771)	0.108 (0.795)	–1.894 (0.005)	–10.614 (0.001)
East coast of Korea (EK)	11.087	3.526	0.016	2.848	0.014 (0.923)	0.031 (0.897)	–0.171 (0.454)	–2.674 (0.194)

τ, expansion parameter; obs. mean, mismatch observed mean; θ_0, θ_1, mutation parameter before (θ_0) and after (θ_1) expansion
SSD, sum of squared deviations, observing a less good fit between the model and the observed distribution by chance
Ragged, raggedness index of Harpending (1994)

Peninsula, which must be equivalent with the "EK" population of Okiyama (1970). Other five samples east of *Oki* are therefore grouped into an independent population, called here the "west coast of Japan" (WJ) population; comparing to Okiyama (1970), our WJ population is composed of his WH + NM (but his WM was supposed to be a mixture of EK and NM). Our hypothesis, regarding two populations within the Sea of Japan, corresponds to the conclusions of Fujino and Amita (1984) based on their isozyme research and Watanabe et al. (2004) on fishery statistics for years from 1978 to 1999. Fujino and Amita (1984) regarded the boundary of the two populations as from Hyogo Prefecture to the Oki Islands and Watanabe et al. (2004) as from Wakasa Bay to the Noto Peninsula; our results found it at the west of the Oki Islands. Such a difference should not be categorical, because the boundary must be not so stable because of the stock condition of both populations and/or their seasonal migration (Okiyama, 1970). In this analysis, we failed to find three or more distinct populations within the Sea of Japan.

On the single-population hypothesis of Yanagimoto (2004), we found that his conclusion is based on insufficient PCR-RFLP data. Yanagimoto (2004) performed PCR-RFLP analysis of two mitochondrial regions, sized 1.4 kb (including the whole control region) and 1.5 kb (almost all 12SrDNA and the anterior part of 16SrDNA). He detected only four differences in the region we studied (only 16% of our data). We also noticed that the author extracted twice at the same site [both *Scr*F I (CCNGG) and *Msp* I (CCGG) digested the site of our 265th–268th nucleotide (CCYGG)] and might have failed to separate the fragments in *Scr*FI digestion.

The WJ population is distributed from the San'in district to the west coast of Hokkaido, and it is considerably broader in distribution than the other known populations. Yanagimoto (2004) regarded the positive genetic exchanges within his Japan Sea population for the following reasons: (1) individuals can migrate to the Sea of Okhotsk because of the Tsushima Current flowing along the Japanese Archipelago, and (2) the NM population, which was considerably larger in size during the decade from the 1960s to the 1970s, might have expanded its own migratory limit and spread to the neighboring areas. The present mt-CR data only showed the existence of a large WJ unit and cannot provide either agreement or opposition to Yanagimoto's opinion. However, we have to mention here that such a large migration or expanse of domicile was not observed at all for these decades.

The population in a genetic sense is often not equivalent to the fishery stock. In the stock condition, the WJ population seems to have been separated into two or more subgroups (Okiyama, 1970). As shown in Fig. 1C, catches have greatly increased in the 1960s in NM but not in the neighboring WH and WM areas. After 1975, catches in NM suddenly decreased, and the two latter populations did not show fluctuations in parallel to NM. In the west coast of Hokkaido, a moderate-sized spawning ground has been known in Ishikari Bay for the WH population, and this population seems to be independent from the NM one; one of the reasons is that scarcely any sandfish were caught during the recent 50 years or more in the Hiyama district, the west coast of the Oshima Peninsula, Hokkaido (Kunihiro, 2004).

History of population formation in the Sea of Japan. We noticed two results among the present analyses as a clue to understanding the formation of two genetic populations in the Sea of Japan. One is a sign of the recent population expansion detected in WJ population by neutrality tests. The estimate $\tau = 1.438$ in the mismatch distribution analysis (see Table 6) implies that a population expansion of the WJ population had begun several tens of thousands of generations ago [$\tau/(2u)$: u, mutation rate] when a generation of sandfish is 2.5 years (the first participation in reproduction is seen 2 or 3 years after being spawned: Kiyokawa, 1991). For example, when the nucleotide divergence rate is supposed be to 2%/Myr (million years), 3.6×10^4 generations (about 90 Ka, 90 thousand years ago) is calculated. Another key is many rare haplotypes closely related to each other within the Sea of Japan are found in the haplogroup A stemming from prevalent haplotypes a01 and a15 (see Fig. 3). Such a pattern of network should indicate the rapid haplotype differentiations caused by population expansion (Shields and Gust, 1995; Grant and Bowen, 1998; Zardoya et al., 2004).

The invasion of a small ancestral population from a neighboring area and demographic expansion afterward under new environmental conditions has often been suggested to have an important role in population formation and/or speciation (Avise, 2000). Nishimura (1978) considered that a large part of the demersal fishes within the Sea of Japan have invaded from the Sea of Okhotsk during a recent geographic period correlated with the Riss–Würm Interglacial Stage (about 150–72 Ka). Up to now through the last glacial maximum (ca. 18 Ka), the palaeoenvironmental conditions of the Sea of Japan have changed dramatically over the past several tens of thousand years (Masuzawa and Kitano, 1984; Oba et al., 1991; Ishiwatari et al., 1994). According to these authors, in the Sea of Japan the seafloor was severely anoxic before 20 Ka; since then (to 10 Ka), the Oyashio (Kurile) Current has flowed into the Sea of Japan, reestablishing deep-water circulation. About 10 Ka, the warm Tsushima Current started to flow into the Sea of Japan to establish the modern oceanographic regime that has existed since 8 Ka. Adults of sandfish in the Sea of Japan are mainly distributed about 180–300 m deep, and such environmental changes must have impacted the genetics of the ancestral population. Such geographic hypotheses suggest, as one possibility, that during the last glacial period or after, the present Japan Sea populations of sandfish were formed through the invasion of a small ancestral stock to the Sea of Japan and its population expansion.

Yanagimoto (2004) found low haplotype diversity throughout the Sea of Japan by his PCR-RFLP analyses. He considered that "the bottleneck effects caused by the recent decrease of resource abundance" was the reason for this and further pointed out the influence of extensive fry releases afterward. The sandfish stock in the northern area of Honshu decreased suddenly in the middle of the 1970s and almost collapsed in the 1980s (for the commercial catch, see Fig. 1C); in the latter half of the 1990s and thereafter, it has gradually increased, partly because of the fishing moratorium in Akita Prefecture for 3 years (Sakuramoto et al., 1997; Watanabe et al., 2005). During the fluctuation of population size, extensive fry releasing has been performed; it was said to reach 5 million fishes per year or more (http://www.pref.akita.jp/). We must consider the resource conditions of the WJ population, where much effort has been made for its recovery (Okiyama, 1990). However, our present analyses did not suggest a serious influence of the fluctuation for these two or three decades, such as rapid loss of rare haplotypes.

Haplogroup A of each sampling site within the Sea of Japan was composed of a01 and various rare haplotypes (see Fig. 4). Most of the latter should be the result of the population expansion, as discussed earlier, and are therefore considered to have spread into the whole of the Sea of Japan after the expansion. However, the EK population did not show distinct evidence of a recent population expansion. How must we consider this? Samples of this population shared haplogroup C in a higher frequency. This type of haplotype, inherent in the Sea of Japan, differed from sympatric haplogroup A by six mutations or more. Furthermore, its haplotypes (c01–c05) were one to seven mutations removed from each other, not showing a starlike topology. These characteristics mean that haplogroup C might have been absent in the expanded population estimated earlier but should be present in another population. In other words, we can estimate that haplogroup C was primarily distributed in the Sea of Japan and mingled with haplogroup A after the invasion and rapid differentiation of the latter. Therefore, the EK population, influenced by such a mixture, will possess haplogroup C in a higher frequency and a different haplotype composition from the WJ population. Further analyses using nuclear DNA markers will provide much more information about this problem.

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