RFLP analysis of the mitochondrial DNA control region in minke whales sampled during the 1996 JARPN.

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ABSTRACT

A restriction fragment length polymorphisms (RFLP) analysis of the mitochondrial DNA (mtDNA) control region is used to examine the genetic difference of minke whales taken during the 1996 JARPN. A total of 77 whales is examined (16 from sub-areas 8 taken in July and August, 31 from sub-area 7 in July, August and September and 30 from sub-area 11 in August). In addition, two skin biopsy samples from sub-area 11 taken in August are included in the analysis. Homogeneity tests were conducted using AMOVA. In general, the pattern of variation found is similar to that reported in a previous paper. Haplotype frequencies in the samples from sub-areas 7 and 8 are not significantly different from those of the 'O' stock. Analysis of monthly variation in sub-area 11 (now including samples from August) suggested significant differences attributed to the April sample. Although not statistically significant, the main haplotypes of the J stock (haplotypes '3' and '5') were present in the August sample in relatively high frequencies (haplotype '3' was 9.4%; haplotype '5'was 15.6%). Further analyses on a monthly basis will be necessary in order to investigate whether the 'J' stock is present in months other than April in sub-area 11.

INTRODUCTION

Goto and Pastene(1997) investigated the population structure of the western North Pacific minke whales using RFLP analysis of mtDNA control region. They showed remarkable mtDNA differences between whales from coastal Korea and those of the eastern side of Japan. They also showed that both populations temporarily mix each other in the southern part of Okhotsk Sea in April. In addition, no significant differences between coastal and offshore samples in the Pacific side of Japan were found.

In 1996, the Japanese Whale Research Program under Special Permit in the North Pacific (JARPN) covered sub-areas 7, 8 and 11 between July and September and 77 minke whales were

sampled (Fujise *et al.*, 1997). Of particular interest are the samples obtained from sub-area 8, from where no samples had been available previously, and samples from sub-area 11 taken late in the season (August).

We present here the results of a mtDNA analysis conducted on the 1996 JARPN samples. Analysis of samples from sub-area 8 is important to elucidate whether one or more stocks are distributed in the Pacific side of Japan. Analysis of samples from sub-area 11 taken late in the season is also important to investigate the temporal mixing of J and O stocks in that sub-area. The analysis of the 1996 samples is conducted in conjunction with the analysis of previous data (Goto and Pastene, 1997).

MATERIALS AND METHODS

Samples and localities

Minke whales used in this study were caught in the past Korean and Japanese coastal small-type whaling operations during 1982-1987. Other samples were from the JARPN surveys between 1994-1996. Five regions were defined using the geographical position of the samples taken (Fig. 1): Korea (sub-area 6 defined by the Working Group on North Pacific Minke Whale Management Trials, including one individual from sub-area 5), Pacific coast of northern Japan (sub-area 7), southern part of Okhotsk Sea (sub-area 11) and two offshore areas (sub-area 8 and 9). The offshore samples from sub-area 9 were obtained during the 1994 and 1995 JARPN surveys. During the 1996 JARPN conducted from July to September, a total of 77 minke whales was taken from sub-area 7 (n=31), sub-area 8 (n=16) and sub-area 11 (n=30). In addition, two skin biopsy samples obtained from sub-area 11 were used. The number of samples collected during 1996 JARPN and the total number of samples examined until now, are shown in Table 1 and 2 by sub-area and month, respectively. Th geographical localities and sample size examined for this analysis are showed in Fig.1.

Tissue samples, DNA extraction and amplification of the mtDNA control region

Using established protocols (Sambrook *et al.*, 1989), genomic DNA (mtDNA + nuclear DNA) was isolated from liver or muscle tissue. The control region of the mitochondrial genome was amplified by using the polymerase chain reaction (PCR)(Hoelzel, 1992). Primers for amplifying the 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'-CAAGGAAGAAGT-ATTACACTCCACCA-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. The statistical analysis of mtDNA RFLP data is as described in our previous paper (Goto and Pastene, 1997).

RESULTS

Haplotype frequency

Five of the eight enzymes (*Afa*I, *Dde*I, *Hae*III, *Hin*fI and *Sau*96I) revealed polymorphic patterns among the samples. A total of six polymorphic sites were detected defining eight unique haplotypes among 556 minke whales (see Goto and Pastene, 1997).

Haplotype frequencies of samples collected in 1996 JARPN by sub-area is shown in Table 3. Haplotype '1' was the predominant haplotype in the Pacific coastal region (sub-area 7) and in the offshore region (sub-area 8). Although the predominant haplotype in sub-area 11 was haplotype '1' there is a relatively high frequency of haplotypes '3' and '5', which are the main haplotypes in whales from the J stock.

The frequencies of each haplotype in the total sample are shown in Table 4, by sub-areas. Haplotype '1' was the predominant haplotype in the coastal Japanese sampling region (sub-areas 7 and 11) and in the offshore region (sub-areas 8 and 9). In contrast, this haplotype was not observed in 30 individuals from the coastal sub-areas of Korea (sub-areas 5 and 6). Instead, the predominant haplotype in the Korean sample was haplotype '5', followed by haplotype '3'. With the exception of sub-area 11, haplotypes '3' and '5' were present but in low frequencies in sub-areas 7 and 9, and they were not observed in sub-area 8.

The nucleon diversity (h) estimated in the sub-areas 7, 8 and 9 were 0.142, 0.121 and 0.142, respectively (Table 4). In contrast, the h in the sub-areas 6 (0.414) and 11 (0.420) were relatively higher than in the other sub-areas. In sub-area 11, the h estimated excluding April data (0.283) also showed a relatively high value (figure in parenthesis in Table 4).

Monthly distribution of haplotypes in sub-areas 7, 9 and 11.

Haplotype frequencies by months in all sub-areas are shown in the appendix. The results of a statistical test for monthly heterogeneity within each sub-area are shown in Table 5. There were no significant differences in haplotype frequencies among months in sub-area 7 (PHIst=-0.003, P=0.5447) and sub-area 9 (PHIst=-0.014, P=0.8076). In sub-area 11 haplotypes '3' and '5' were observed at moderate frequency in the April and August samples, but they were scarcely represented in the rest of the months for this region (see appendix c). The results of the AMOVA for temporal heterogeneity in this region indicated significant differences in haplotype frequencies among months (PHIst=0.095 and P=<0.0005). If the April sample is excluded the results of the AMOVA show no significant differences among months in this sub-area.

Comparison among sub-areas (April data excluded from sub-area 11).

Minke whales distributed in sub-area 11 in April could be a mixture from two different populations (Goto and Pastene, 1997). Therefore we have excluded April data from sub-area 11 for comparison among sub-areas. Results of the nested analysis by AMOVA are summarized in Table 6. Of the total molecular variance 82.44% was due to Korea-eastern side of Japan division, which was highly significant; though samples from the eastern side of Japan including sub-area 8 showed no significant amount of the pairwise variation. This indicates that sub-areas 11, 7, 9 and 8 are highly divergent from minke whales from Korea (sub-areas 5 and 6).

DISCUSSION

In this study, samples collected in 1996 JARPN were analyzed using RFLP analysis of the mtDNA control region. For doing that, we compared the pattern of mtDNA variation in these samples with those found in our previous study (Goto and Pastene, 1997)

The pattern of genetic variation derived from this study is similar to that reported in the previous paper (Goto and Pastene, 1997). The results suggest that whales from sub-area 8 have a similar mtDNA composition to that of sub-area 7 and 9. Then we found no evidence to support the occurrence of more than one stock in the Pacific side of Japan and then we can not reject the hypothesis that the same O stock distribute in both coastal and offshore areas in the Pacific side of Japan. Wada (1997) analyzed minke whales from sub-areas 7, 8, 9 and 11 using allozymes. He suggested that samples from sub-area 9 were not from a mixed population and that these samples belong to the same stock as whales from sub-areas 7 and 11. Abe *et al.* (1997) used four microsatellite loci to compare samples from sub-areas 7 and 9. No significant differences were found between these two sub-areas. In contrast they differed significantly from whales from sub-area 6 (Korean).

On the other hand, analysis of monthly variation in sub-area 11 suggested significant differences attributed to the April samples (Goto and Pastene, 1997). Although not statistically significant, the representative haplotypes of the J-stock (haplotype '3' and '5') were present in the August sample in relatively high frequencies. Pastene *et al.* (1997) calculated the monthly mixing proportion of the J and O stocks in sub-area 11 using mtDNA haplotype data. They estimated the proportion of the J stock in that sub-area as 0.4032 (S.E.0.0770) in April and 0.1837 (S.E.0.0700) in August. In addition, these results showed a marked segregation by sex in that sub-area, that is, the contribution of female of the J stock was estimated 0.4075 (S.E.0.0806) in April while the contribution of male was 0.3147 (S.E. 0.1160) in August.

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Table 1: Number of samples collected in 1996 JARPN by sub-area and month. Samples in sub-area 11 are including two biopsy samples.

Sub-areas	Months			Total
	7	8	9	
Sub-area 7	1	15	15	31
Sub-area 8	11	5	0	16
Sub-area11	0	32	0	32
Total	12	52	15	79

Table 2: Number of samples examined in RFLP analysis of mtDNA control region by sub-area and month.

Sub-areas	Months							Total
	4	5	6	7	8	9	10	
Sub-area 6	0	0	0	0	0	19	11	30
Sub-area 7	44	30	24	26	22	38	0	184
Sub-area 8	0	0	0	11	5	0	0	16
Sub-area 9	0	0	14	69	34	4	0	121
Sub-area11	57	66	31	5	40	6	0	205
Total	101	96	69	111	101	67	11	556

Table 3: Haplotype frequency in samples collected in the 1996 JARPN, by sub-area.

Hap.	Sub-area		
	7	8	11
1	30(96.8)	15(93.8)	23(71.9)
2		1(6.2)	1(3.1)
3			3(9.4)
4			
5			5(15.6)
6	1(3.2)		
7			
8			
Total	31	16	32

Table 4: Haplotype frequency for the total sample and nucleon diversity (h) by sub-area. In parenthesis is the nucleon diversity for sub-area 11 estimated excluding April data.

Hap.	Sub-area					Total
	6	7	8	9	11	
1	0	170	15	112	154	451
2	1	4	1	2	9	17
3	7	4	0	4	15	30
4	0	0	0	0	1	1
5	22	3	0	2	19	46
6	0	3	0	1	3	7
7	0	0	0	0	2	2
8	0	0	0	0	2	2
Total	30	184	16	121	205	556
h	0.414	0.142	0.121	0.142	0.420 (0.283)	

Table 5: Nested statistical test for monthly heterogeneity within each sub-area by AMOVA. V(A) and V(B) are the molecular variances between and within months, respectively.

	V(A)	V(B)	PHIst	P
Sub-area 7	-0.26	100.26	-0.003	0.5447
Sub-area 9	-1.44	101.44	-0.014	0.8076
Sub-area 11*1	9.49	90.51	0.095	<0.0005
Sub-area 11*2	2.11	97.89	0.021	0.1309

Note: Sub-area 7 : Apr./May/June/July/Aug./Sep.
Sub-area 9 : June/July/Aug.+Sep.
Sub-area 11*1: Apr./May/June/July/Aug./Sep.
Sub-area 11*2: May/June/July/Aug./Sep.

See Appendix.

Table 6: Statistical analysis by AMOVA including all the geographical sub-areas (April data excluded in sub-area 11). In parenthesis are the values excluding 1996 data.

	df	total variance (%)	PHI	P
Among Korea/ eastern side of Japan	1	82.44	CT: 0.824	<0.0005
Among areas/ eastern side of Japan	4	0.06 (-0.10)	SC: 0.003 (-0.006)	0.2049 (0.9290)
Within areas	493	17.50	ST: 0.825	<0.0005

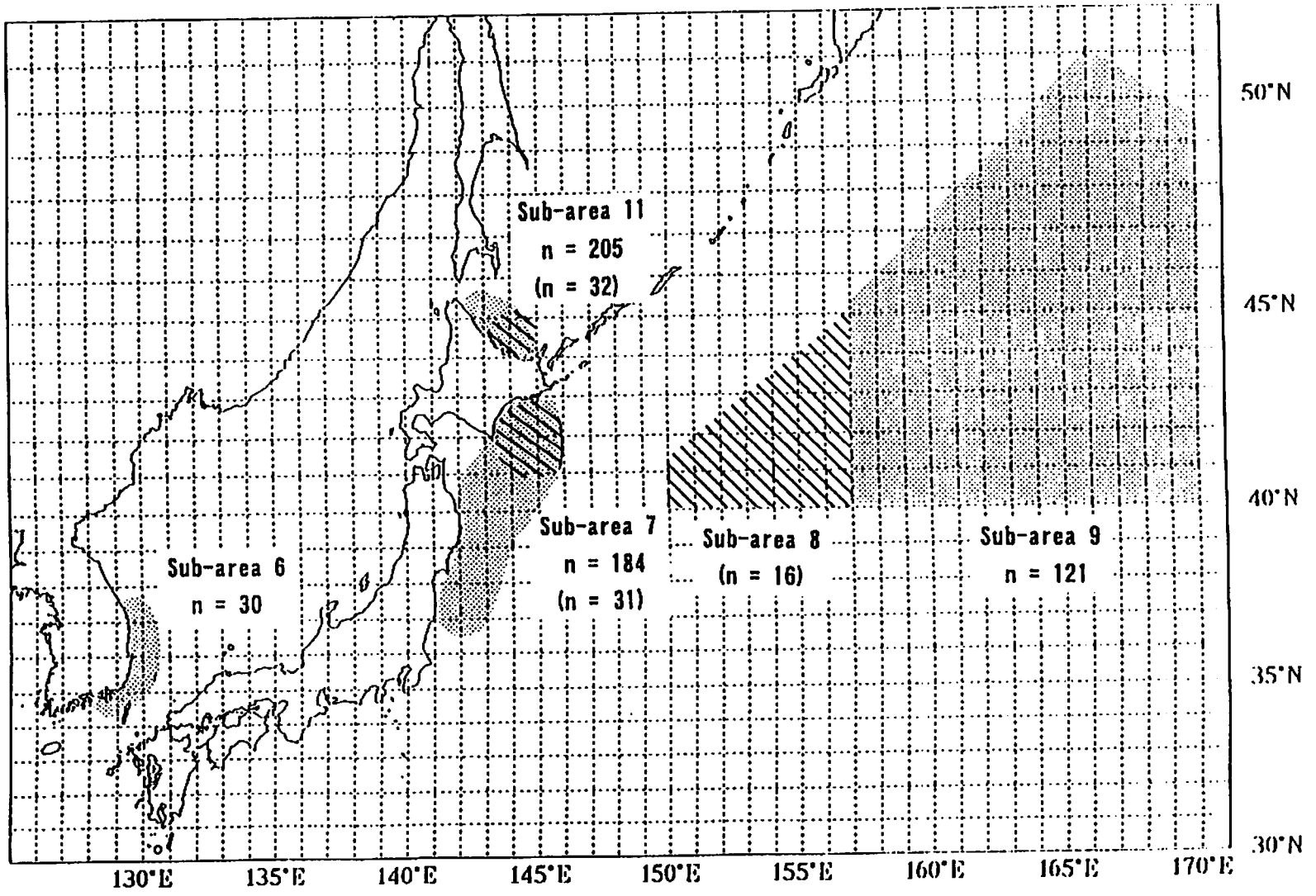

Fig. 1. Geographical localities and sample size examined for this analysis. The oblique-lined areas and the number in parenthesis are the sampling area and sample size in the 1996 JARPN, respectively.

Appendix : Haplotype frequency in each sub-area, by month.

a) Sub-area 7

Hap.	Months						Total
	4	5	6	7	8	9	
1	42	27	22	23	21	35	170
2	0	2	2	0	0	0	4
3	1	0	0	2	0	1	4
4	0	0	0	0	0	0	0
5	1	1	0	0	0	1	3
6	0	0	0	1	1	1	3
7	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0
Total	44	30	24	26	22	38	184

b) Sub-area 9

Hap.	Months				Total
	6	7	8	9	
1	14	63	32	3	112
2	0	2	0	0	2
3	0	3	0	1	4
4	0	0	0	0	0
5	0	1	1	0	2
6	0	0	1	0	1
7	0	0	0	0	0
8	0	0	0	0	0
Total	14	69	34	4	121

c) Sub-area 11

Hap.	Months						Total
	4	5	6	7	8	9	
1	29	59	27	3	30	6	154
2	5	1	2	0	1	0	9
3	9	1	1	1	3	0	15
4	0	1	0	0	0	0	1
5	12	1	1	0	5	0	19
6	1	1	0	0	1	0	3
7	0	2	0	0	0	0	2
8	1	0	0	1	0	0	2
Total	57	66	31	5	40	6	205