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Population Structure in the Western North Pacific Minke Whale Inferred from Microsatellite Analysis

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ABSTRACT

The genetic variability and population structure of the western North Pacific minke whale (*Balaenoptera acutorostrata*) was investigated by statistical analysis of eight microsatellite loci. A total of 535 individuals were collected from five geographic sub-areas by JARPN surveys (n=496; sub-areas 7, 8 ,9 ,11), by-catch (n=10; sub-area 6) and Korean former commercial whaling (n=29; sub-area 6). An average of 11.75 alleles per locus was detected and the average heterozygosity was 0.7423 with a range of 0.4310 - 0.8812. Significant deviations from the Hardy-Weinberg equilibrium were detected both in 1996 and 1999 JARPN data sets, which were attributable to samples from sub-area 11. While no significant deviation from the Hardy-Weinberg equilibrium was detected in sub-areas 7, 8 and 9. Population differentiation test also revealed genetic heterogeneities in samples from sub-area 11. Genetic distance, calculated over all loci for all sub-areas, showed that sub-area 11 animals had a close genetic relationship with samples obtained from sub-area 6 and they were differentiated from sub-areas 7, 8 and 9. These results support the hypothesis derived from previous allozyme and mitochondrial DNA (mtDNA) analyses, which suggest mixing of J and O stocks in sub-area 11. However, our results presented here cannot support the existence of the W stock in the offshore areas of the North Pacific. Based on Nei's standard distance and $(\delta\mu)^2$, divergence time between J and O stocks was estimated to be around early Holocene.

INTRODUCTION

In the western North Pacific the occurrence of two distinct populations—J stock (the Sea of Japan, Yellow Sea and East China Sea) and O stock (the Pacific coast of Japan and Sea of Okhotsk) of minke whales has been strongly supported by genetic (Goto and Pastene, 1997; Wada, 1983; 1984) and morphological studies (Ohsumi, 1983; Kato *et al.*, 1992). The International Whaling Commission (IWC) regarded these distinguishable populations as two independent 'stocks' even though these populations might mix in the southern part of the Sea of Okhotsk (sub-area 11) in a certain proportion (Pastene *et al.*, 1998). In 1993, however, a Working Group on North Pacific Minke Whale Management Trials proposed a new stock structure scenario in the western North Pacific. The group proposed the occurrence of an additional new stock, W stock (West Pacific) in offshore areas, and they also assumed that O stock should be divided into several sub-stocks (IWC, 1994).

To check these hypotheses, two different kinds of genetic markers (mtDNA and microsatellite DNA) were employed and minke whales from the coastal Japan (sub-area 7) were compared to those distributed in the offshore area (sub-area 9). However, neither of these genetic analyses showed significant differences between coastal and offshore samples (Goto and Pastene, 1997; Abe *et al.*, 1997), although additional samples and microsatellite loci were required to reach conclusion about this issue (Abe *et al.*, 1997).
Microsatellite loci are used with success to investigate the population genetics of a growing number of organisms (e.g. Goodman, 1998) and rapidly becoming the dominant source of nuclear genetic markers. It differs from mtDNA in that it is not limited to the female component of population and therefore should be an appropriate to examine sex-related differentiation in dispersal patterns. Genetic information from nuclear markers is required for a more detailed analysis of population genetic structure in the western North Pacific minke whale.
The purpose of this paper is to conduct a new microsatellite analysis on the western North Pacific minke whales using additional microsatelite loci and samples derived mainly from the JARPN surveys during 1994 - 1999 seasons. Statistical tests are applied to clarify whether our results can be in accordance with hypotheses described above. In addition, this paper describes the divergence time between J and O stocks according to two different parameters, Nei's standard genetic distance and $(\delta\mu)^2$. These data together with paleoenvironmental knowledge will provide a new insight into historical immigration of the western North Pacific minke whales.

MATERIALS AND METHODS

Samples and DNA Extraction

Sampling localities and the number of specimen used in this study are shown in Fig.1. A total of 535 minke whales were collected from five geographic sub-areas by 1994 - 1999 JARPN surveys (n = 496; sub-areas 7, 8 ,9 ,11), by-catch (n = 10; sub-area 6) and Korean commercial whaling (n = 29; sub-area 6). Genomic DNA was extracted from several kinds of tissues (e.g. muscle, liver, skin) using standard proteinase K, phenol-chloroform procedure (Sambrook *et al.*, 1989).

Genotyping

More than 30 microsatellite primer sets, which were derived primary from humpback whales were tested to estimate their applicability for population genetic study of the western North Pacific minke whales. Eight of them turned out to yield polymorphic and reasonable alleles by polymerase chain reaction (PCR; Saiki *et al.*, 1988). Five of eight microsatellite loci contained dinucleotide motifs [GT023, GT211, GT509 (Bérubé et al. *in prep*), EV37*Mn* and EV104*Mn* (Valsecchi and Amos, 1996)] and the others composed of tetranucleotide motifs [GATA028, GATA098, GATA417 (Palsbøll *et al.*, 1997)]. Microsatellite polymorphisms were detected fluorescently using end-labeled primers with either of 6-carboxyfluorescein (6-FAM), 4,7,2',7',-tetrachloro-6-carboxyfluorescein (TET) or 4,7,2',4',5',7',-hexachloro-6-carboxyfluorescein (HEX) dye at 5' of a primer. PCR amplifications were carried out in 15 μl reactions containing 5 pmol of each labeled and unlabeled primer, 0.625 units of Ex Taq polymerase (Takara Shuzo Co.), 2 mM of each dNTP, reaction reagent [reaction buffer comprises of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM

$MgCl_2$, 0.01%($^{w}/_{v}$) gelatin] and 10 - 100 ng of genomic DNA. Multiplex PCR (Chamberlain *et al.*, 1988) was performed in three PCR classes, set A - GT023, GATA098, GATA417; set B - GT509, EV104*Mn*; set C - GATA028, EV37*Mn*. Annealing temperatures for multiplex sets in the two-stage thermal cycling program are: set A and set B, 52.5℃; set C, 47.5℃; GT211, 52.5℃. Each PCR product was electrophoresed with internal size standard (N,N,N',N',-tetrametyl-6-carboxyrhodamine; TAMRA 500) through 5% polyacrylamide denaturing gel (Long Ranger) using an ABI 377 DNA Prism sequencer (PE Biosystems Japan Ltd.).

Data Analysis

Genetic Variability

Genetic variation was quantified by calculating observed and expected heterozygosity for each locus over the total JARPN samples. These calculations were performed using POPGENE (ver. 1.31) PC software package. In addition, the probability of identity (PI) was estimated in each locus and across all loci. PI is the probability that two unrelated individuals have the same genotype at a single locus, or across all loci (obtained by simple multiplication of the estimated PI's for all loci).

Homogeneity Tests

Deviations from Hardy-Weinberg (HW) equilibrium at each locus in each geographic area were investigated using χ^2 test, as implemented in the POPGENE program. Population differentiation test was conducted using the GENEPOP (ver.3.1) program (Raymond and Rousset, 1995). For each locus, an unbiased estimate of the P-value was obtained by Markov-chain Monte-Carlo simulations. P-values from the eight loci were combined into a single P-value as described by Sokal and Rohlf (1995, p.795).

Genetic Distance

We first examined the genetic relationships between by-catch and Korean commercial samples in sub-area 6 and confirmed that there was no meaningful difference in genetic traits between these two sets of samples. Therefore we considered by-catch and Korean commercial samples in sub-area 6 as representative of the J stock. Both the Nei's standard (Nei, 1972) and the unbiased (Nei, 1978) genetic distances were calculated using POPGENE. While the Goldstein *et al.*'s (1995) $(\delta\mu)^2$ was estimated using RSTCALC (ver. 2.1) program. Regarding to RSTCALC, we had to eliminate genotypes containing microvariant alleles (Puers *et al.*, 1993) from the input data files because this program must be calculated from allele size in terms of repeat number (Goodman, 1997). Dendrogram was constructed according to Nei's unbiased genetic distance between sub-areas using UPGMA algorithm in NEIGHBOR program supplied with PHYLIP 3.5c (Felsenstein, 1993). These estimators of genetic distance were also used to estimate divergence time (Takezaki and Nei, 1996) between minke whale populations in the eastern and western side of Japan. The isolation by distance analysis could not be performed, simply because sampling localities showed a scattered distribution in the North Pacific (see, Fig.1).

RESULTS

Genetic Variability

In the North Pacific, an average of 11.75 alleles per locus were detected and the average heterozygosity was 0.7423 with a range of 0.4310 - 0.8812 (Table 1). While the average

heterozygosities in sub-area 6 were slightly lower than those observed in the North Pacific. We could hardly find difference in allele frequencies between the Sea of Okhotsk (sub-area 11) and the North Pacific sub-areas (data not shown). Theoretically the overall PI in each population showed low value enough to identify all individuals in each region (Table 1).

Homogeneity Tests

Significant deviations from H-W equilibrium were observed in the total samples of 1996 and 1999 JARPN surveys (Table 2). In these cases, however, no departure from H-W expectation at 5% level was observed in the individual sub-area. Furthermore, the P-value for all loci for the combined sub-area 11 was significant ($P<0.001$), while no significant deviation from H-W equilibrium was detected in the combined data set from sub-areas 7, 8 and 9. Pairwise comparisons among sub-areas clearly showed genetic heterogeneity in sub-area 11, whereas no significant differences were found among sub-areas 7, 8, 9 (Table 3). Hence, sub-areas 7, 8 and 9 were combined and tested against sub-area 11, and the result showed a highly significant level of differentiation.

Genetic Distance

Nei's unbiased genetic distances among five sub-areas are shown in Table 4. A dendrogram based on these genetic distances was constructed using the UPGMA algolism (Fig.2a,b). The tree showed a close genetic relationship among sub-areas 7, 8 and 9, while genetic component of sub-area 11 are divergent from those of sub-areas 7, 8 and 9. To estimate divergence time between J and O stocks, Ds and $(\delta\mu)^2$ were calculated between samples derived from the Sea of Japan (sub-area 6) and combined data from sub-areas 8 and 9. The values of Ds and $(\delta\mu)^2$ were 0.1496 and 0.1230 respectively, and assuming a mutation rate of 8.0×10^{-5} [as estimated in porcine microsatellite (Ellegren, 1995)] and a generation time of 10 years (Mitchell, 1986), the divergence times between them were estimated to be 7,688 - 9,350 years ago.

DISCUSSION

Population Structure of Minke Whales in the Western North Pacific

Our study confirmed the occurrence of two stocks in the western North Pacific, and the geographic distributions of J and O stocks overlap in the southern part of the Sea of Okhotsk. However, these findings provide two alternative possibilities: a spatial/temporal factor might simply affect the geographical overlap of two stocks in this region or reciprocal gene flow between them might have occurred in the mixing zone. Previous study (Omura and Sakiura, 1956; Kato, 1992) on biological features of the western North Pacific minke whales indicated that different peaks in the breeding season could be detected between J stock (autumn breeding stock) and O stock (winter breeding stock). Furthermore, Pastene *et al.* (1998) suggest that a sexual segregation does exist in the pattern of mixing of two stocks in sub-area 11. These observations implies that discrepancy in breeding phase might play an important role to prevent gene flow between two stocks, even though this biological barrier might not be perfect.

Depending on mtDNA haplotype data (Goto *et al.*, this meeting), individuals from sub-area 11 are hypothetically classified into J and O stock animals represented by sub-area

11[J] and sub-area 11[O], respectively. Of 80 minke whales originated from sub-area 11, 24 individuals were characterized by haplotypes in which J stock animals predominated. Nei's genetic distance indicates that sub-area 11[J] is genetically different from sub-area 11[O] and belongs to the same clade with the Korea plus by-catch group, while sub-area 11[O] is included internal branches among sub-areas 7, 8 and 9 (Fig 2b). These findings suggest that the judgment based on mtDNA haplotypes reflects the nature of genetic difference between two stocks, although sub-area 11[J] cannot be regarded as pure J stock animals. It is conceivable that discrepancy of genetic traits may be detected between two component of DNA (mitochondria and nuclear DNA) in a single individual because diploid markers (e.g. microsatellite) can rapidly disperse within population as compared to maternally inheritable markers such as mtDNA.

On the other hand, we could not find any hierarchical structure among the North Pacific populations, which were thought to be a panmictic group based on H-W test for significance. Therefore our results cannot support the existence of the W stock in the offshore areas of the North Pacific. Lack of biological boundary in this region as well as high mobility of marine mammals is one of the critical factors preventing them from accumulating distinct genetic characters.

Divergence Time between Two Stocks

We have used *Ds* and $(\delta\mu)^2$ to estimate divergence time between J and O stocks and obtained estimates of 9,350 and 7,688 years, respectively. These values seem to be adequate because *Ds* and $(\delta\mu)^2$ are better than other distance (e.g. Cavalli-Sforza and Edward's chord distance) in branch length estimation (Takezaki and Nei, 1996). Furthermore, these estimations are in concordance even though *Ds* and $(\delta\mu)^2$ could be calculated depending on different models, the infinite-allele model (Kimura and Crow, 1964) and the stepwise mutation model (Ohta and Kimura, 1973), respectively. However, it should be emphasized that the following conditions must be carefully considered in determining divergence time between them.

First fidelity of divergence time depends upon the level of gene flow between two populations. Our estimation could be correct if direct and/or indirect gene flow between J and O stock animals were completely restricted after they had once established their genetic characters, whereas if successive gene flow between two stocks occurred in evolutionary history of the western North Pacific minke whales, the inferred date must be greater according to the amount of gene flow between them.

Second we applied Ellegren's mutation rate (8.0×10^{-5}) for porcine to this calculation, simply because several genetic approaches have shown that cetaceans are closely related to artiodactyl (Shimamura *et al.*, 1997; Graur and Higgins, 1994). However, we can not get rid of the possibility that mutation rate applied to cetacean evolution should be different from that used in terrestrial mammals.

It is meaningful to take a history of the Sea of Japan into consideration from the paleoenvironmental point of view. The Sea of Japan had experience of being completely isolated from the North Pacific Ocean by Japanese land mass at the middle of Pleistocene (Fujii, 1990). Furthermore, during glacial lowstands, salinity of the surface water decreased probably due to excess input of fresh water over evaporation (Tada, 1994). It is conceivable that such a severe and isolated environment could not allow the minke whale to endure in this area. Our estimation of divergence time is consistent with the data derived from geological study, and one plausible scenario could be drawn based on these

findings. As sea level rises after last glaciation, founder population could invade into the Sea of Japan, notwithstanding the strait they passed though into this area could not be determined. This initial population may have been influenced by population bottleneck and founder effect (Sage and Wolff, 1986) following the recovery of population size. In more recent years, some of J stock animals could penetrate into the Sea of Okhotsk and geographically mix with O stock animals. Our hypothesis presented here is somewhat analogous to those of the other genetic studies, suggesting that present-day patterns of genetic structure may reflect effects of Pleistocene glaciations and post-glacial range expansions (Larson *et al.*, 1984; Merila *et al.*, 1997; Matsuhashi *et al.*, 1999).

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Table 1. Summary of Heterozygosity Statistics for All Loci

JARPN samples (n=496; sub-areas 7,8,9 and 11)

Loci	NA*	Size (bp)	Obs_Het	Exp_Het	Ave_Het	PI #
GT 023	14	88 - 120	0.8482	0.8841	0.8812	0.025
GATA 028	16 ¶	148 - 230	0.8296	0.8352	0.8309	0.044
GATA 098	6	100 - 120	0.6154	0.6338	0.6301	0.183
GT 211	16 ¶	95 - 126	0.8891	0.8780	0.8742	0.028
GATA 417	12 ¶	196 - 224	0.7175	0.7442	0.7405	0.102
GT 509	16	182 - 222	0.8295	0.8525	0.8484	0.034
EV37 *Mn*	11	179 - 209	0.7039	0.7081	0.7020	0.120
EV104 *Mn*	3	151 - 155	0.4222	0.4369	0.4310	0.404
Overall PI						9.248E-10

Korea / by-catch samples (n=39; sub-area 6)

Loci	NA*	Size (bp)	Obs_Het	Exp_Het	Ave_Het	PI #
GT 023	9	94 - 116	0.8611	0.7973	0.8629	0.065
GATA 028	5	198 - 218	0.5405	0.6775	0.7962	0.172
GATA 098	4	104 - 120	0.5	0.4775	0.6082	0.337
GT 211 §	4	102 - 114	0.625	0.6583	0.8170	0.222
GATA 417	5 ¶	208 - 220	0.7105	0.6081	0.7084	0.231
GT 509 §	6	200 - 218	0.75	0.8667	0.8422	0.062
EV37 *Mn*	5	179 - 207	0.5833	0.7011	0.6900	0.158
EV104 *Mn* §	2	151, 153	0.2222	0.3660	0.3987	0.488
Overall PI						9.263E-07

* Number of alleles

Probability of identity (see Paetkau and Strobeck, 1994)

¶ Microvariant alleles (see Puers, 1993) were included

§ Only by-catch samples were examined

Table 2. Chi-square Test for Hardy-Weinberg Equilibrium in Each Study Sub-area

Samples	Sub-area	N	P-value
KOREA*	6	29	0.3660
Bycatch	6	10**	0.6486
94NP	9	21	0.8135
95NP	9	100	0.8404
96NP	7	31	0.2479
96NP	8	16	0.9580
96NP	11	30	0.3051
96NP	7,8,11	77	0.0334
97NP	9	67	0.7694
97NP	8	31	0.7758
97NP	8,9	98	0.7215
98NP	7	56	0.3839
98NP	8	44	0.3518
98NP	7,8	100	0.7395
99NP	7	50	0.5596
99NP	11	50	0.0564
99NP	7,11	100	0.0048
Sub-area 6*	-	39**	0.9602
Sub-area 7	-	137	0.1592
Sub-area 8	-	91	0.8200
Sub-area 9	-	188	0.8853
Sub-areas 7,8,9	-	416	0.5926
Sub-area 11	-	80	<0.001

* Only five microsatellite loci were analysed

** Including one individual originated from sub-area 10

Table 3. P-values of Population Differentiation Test among Sub-areas 7, 8, 9 and 11

Sub-areas	GT023	GATA028	GATA098	GT211	GATA417	GT509	EV37*Mn*	EV104*Mn*	Combined
Sub-area 8 & Sub-area 7	0.2680	0.0149	0.4474	0.7764	0.5062	0.6271	0.2739	0.0527	0.0910
Sub-area 9 & Sub-area 7	0.5382	0.0631	0.2284	0.9132	0.6879	0.4808	0.8030	0.0848	0.3547
Sub-area 9 & Sub-area 8	0.3005	0.2710	0.8532	0.6788	0.7363	0.1079	0.3000	0.6745	0.5712
Sub-area 11 & Sub-area 7	0.0022	0.8237	0.2613	0.1890	0.5452	0.0146	0.1649	0.1227	0.0028
Sub-area 11 & Sub-area 8	0.3609	0.4236	0.8048	0.1124	0.2685	0.0974	0.0857	0.1517	0.0785
Sub-area 11 & Sub-area 9	0.0548	0.3021	0.8281	0.0922	0.4092	0.0056	0.1405	0.3776	0.0120
Sub-area 11 & Sub-areas 7,8,9	0.0113	0.7355	0.6593	0.0448	0.2556	0.0046	0.0423	0.3370	0.0012

Table 4. Nei's Unbiased Measures of Genetic Identity (above diagonal) and Genetic Distance (below diagonal) between Each Pair of Populations

Sub-areas	6	7	8	9	11	11[O]	11[J]
6	–	0.8700	0.8638	0.8774	0.9133	0.8844	0.9423
7	0.1392	–	0.9916	0.9913	0.9828	0.9931	0.9269
8	0.1464	0.0085	–	0.9970	0.9888	0.9946	0.9421
9	0.1308	0.0087	0.0030	–	0.9919	0.9972	0.9462
11	0.0907	0.0173	0.0113	0.0081	–	–	–
Sub-area 11[O]	0.1229	0.0069	0.0055	0.0028	–	–	0.9489
Sub-area 11[J]	0.0594	0.0759	0.0596	0.0553	–	0.0524	–

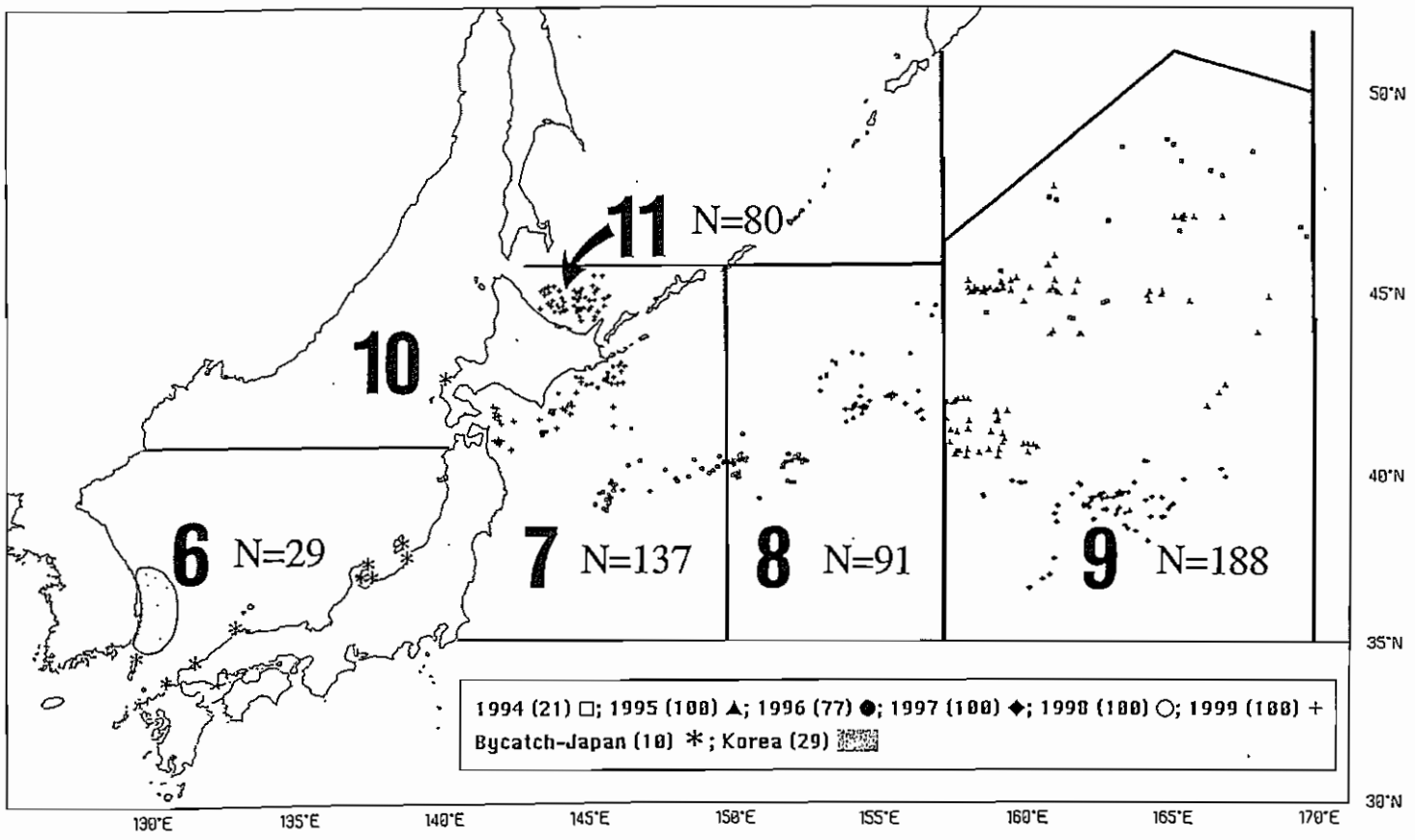

Fig 1. Geographic origin of the samples. The numbers shown in bold denote the sub-areas defined by the Working Group on North Pacific Minke Whale Management Trials (IWC, 1994).

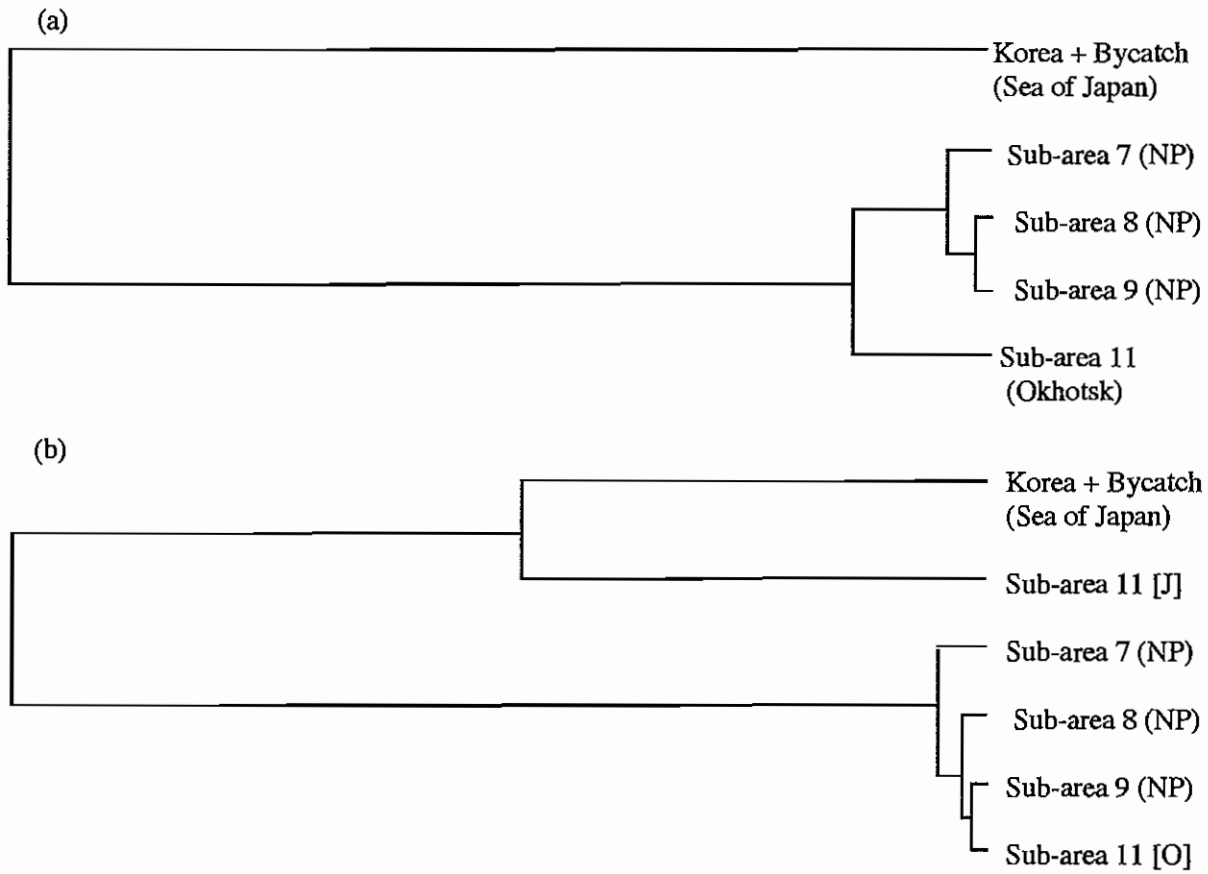

Fig 2. Dendrograms based on the Nei's genetic distance. (a) between sub-areas (b) samples obtained from sub-area 11 were hypothetically classified into two groups, sub-area 11[J] and sub-area 11[O] according to the judgement made by mtDNA haplotypes. NP; North Pacific