Population structure in the western North Pacific minke whale based on RFLP and sequencing analyses of mtDNA control region, using data from the 1994-1999 JARPN surveys

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ABSTRACT

Restriction fragment length polymorphism (RFLP) analysis of the whole mtDNA control region and sequencing of a 487 bp segment of the control region are used to examine stock structure in the North Pacific minke whales. A total of 418 samples obtained from JARPN surveys during 1994 to 1999 (sub-area 7: n=139, sub-area 8: n=91, sub-area 9: n=188) was used in this analysis. Samples from past coastal commercial whaling in Korea were used as outgroup (sub-area 6, n=29). The RFLP analysis, which used eight restriction enzymes, detected five haplotypes. In the sequencing analysis, a total of 62 haplotypes were defined by 33 variable sites. Nucleotide diversity was considerably lower in sub-area 6. Homogeneity tests were based on the haplotype (Fst) and sequence statistics (PHIst) of the Analysis of Molecular Variance (AMOVA). Results of these tests showed that whales from sub-area 6 are discriminated from whales from the Pacific side of Japan. However no significant statistical differences were found among sub-areas 7, 8 and 9.

INTRODUCTION

The Japanese Whale Research Program under Special Permit in the North Pacific (JARPN) began with feasibility studies in 1994 and 1995. The full research was conducted between 1996 and 1999. The main objective of the JARPN was to elucidate the stock structure of the western North Pacific minke whale. Since 1996 a new objective related to the feeding ecology of this species, was added.

The research areas of the JARPN surveys were defined as the sub-areas specified by the IWC Scientific Committee in 1993 (IWC, 1994, Fig. 1). In 1994 and 1995 the surveys covered the offshore sub-area 9, from where no samples were available. In the subsequent years, apart from sub-area 9, offshore sub-area 8 and coastal sub-areas 7 and 11 were also covered (Fujise, this meeting).

Previous to the JARPN, analyses of stock structure in the western North Pacific were based on samples collected in coastal areas of Korea and Japan. Studies based on genetics (allozymes) as well on other kind of biological parameters, showed that two biological stocks occur on either side of Japan (see review by Pastene *et al.*, this meeting), with a temporal mixing of stocks in sub-area 11.

Under the JARPN, samples from offshore areas of the western North Pacific became available and then comparison between coastal and offshore samples in the eastern side

of Japan was possible. On the other hand, new sets of coastal samples became available and then yearly variation in coastal sub-areas was also possible.

During the 1999 meeting, the Scientific Committee decided to conduct a review of the JARPN. Terms of references of the review and objectives of this research program were specified in the Annex Q of the 1999 Scientific Committee meeting report (IWC, 1999). The objectives of the JARPN indicated there were: a) to determine whether or not the W stock exists, and if so to estimate mixing rates between the O and W stocks, and b) determine the feeding ecology of minke whale in the North Pacific.

Under the JARPN several approaches have been used to study stock structure. Among them, genetic analyses based on DNA have been considered an important tool for such purpose. Several reports based on mtDNA analyses have been presented to the IWC Scientific Committee in each year. We presented here the results of a mtDNA analysis (RFLP and sequencing of the mtDNA control region), based on the total samples available from the JARPN surveys between 1994 and 1999 for sub-areas 7, 8 and 9. The analysis is focused to determine whether or not the 'W'-stock exist in the eastern side of Japan. Past commercial catch samples from the Sea of Japan are used in our analysis as outgroup.

MATERIALS AND METHODS

Samples and localities

Minke whales used in this study were sampled during the JARPN surveys between 1994-1999. As an outgroup we used 29 Korean minke whales caught in the past by Korean coastal whaling operations in September and October 1982 (28 used in the sequencing analysis). Four regions were defined using the geographical position of the samples taken (Fig. 1): Sea of Japan (sub-area 6 defined by the Working Group on North Pacific Minke Whale Management Trials), Pacific coast of northern Japan (sub-area 7) and two offshore areas (sub-areas 8 and 9). A total of 447 samples (n=29 in sub-area 6, n=139 in sub-area 7, n=91 in sub-area 8 and n=188 in sub-area 9) was examined. The total number of JARPN samples examined in this study by sub-area, period, month and sex is shown in Table 1. Samples from sub-area 11 collected during the 1996 and 1999 JARPN surveys, were excluded from this study, because J and O stock animals temporary mix within this sub-area (Pastene *et al.*, 1998; Goto *et al.*, this meeting) and then our analysis was focused to the eastern side of Japan.

Tissue samples and DNA extraction

Using established protocols (Sambrook *et al.*, 1989), genomic DNA (mtDNA+nuclear DNA) was isolated from liver, muscle or skin tissue that had been frozen at –20° for several months or at room temperature in 70% ethanol solution. Briefly, about 50mg of tissue was mixed with 500ul of homogenization solution (1% SDS and 1mg proteinase K). The mixture was incubated overnight at 37°. After incubation, crude DNA was extracted by established phenol/chloroform methods. The genomic DNA were dissolved in 500ul TE buffer and stored at –20° until use.

RFLP analysis

The control region of the mitochondrial genome was amplified by using the polymerase chain reaction (PCR) (Hoelzel, 1992). Primers for amplifying the about 1,050bp minke whale mtDNA control region were designed from mtDNA sequences of minke whale (Hori *et al.*, 1994) and sperm whale (Dillon and Wright, 1993): light-

strand Primer-1 (*5'*-CAAGGAAGAAGTATTACACTC-CACCA-*3'*) and the heavy-strand Primer-2 (*5'*-CAGAATTGGAATTCATTTTCAGTGTCTTGTTT-*3'*). These primers annealed to $tRNA^{pro}$ and $tRNA^{phe}$ regions, which flank the control region.

After amplification of mtDNA control region, one tenth (5ul) of the PCR-products was digested with two units of the following four-base recognition restriction enzymes: *Afa*I, *Dde*I, *Hae*III, *Hin*fI, *Mbo*I, *Msp*I, *Sau*96I and *Scr*FI using conditions recommended by manufacture. The restricted PCR products were electrophoresed on a 2.5% agarose gel and stained with 250 ng/ml ethidium bromide. The restriction fragment patterns were visualized under short-wave ultraviolet light. The size of each fragment was estimated by comparing their relative mobilities with known-sized fragments of a molecular standard marker (pHY marker, Takara Shuzo Co.). Distinctive restriction fragment patterns produced by each enzyme were assigned letters. Individuals were assigned haplotypes consisting of the series of the letters designating the fragment profiles produced by each of the eight restriction enzymes. Thus, the composite haplotype for each individual consisted of a string of eight letters.

Sequencing analysis

The first half of control region of the mitochondrial genome was amplified by using primers light-strand MT4 (*5'*-CCTCCCTAAGACTCAAGGAAg-*3'*) and heavy-strand Dlp 5R (*5'*-CCATCGAGATGTCTTATTTAAGGGGAAC-*3'*). PCR products were purified by MicroSpin S-400HR columns (Pharmacia Biotech). Cycle sequencing were performed with the same primers, using AmpliTaq FS Sequencing Kit (Perkin-Elmer, Inc). The cycle sequencing products were purified by AutoSeq G-50 spin Columns (Pharmacia Biotech) and then sequenced on an ABI 377 Automated DNA Sequencer (Applied Biosystems, Inc), following the protocols of the manufacturer. For each sample both strands were sequenced. Sequences were aligned using Sequence Navigator (Applied Biosystems, Inc).

Data analysis

Grouping of samples

Samples examined in both RFLP and sequencing analyses from sub-areas 7, 8 and 9 were grouped into year/sub-area/temporal groups. Temporal groups involved an 'Early' and 'Late' group. 'Early' refers to whales sampled in May and June. 'Late' refers to whales sampled in July, August and September. Samples from sub-area 6 were used as an outgroup.

Genetic variability

Pairwise genetic distances among eight RFLP haplotypes were calculated using maximum likelihood methods (Nei and Li, 1979; Nei and Tajima, 1983). The Kimura's two parameters method (Kimura, 1980) was used for estimating genetic distances between two unique sequences. We used sequence data to estimate index of nucleotide diversity (Nei, 1987 pp. 256) in each sub-area. The net genetic distances between sub-areas were estimated from equation 10.21 of Nei (1987).

Homogeneity test

Quantification of the geographical and temporal mtDNA subdivision was carried out using the Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992). We used both the PHIst and Fst statistics. The former statistics consider genetic distance information among haplotypes while the latter is based only in frequency data of each haplotype. The significance of the variance components was tested by resampling 10,000 times the original data set.

We tested first for yearly variation in the temporal groups ('early' and 'late' groups) in each sub-area. Next we tested for temporal variation within sub-area ('early' against 'late' groups in each sub-area) and finally we tested for differences among sub-areas.

Phylogenetic analysis
A phylogenetic tree among sub-areas was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) as implemented in the PHYLIP package (Felsenstein, 1993). The tree was based in the net inter-populational distances among sub-areas.

Effective dispersal rate
The dispersal rate between sub-areas were examined by the following equation:

$$Fst = 1/(1 + 2N_e d) \qquad (1)$$

where N_e is the effective population size and d is dispersal rate. Equation 1 assumes that there are equal abundances in both sub-areas and that the abundance is expressed as the effective population size (approximately the number of breeding females) (Wright, 1951). Data on effective population size was obtained from SCWP7 of the 1996 SC meeting.

RESULTS

RFLP analysis

Genetic variability
A previous study had discriminated a total of eight mtDNA RFLP haplotypes in the western North Pacific minke whale (Table 2). Of them five (haplotypes 1, 2, 3, 5 and 6) were identified in this study. Genetic distances among five haplotypes ranged from 0.0038 to 0.0150.

Geographical distribution of haplotypes
Haplotype frequencies by period, sub-area and year are shown in Table 3. Haplotype '1' was the predominant haplotype in the Pacific side of Japan (sub-areas 7, 8 and 9). In contrast, the frequency of this haplotype was zero in sub-area 6. Instead, the predominant haplotype in sub-area 6 was haplotype '5', followed by haplotypes '3'.

Homogeneity test
In the sub-area/period groups of 7 early (sa7E), 8 late (sa8L), 9 early (sa9E) and 9 late (sa 9L), there was no significant yearly variation (Table 5a). In groups 7 late (sa 7L) and 8 early (sa 8E) the test for yearly variation could not be conducted because samples from only a single year was available.

Table 6a shows the result of the temporal variation within each sub-area by the PHIst and Fst statistics. No significant differences were found between early and late groups in the sub-areas. A close-to-significant value was found for the PHIst. However none of the other statistics (Fst of RFLP and PHIst and Fst of sequencing) were significant. Thus we disregarded that close-to-significant estimate.

Results of the nested analysis using both the PHIst and Fst statistics are summarized in Table 7a. Of the total molecular variance 80.82% (Fst) and 85.41% (PHIst) were due to sub-area 6/sub-areas 7, 8 and 9 division, which was highly significant; however,

stratifying the samples from sub-areas 7, 8 and 9 accounted for a no significant amount of the pairwise variation. This indicates that samples from the Pacific side of Japan (i.e. sub-areas 7, 8 and 9) are highly divergent from minke whales from Sea of Japan (sub-areas 6).

Sequencing analysis

Genetic variability

A 487-base pairs of the mtDNA control region (the 5'-end) was analyzed. A total of 33 polymorphic sites defined 62 haplotypes. Except for two in/del and one transversion sites, all substitutions were transitions. Genetic distances among 62 sequencing haplotypes range from 0.0021 to 0.0274.

Geographical distribution of haplotypes

The sequence haplotype frequencies by period, sub-area and year are shown in Table 4. In the 28 individuals from sub-area 6, five haplotypes were detected. Haplotype '1' was the predominant haplotype in this sub-area, while the frequency of this haplotype was relatively low in the Pacific side of Japan (sub-areas 7, 8 and 9). Three haplotypes were specific to this sub-area. In the 139 individuals from sub-area 7, 47 haplotypes were detected, and eleven haplotypes were found in single specimens. In the 91 individuals from sub-area 8, thirty-six haplotypes were detected, and twenty haplotypes were found in single specimens. In the 188 individuals from sub-area 9, forty haplotypes were detected, and fifteen haplotypes were found in single specimens. Seven, eight and ten haplotypes were specific to sub-areas 7, 8 and 9, respectively.

Homogeneity test

No significant yearly differences were found in sa7E, sa8L, sa9E and sa9L using both the PHIst and Fst statistics (Table 5b).

Table 6b shows the result of the test for temporal variation within each sub-area by the PHIst and Fst statistics. No significant differences were found between early and late groups in the sub-areas.

Results of the nested analysis using both the PHIst and Fst statistics are summarized in Table 7b. Both statistics separated clearly sub-area 6 from the three sub-areas in the Pacific side of Japan (7, 8 and 9). No significant differences were found among sub-areas 7, 8 and 9, although the probability of Fst of the test among sub-areas 7, 8 and 9 was close to significant (P=0.0832).

Intra- and inter- populational distances and phylogenetic tree

Table 8 shows the intra- and inter-populational distances among sub-areas. The intra-populational distances (nucleotide diversity) were 0.00457+/-0.00090, 0.00843+/-0.00040, 0.00832+/-0.00050 and 0.00727+/-0.00028 for sub-areas 6, 7, 8 and 9, respectively. Estimated nucleon diversity was 0.6032 for sub-area 6, 0.9543 for sub-area 7, 0.9602 for sub-area 8 and 0.9435 for sub-area 9. The net inter-populational distance between sub-area 6 and Pacific side of Japan ranged from 0.00692 to 0.00704. On the other hand, the net interpopulational distance among sub-areas in the Pacific side of Japan ranged from negative value to 0.00001. Intrapopulational distances within sub-areas were almost equal to the interpopulational distances among sub-areas in the Pacific side of Japan. The phylogenetic tree among sub-areas is shown in Fig.2. This tree showed a close relationships among sub-areas 7, 8 and 9. These three sub-areas are distant from sub-area 6.

Estimation of effective dispersal rate
The largest Fst or PHIst value among sub-areas 7, 8 and 9 was 0.003 by the hierarchical analysis using AMOVA (Fst of sequencing analysis in Table 7). Under the assumptions of Wright's island model of population structure, the number of disperse animals was estimate to be 131.2 female per generation.

DISCUSSION

In this study the total samples collected during the JARPN surveys between 1994 and 1999 in sub-areas 7, 8 and 9, were analyzed using two independent mtDNA methods, RFLP and sequencing analyses of the mtDNA control region. As established earlier, the analysis was focused to find evidence for the occurrence of the W stock in the eastern side of Japan.

Statistics

Our homogeneity tests were based on the haplotype (Fst) and sequence (PHIst) statistics of the AMOVA. Although none of our comparisons yielded a significant Fst or PHIst value, generally we found that the Fst values were higher than the PHIst values and some of the Fst values were near to significant. This suggests that Fst statistic could be more sensitive than the PHIst to discriminate among populations. A similar result was found by Pastene and Baker (1997), whose used both statistics to examine population structure in the southern humpback whale.

Population structure

In concordance with previous morphological, conception dates and genetic studies, we found striking genetic differences between minke whales from Korean waters (sub-area 6) and those in the eastern side of Japan. We found a marked segregation of haplotypes between sub-areas 6 and the sub-areas 7, 8 and 9. These results are consistent with the previous view of two populations on either side of Japan. In contrast, the statistics used were unable to discriminate among whales from sub-areas 7, 8 and 9.

These results are similar to those found by Abe *et al.* (this meeting) whose used eight microsatellite loci to examine a similar data set. In that study no significant deviation from Hardy-Weinberg equilibrium was observed in the sub-areas of the eastern side of Japan. The homogeneity test showed striking differences between sub-areas 6 and the other sub-areas, but it showed no significant differences among sub-areas 7, 8 and 9.

Thus the results of uni and biparental inherited genetic markers are similar and they found no evidence for additional stock structure in the eastern side of Japan (i.e. W stock). These results should be considered to be robust because a) microsatellite and mtDNA control region sequencing analyses are considered two powerful tools for investigating population structure and b) our analysis was based in large sample sizes, at least larger than those used in most of the genetic studies on marine mammals. In addition the dispersal rate obtained for sub-areas 7, 8 and 9 using mtDNA data was high. Thus genetic data imply sufficient dispersal between animals in sub-areas 7, 8 and 9 to maintain the observed degree of genetic homogeneity.

There is a necessity, however, to define a geographical boundary for the O stock in the eastern side of Japan. In a first approach to this issue, Goto and Pastene (1999) examined the mtDNA control region sequences of six eastern North Pacific minke whales. Surprisingly, each of these animals presented a different sequence, all of which

were represented in sub-areas 7, 8 and 9 in the central and western parts of the North Pacific. Such results suggest that minke whales of North Pacific could belong to one large population, or that the degree of genetic differentiation among putative stocks in the North Pacific could be very low. In this regard it is necessary further analysis of more samples from the east of sub-area 9.

We did not conduct an estimation of the statistical power of our mtDNA analysis. The statistical power will depend on the expected degree of genetic differentiation between populations and on sample size. As mentioned earlier, our analysis was based on JARPN samples only and involved large sample sizes. As samples were available from former coastal commercial whaling operations for sub-area 7, we examined these samples to investigate the effect in our results of including larger sample size in this sub-area. We conducted the same hierarchical analysis by AMOVA but the sample size for sub-area 7 increased from 139 to 285. The results for both RFLP and sequencing are shown in the appendix. Even by increasing the sample size no significant differences were found among sub-areas 7, 8 and 9.

Genetic diversity

Nucleotide diversity was lower in the Korean minke whales (sub-area 6) than in minke whales from the eastern side of Japan. The low genetic diversity in the Korean sample is in agreement with the results of the microsatellite analysis (Abe *et al.*, this meeting), which found a lower heterozygosity in the Korean sample. The levels of mtDNA diversity found are well correlated with the population sizes of the J and O stocks, the former being substantially smaller that the O stock.

Nucleotide diversity in the North Atlantic minke whale was estimated at 0.0064 (Bakke *et al.*, 1996). Then this value is lower than North Pacific sub-areas 7, 8 and 9 but higher than sub-area 6 (Korea). Nucleotide diversity in the Antarctic minke whale (0.0159) is higher than in North Pacific and North Atlantic (Bakke *et al.*, 1996). These authors explained that the higher value of nucleotide diversity in the Antarctic than in the North Atlantic reflects a larger long-term effective population size of the Antarctic minke whale compared to the North Atlantic minke whale. In the Antarctic humpback whale nucleotide diversity was larger ranging from 0.0238 to 0.0323 (Pastene *et al.*, 1997a). The range of nucleotide diversity in the North Pacific Bryde's whale (0.0077 to 0.0091) (Pastene *et al.*, 1997b) is similar to that of North Pacific minke whale of the eastern side of Japan.

Phylogenetic relationships

The phylogenetic tree among sub-areas showed a close relationship among sub-areas 7, 8 and 9. They were clearly divergent from whales from sub-area 6 (Fig.2). This result supported the results of our homogeneity test.

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Table 1. Number of samples collected during the JARPN surveys from 1994 to 1999 by sub-area, year, month, period and sex. All these were examined in this study for RFLP and sequencing analyses except sub-area 11.

Sub-area	Year	Month										Total
		Early				Late						
		May		June		July		August		September		
		Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	
7	1996					1			15	2	13	31
	1997				2							2
	1998	7	49									56
	1999			7	43							50
8	1996						11		5			16
	1997					1	30					31
	1998	1	7	3	33							44
9	1994					2	6	1	8		4	21
	1995				14	5	56	4	21			100
	1997	7	20	5	35							67
11	1996							11	19			30
	1999					22	28					50
	Total	15	76	15	127	31	131	16	68	2	17	498

Table 2: Composite pattern of eight western North Pacific minke whale mtDNA control region RFLP haplotypes and a matrix of presence or absence of six restriction sites (from Goto and Pastene, 1997). Letters from left to right refer to the digestion pattern for *Afa*I, *Dde*I, *Hae*III, *Hin*fI, *Mbo*I, *Msp*I, *Sau*96I and *Scr*FI.

Haplotype	Restriction site matrix					
1-CCCCNNCN	1	1	0	1	0	0
2-BCCCNNCN	0	1	0	1	0	0
3-CCBCNNCN	1	1	0	0	0	0
4-CCCCNNDN	1	1	0	1	0	1
5-BCBCNNCN	0	1	0	0	0	0
6-CCDCNNDN	1	1	1	1	0	1
7-CCBDNNCN	1	1	0	0	1	0
8-CBCCNNCN	1	0	0	1	0	0

Table 3: mtDNA RFLP haplotype frequency of JARPN samples by sub-area and year.

Hap.	Sub-area 6	Sub-area 7				Sub-area 8			Sub-area 9			
		Early			Late	Early		Late	Early		Late	
		1997	1998	1999	1996	1998	1996	1997	1995	1997	1994	1995
1	0	1	49	47	30	41	15	26	14	65	19	79
2	1	0	3	0	0	2	1	2	0	0	0	2
3	7	1	1	0	0	0	0	0	0	1	1	3
4	0	0	0	0	0	0	0	0	0	0	0	0
5	21	0	3	3	0	1	0	2	0	1	1	1
6	0	0	0	0	1	0	0	1	0	0	0	1
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
Total	29	2	56	50	31	44	16	31	14	67	21	86

Table 4: mtDNA sequence haplotype frequency of JARPN samples by sub-area and year.

	Sub-area 6	Sub-area 7				Sub-area 8			Sub-area 9			
		Early			Late	Early		Late	Early		Late	
Hap.		1997	1998	1999	1996	1998	1996	1997	1995	1997	1994	1995
1	18	0	3	3	0	0	0	1	0	1	1	0
2	2	0	0	0	0	0	0	0	0	0	0	0
3	6	0	0	0	0	0	0	0	0	0	0	0
4	1	0	0	0	0	0	0	1	0	0	0	0
5	1	0	0	0	0	0	0	0	0	0	0	0
6	0	0	3	4	1	1	0	0	0	3	2	4
7	0	1	5	6	3	3	2	5	1	6	0	8
8	0	0	1	3	2	0	0	1	1	0	0	2
9	0	0	6	5	2	5	1	1	3	9	3	15
10	0	0	0	0	1	0	0	0	0	0	0	0
11	0	0	0	4	1	3	1	1	2	1	1	6
12	0	0	1	2	1	1	0	2	0	3	0	4
13	0	0	4	6	3	6	2	0	0	2	0	2
14	0	0	2	2	2	3	0	3	1	5	1	4
15	0	0	3	0	1	3	0	1	0	4	0	2
16	0	0	3	2	3	3	1	0	3	5	1	2
17	0	0	2	3	1	2	0	3	0	6	2	8
18	0	0	1	0	1	1	1	0	0	0	1	2
19	0	0	0	1	1	0	0	1	0	1	0	0
20	0	0	1	0	2	0	0	0	0	0	0	0
21	0	0	2	0	1	0	0	0	1	1	1	0
22	0	0	0	0	1	0	0	0	0	0	0	0
23	0	0	0	0	1	0	0	0	0	0	0	1
24	0	0	0	0	1	0	0	0	0	0	0	0
25	0	0	0	0	1	0	2	0	0	0	2	2
26	0	0	0	0	1	1	0	0	0	0	0	0
27	0	1	0	0	0	0	0	0	0	0	1	0
28	0	0	1	0	0	0	0	0	0	0	0	0
29	0	0	1	1	0	1	1	2	0	4	1	0
30	0	0	3	4	0	0	0	1	1	3	3	2
31	0	0	2	0	0	0	0	0	0	0	0	2
32	0	0	1	0	0	0	0	0	0	0	0	0
33	0	0	1	1	0	0	0	0	0	0	0	0
34	0	0	2	0	0	3	1	0	0	0	1	2
35	0	0	2	0	0	0	0	1	0	0	0	0
36	0	0	3	0	0	2	1	0	0	0	0	1
37	0	0	1	1	0	0	0	0	0	1	0	1
38	0	0	1	1	0	1	0	0	1	0	0	0
39	0	0	1	0	0	0	1	1	0	2	0	3
40	0	0	0	0	0	0	1	0	0	0	0	0
41	0	0	0	0	0	0	1	0	0	0	0	0
42	0	0	0	0	0	0	0	1	0	0	0	0
43	0	0	0	0	0	0	0	1	0	0	0	0
44	0	0	0	0	0	0	0	1	0	0	0	0
45	0	0	0	0	0	1	0	1	0	0	0	0
46	0	0	0	0	0	0	0	1	0	0	0	0
47	0	0	0	0	0	0	0	1	0	0	0	0
48	0	0	0	0	0	1	0	0	0	0	0	1
49	0	0	0	0	0	1	0	0	0	1	0	0
50	0	0	0	0	0	1	0	0	0	1	0	0
51	0	0	0	0	0	1	0	0	0	1	0	0
52	0	0	0	0	0	0	0	0	0	2	0	3
53	0	0	0	0	0	0	0	0	0	1	0	3
54	0	0	0	0	0	0	0	0	0	0	0	1
55	0	0	0	0	0	0	0	0	0	0	0	1
56	0	0	0	0	0	0	0	0	0	0	0	1
57	0	0	0	0	0	0	0	0	0	0	0	1
58	0	0	0	0	0	0	0	0	0	0	0	1
59	0	0	0	0	0	0	0	0	0	1	0	1
60	0	0	0	0	0	0	0	0	0	2	0	0
61	0	0	0	0	0	0	0	0	0	1	0	0
62	0	0	0	1	0	0	0	0	0	0	0	0
Total	28	2	56	50	31	44	16	31	14	67	21	86

Table 5: Yearly variation examined by Fst and PHIst statistics in four sub-area/temporal groups of JARPN minke whales. a) RFLP and b) sequencing. In parenthesis is the sample size. Early: May and June, Late: July, Aug. and Sep. P=probability.

a) RFLP

Subarea/temporal group	Year	Fst	P	PHIst	P
Sub-area 7 Early	1997, 1998, 1999 (2), (56), (50)	0.058	0.0640	0.018	0.2019
Sub-area 8 Late	1996, 1997 (16), (31)	-0.016	0.4761	-0.019	0.5574
Sub-area 9 Early	1995, 1997 (14), (67)	-0.031	0.3425	-0.029	0.3374
Sub-area 9 Late	1994, 1995 (21), (86)	-0.023	0.8791	-0.017	0.6474

b) Sequence

Subarea/temporal group	Year	Fst	P	PHIst	P
Sub-area 7 Early	1997, 1998, 1999 (2), (56), (50)	-0.006	0.7833	-0.018	0.9235
Sub-area 8 Late	1996, 1997 (16), (31)	0.002	0.3898	-0.011	0.6356
Sub-area 9 Early	1995, 1997 (14), (67)	0.000	0.4146	0.028	0.1416
Sub-area 9 Late	1994, 1995 (21), (86)	-0.002	0.5200	-0.008	0.5902

Table 6: Temporal variation examined by Fst and PHIst statistics in each sub-area. a) RFLP and b) sequencing.
Early: May and June, Late: July, Aug. and Sep. P=probability.

a) RFLP

Sub-area	Sample size		Fst	P	PHIst	P
	Early	Late				
Sub-area 7	(108)	(31)	0.010	0.1657	0.035	0.0508
Sub-area 8	(44)	(47)	-0.009	0.5944	-0.012	0.6828
Sub-area 9	(81)	(107)	0.010	0.1276	0.000	0.3452

b) Sequence

Sub-area	Sample size		Fst	P	PHIst	P
	Early	Late				
Sub-area 7	(108)	(31)	-0.006	0.8400	0.015	0.0996
Sub-area 8	(44)	(47)	0.001	0.4070	-0.006	0.6313
Sub-area 9	(81)	(107)	-0.002	0.6896	-0.002	0.5266

Table 7: Results of the nested analyses by Fst and PHIst statistics for four sub-areas. a) RFLP and b) sequencing.

a) RFLP

Fst

	df	%total variance	Fst	P
Among SA6/SA7,8,9	1	80.82	CT: 0.808	<0.0001
Among SA7,8,9	2	0.01	SC: 0.000	0.3001
Within sub-areas	443	19.17	ST: 0.808	<0.0001

PHIst

	df	%total variance	PHIst	P
Among SA6/SA7,8,9	1	85.41	CT: 0.854	<0.0001
Among SA7,8,9	2	0.03	SC: 0.002	0.2121
Within sub-areas	443	14.55	ST: 0.854	<0.0001

b) Sequence

Fst

	df	%total variance	Fst	P
Among SA6/SA7,8,9	1	19.51	CT: 0.195	<0.0001
Among SA7,8,9	2	0.21	SC: 0.003	0.0832
Within sub-areas	442	80.28	ST: 0.197	<0.0001

PHIst

	df	%total variance	PHIst	P
Among SA6/SA7,8,9	1	47.04	CT: 0.470	<0.0001
Among SA7,8,9	2	0.04	SC: 0.001	0.3525
Within sub-areas	442	52.92	ST: 0.471	<0.0001

Table 8: Intra- and inter-populational distance among sub-areas.
The diagonal show the intra-populational distance (i.e. nucleotide diversity) and above diagonal show the net inter-populational distance.

	Sub-area 6 (36)	Sub-area 7 (139)	Sub-area 8 (91)	Sub-area 9 (188)
Sub-area 6	0.00457	0.00701	0.00704	0.00692
Sub-area 7		0.00843	-0.00003	0.00001
Sub-area 8			0.00832	0.00001
Sub-area 9				0.00727

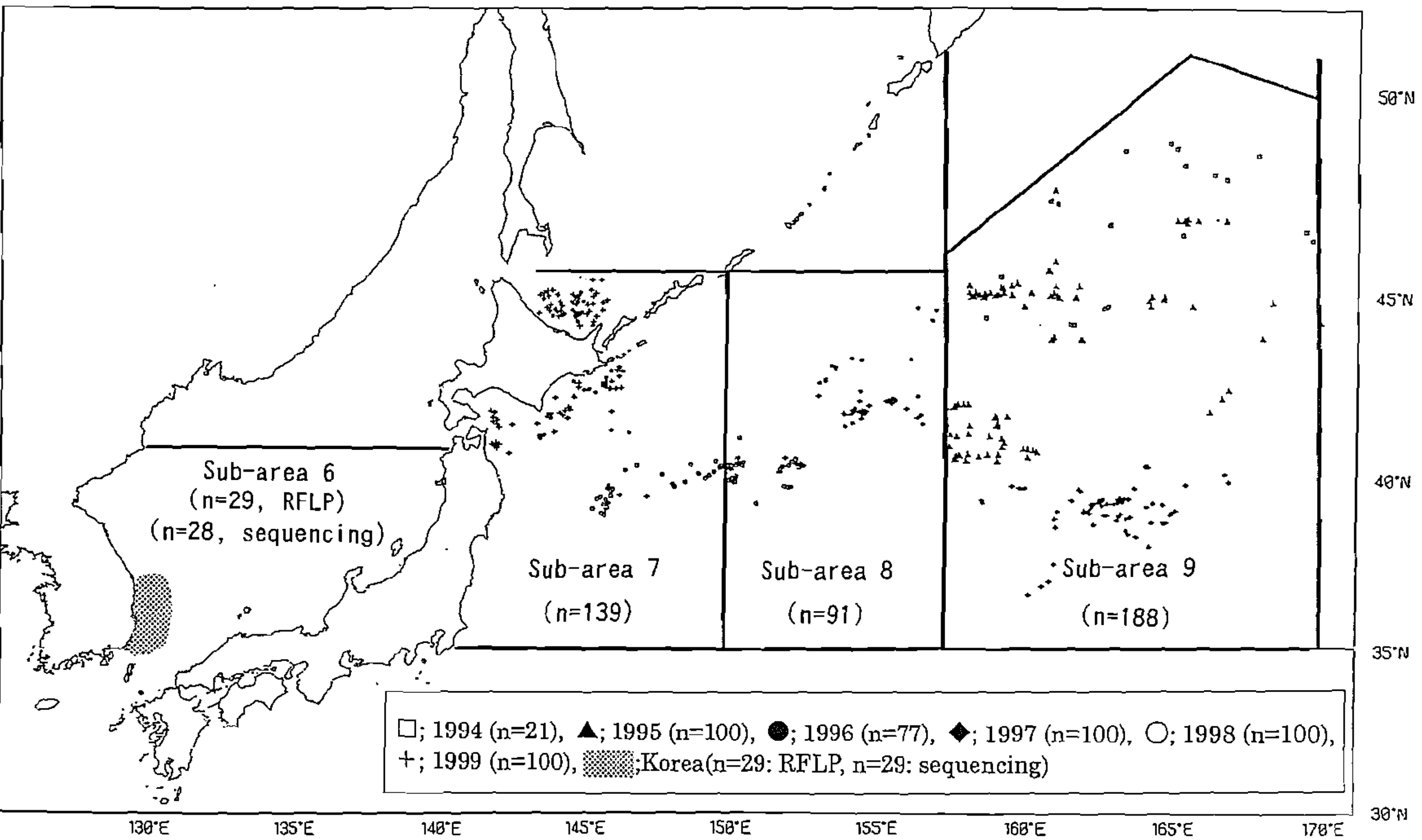

Fig.1. Geographical localities and sample size used in the RFLP and sequencing analyses of the mtDNA control region.

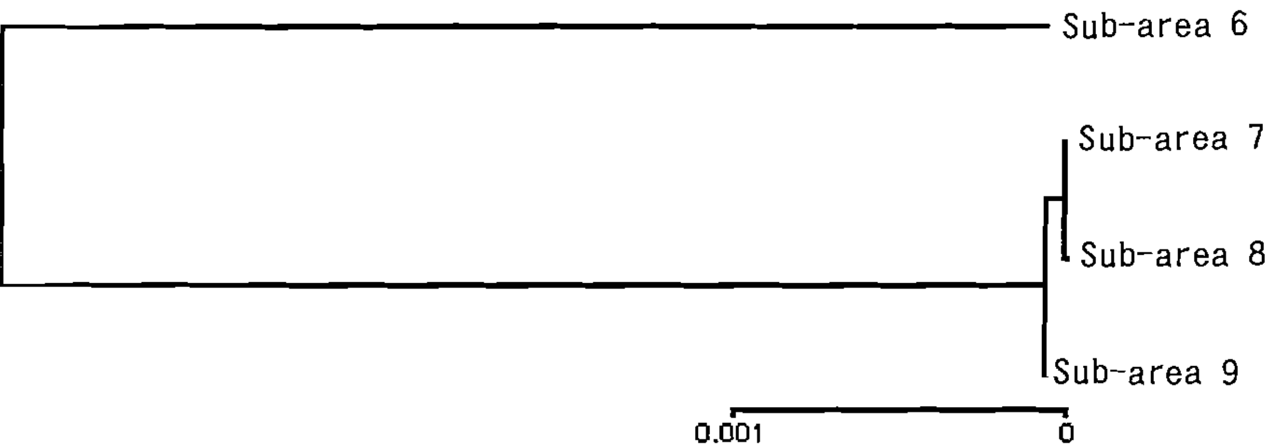

Fig. 2. Phylogenetic relationship among four sub-areas constructed by the neighbour-joining method. SA denote sub-area.

Appendix

Results of the nested analyses by PHIst and Fst for four sub-areas. a) RFLP and b) sequencing. Commercial data were including in sub-area 7. Sample size for sub-area 7 was increased from 139 (JARPN samples) to 285 (JARPN samples + commercial samples).

a) RFLP

Fst

	df	%total variance	Fst	P
Among SA6/SA7,8,9	1	80.67	CT: 0.807	<0.0001
Among SA7,8,9	2	0.00	SC: 0.000	0.3174
Within subareas	589	19.33	ST: 0.807	<0.0001

PHIst

	df	%total variance	PHIst	P
Among SA6/SA7,8,9	1	85.37	CT: 0.854	<0.0001
Among SA7,8,9	2	0.02	SC: 0.001	0.2596
Within subareas	589	14.61	ST: 0.854	<0.0001

b) Sequence

Fst

	df	%total variance	Fst	P
Among SA6/SA7,8,9	1	19.61	CT: 0.196	<0.0001
Among SA7,8,9	2	0.09	SC: 0.001	0.2137
Within subareas	588	80.30	ST: 0.197	<0.0001

PHIst

	df	%total variance	PHIst	P
Among SA6/SA7,8,9	1	45.94	CT: 0.459	<0.0001
Among SA7,8,9	2	-0.03	SC: -0.001	0.5379
Within subareas	588	54.09	ST: 0.459	<0.0001