Population structure in the North Pacific minke whale as revealed by RFLP and sequencing analyses of the mtDNA control region

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ABSTRACT

A restriction fragment length polymorphism (RFLP) analysis of whole control region in the mitochondrial DNA (mtDNA) and sequencing of the first half of same region (487 bp) are used to examine stock structure in the North Pacific minke whales. In the first analysis, a total of 656 samples including 100 whales taken during the 1997 JARPN (67 from sub-areas 9 taken in May and June, 31 from sub-area 8 in July and 2 from sub-area 7 in June), is examined. Homogeneity tests were conducted using the Analysis of Molecular Variance (AMOVA) as implemented in the computer program Arlequin. The pattern of genetic variation in 1997 JARPN samples is similar to that reported in a previous paper. Haplotype frequencies in the samples from sub-areas 8 and 9 are not significantly different from those of the 'O' stock. In the second analysis, a total of 153 samples (28 from sub-area 6, 30 from sub-area 7 and 95 from sub-area 9) is examined in a preliminary sequencing analysis. In this analysis 41 unique sequences (haplotypes) were discriminated. The results of the homogeneity test by AMOVA showed a clear discrimination between sub-area 6 (Korea) and sub-areas 7 and 9 (eastern side of Japan). No significant heterogeneity was found in the comparison between sub-areas 7 and 9, though the probability was close-to-significant. Further analysis using more samples will be made in near future.

INTRODUCTION

Goto and Pastene(in press; 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 to each other in the southern part of Okhotsk Sea in April and August. The mixing rate in both months in the sub-area 11 were estimated by Pastene *et al.* (in review). In addition, no significant differences between coastal and offshore samples in the Pacific side of Japan were found.

The Working Group on North Pacific Minke Whale Trials agreed that the low proportion of females and smaller males in sub-area 9 was inconsistent with those samples representing a separate population (IWC, 1997). However, the group noted that the seasonal coverage of the samples from sub-area 9 did not include the period from April to May. Furthermore large portion of areas 8 had not been sampled. Then the group suggested that the information presented did not preclude the occurrence of W stock either (IWC, 1997).

In response to this discussion, the Japanese Whale Research Program under Special Permit in the North Pacific (JARPN) covered sub-areas 7, 8 and 9 between May and July in 1997 and 100 minke whales were sampled (Ishikawa *et al.*, 1997). Of particular interest are the samples obtained from sub-area 9 taken early in the season (May and June) and from low latitudinal part of this sub-area where few samples had been available previously.

We present here the results of a mtDNA RFLP analysis conducted on the 1997 JARPN samples and we also examined this data in the context of the previous analysis (Goto and Pastene, in press; 1997). We also present here the results of preliminary sequencing analysis to elucidate stock structure in the North Pacific minke whale. Because the higher resolution of the sequencing analysis, this approach might be useful to detect potentially different stocks with low levels of genetic differentiation.

MATERIALS AND METHODS

Samples and localities

RFLP analysis

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 1994-1997. 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). During the 1997 JARPN conducted from May to July, a total of 100 minke whales was taken from sub-area 7 (n=2), sub-area 8 (n=31) and sub-area 9 (n=67). The number of samples collected during 1997 JARPN and the total number of samples examined until now, are shown in Table 1 and 2 by sub-area and month, respectively. The geographical localities and sample size examined for this analysis are showed in Fig. 1.

Sequencing analysis

A total of 153 samples (28 from sub-area 6 sampled during past Korean commercial whaling, 30 from sub-area 7 sampled during the 1996 JARPN and 95 from sub-area 9 sampled during the 1995 JARPN) was examined. The number of samples by sub-area and their geographical localities are showed in Table 8 and Fig. 2, respectively.

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

RFLP analysis

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). In order to amplify the approximately 1,050 bp minke whale mtDNA including control region, primers light-strand MT4 (Arnason *et al.*, 1993) (*5'*- CCTCCCTAAGACTCAAGGAAG-*3'*) and heavy-strand P4 (Dillon and Wright, 1993) (*5'*-CAAGGAAGAAGTATTACACTCCACCA-*3'*) were used. These primers annealed to $tRNA^{Pro}$ and $tRNA^{Phe}$ regions, which flank the control region.

Sequencing analysis

Amplified minke whale mtDNA control region was used as template DNA for sequencing analysis. PCR products were isolated by gel electrophoretic excision and purified by Ultra free MCHV spin columns (Nippon Miri-pore Co.). Cycle sequencing were performed with MT4 and internal primer, Dlp 5R (*5'*-CCATCGAGATGTCTTATTTAAGGGGAAC-*3'*) (Baker *et al.*, 1997) using AmpliTaq FS Sequencing Kit (Perkin-Elmer, Inc). The cycle sequencing products were purified by Centri-Sep spin Columns (Perkin-Elmer, Inc) and then sequenced on an ABI 377 Automated DNA Sequencer (Applied Biosystems, Inc), following the protocols of the manufacture. For each sample both strands were sequenced. Sequences were aligned using Sequence Navigator (Applied Biosystems, Inc).

Restriction site map

In order to investigate the polymorphic site of the RFLP analysis, restriction map of whole mtDNA control region was drew up by sequence analysis. One individual representative of haplotypes (1, 2, 4, 6, 7 and 8 of the RFLP analysis was selected. As regard to haplotype 3 and 5, two individuals, one from Korea and one from eastern side of Japan were examined. A total of 10 individuals was examined in this analysis. PCR products by MT4 and P4 were isolated and purified by the same method mentioned above. Cycle sequencing of the first half of mtDNA control region was performed using primer set MT4 and Dlp 5R, For the that of second half we used a newly designed primer DL4G (*5'*-TGAAACCAGCAACCCGCTTGGCA-*3'*) and P4.

Data analysis

Population differentiation was quantified using the Analysis of Molecular Variance procedure (AMOVA; Excoffier *et al.*, 1992) as implemented in the computer program Arlequin (Schneider *et al.*, 1997) . The significance of the variance components and PHI-st were tested by a random permutation procedure available in the program. In each trial, 10,000 randomization of the original data sets were made. The level of significance obtained by this procedure is referred in this paper as P value.

The diversity level of minke whale in sequencing analysis was estimated at both haplotype and nucleotide level (Nei, 1987) using the computer program *Arlequin.* At the haplotype level, diversity and its standard error were calculated without regard to the genetic distance between two sequences of mtDNA. At the nucleotide level, diversity (Nei, 1987) and its standard error for population sampling and stochastic processes were calculated from the pairwise differences between the mtDNA sequences using the Kimura 2- parameter adjustment (Kimura, 1980).

RESULTS

RFLP analysis

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 656 minke whales (see Goto and Pastene, in press). Haplotype frequencies of samples collected in 1997 JARPN by sub-area are shown in Table 3. Haplotype '1' was the predominant haplotype in the offshore region (sub-area 8 and 9).

The frequencies of each haplotype in the all samples are shown in Table 4, by sub-areas. Haplotype '1' was the predominant haplotype in the offshore region (sub-areas 8 and 9) and pacific

coast of Japan (sub-area 7). 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, 8 and 9.

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

Haplotype frequencies by months in all sub-areas are shown in Table 7. 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=-0.0066, P=0.6882+/-0.005) and sub-area 9 (PHIst=-0.0054, P=0.6338+/-0.0048). In the sub-area 11, the result of same test indicated significant difference in haplotype frequency among months (PHIst= 0.0949, P=0.0002+/-0.0001). In previous papers (Goto and Pastene, in press; 1997, Pastene *et al.*, in review), April and August samples in sub-area 11 were showed significant heterogeneity. And July and September data in sub-area 11 have small sample size. Therefore, the heterogeneity test in sub-area 11 was conducted using data of May and June and the result showed no significant difference between both months (PHIst=-0.0034, P=0.4460+/-0.0049). The same heterogeneity test within sub-area 8 was not conducted, because the sample size was very small when the samples were grouped in the same way.

Comparison among sub-areas

Results of the nested analysis by AMOVA are summarized in Table 6. In sub-area 11, the data of May and June were used. Of the total molecular variance 85.13% 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 7, 8, 9 and 11 are highly divergent from minke whales from Korea (sub-areas 5 and 6).

Restriction site map

Fig. 3 shows the restriction map including informative sites which produce 8 kinds of RFLP haplotypes by sequencing of whole mtDNA control region. Polymorphic sites among eight haplotypes were concentrated in the first half of control region. In contrast, only one site which produced haplotype 4 and 6 was detected in the second half. There was no specific site discriminating samples from Korea and that from eastern side of Japan in the whole mtDNA control region.

Sequencing analysis

Haplotype frequency

A 487 base pairs of mtDNA control region (the 5'-end) was analyzed for the total samples of 153 individuals. A total of 25 polymorphic site defined 41 haplotypes (Table 8). Except for one in/del site, all substitutions were transitions. The frequencies of haplotypes in the three sub-areas are also shown in Table 8. In the 28 individuals from sub-area 6, five haplotypes were detected. All five haplotypes were specific in this area and two of which were found only in single specimens. In the 30 individuals from sub-area 7, twenty haplotypes were detected, thirteen of which were found only in the single specimens. In the 95 individuals from sub-area 9, thirty haplotypes were detected, eleven of which were found only in the single specimens. Four-teen haplotypes were shared between sub-area 7 and 9. Six and six-teen haplotypes were specific to sub-area 7 and 9, respectively.

Homogeneity test

Table 9 shows the results of the homogeneity test by AMOVA. This statistics separated clearly sub-area 6 (Korea) from sub-areas 7 and 9 (eastern side of Japan). Although the probability was close-to-significant, no significant heterogeneity was found between coastal (sub-areas 7) and offshore areas (sub-area 9).

mtDNA diversity

Table 10 shows the nucleon diversity and the nucleotide diversity for each of the three sub-areas examined using sequence data. Estimated nucleon diversity was 0.5529+/-0.0930 for sub-area 6, 0.9701+/-0.0154 for sub-area 7 and 0.9438+/-0.0113 for sub-area 9. Nucleotide diversity were 0.00463+/-0.00292, 0.00880+/-0.00499 and 0.00758+/-0.00428 for Sub-areas 6, 7 and 9, respectively.

DISCUSSION

In this study, samples collected in the 1997 JARPN survey were analyzed using a RFLP analysis of the mtDNA control region. The pattern of mtDNA variation in these samples was compared with those found in our previous studies (Goto and Pastene, in press; 1997). Furthermore a preliminary sequencing analysis of the first half of the mtDNA control region was conducted using a part of the samples used in the RFLP analysis.

RFLP analysis

The pattern of genetic variation revealed from the RFLP analysis in this study is similar to that reported in the previous paper (Goto and Pastene, 1997). Our results suggest that whales from sub-area 8 and 9 taken during the 1997 JARPN survey have a similar mtDNA composition to those of sub-areas 7 and 9 of our previous study.

Then the results of our RFLP analysis provide no evidence for the occurrence of more than one stock in the eastern 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 eastern side of Japan at the resolution level of the RFLP analysis.

However, we recognize that the no significant differences among the sub-areas in the eastern side of Japan may mean either that there is no population segregation among these sub-areas or that there is low power to detect segregation. Low power might be the results of low resolution of the genetic technique used (Goto and Pastene, in press).

Sequencing analysis

In order to increase the resolution power of the RFLP analysis, we also conducted a preliminary sequencing analysis using part of the samples used in the RFLP analysis. Sequencing analysis is considered to have larger resolution than RFLP analysis.

Restriction sites map

We constructed a restriction map of the whole mtDNA control region to get information of position of the restriction sites produced in the RFLP analysis. Apart one of the sites, all of the polymorphic sites were concentrated in the first half of the control region. For stock identity analysis in the North Pacific minke whale we concluded that a larger resolution could be obtained by sequencing

the first half of the control region. On the other hand, there was no specific site discriminating individual samples from Korea and from eastern side of Japan using analysis of the mtDNA control region.

Homogeneity test

The result of the homogeneity test using sequencing analysis was consistent with that of the mtDNA RFLP analysis and with that of the microsatellites analysis using six loci (Abe *et al.*, this meeting). This analysis discriminated clearly between Korean (sub-area 6) and eastern side of Japan (sub-areas 7 and 9) minke whales. Also we could not find significant differences between coastal (sub-area 7) and offshore (sub-area 9) areas in the eastern side of Japan. It should be noted, however, that more samples are already available for sub-areas 7, 8 and 9 and that further sequencing analysis using these samples should be conducted in future.

Nucleotide diversity

Nucleotide diversity was lower in the Korean minke whales (sub-area 6: 0.0046) than in minke whales in the eastern side of Japan (sub-area 7: 0.0088; sub-area 9:0.0076). 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.

Nucleotide diversity in the North Atlantic minke whale was estimated at 0.0064 (Bakke *et al.*, 1996). Then this value is similar to North Pacific sub-areas 7 and 9 but higher than sub-area 6 (Korea). Nucleotide diversity in the Antarctic minke whale is higher than in North Pacific and North Atlantic (0.0159) (Bakke *et al.*, 1996). These authors explained that the higher value of nucleotide diversity in the Antarctic than that of 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.

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Table 1: Number of samples collected in 1997 JARPN by sub-area and month.

Sub-areas	Months			Total
	5	6	7	
Sub-area 7	0	2	0	2
Sub-area 8	0	0	31	31
Sub-area 9	27	40	0	67
Total	27	42	31	100

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	26	26	22	38	0	186
Sub-area 8	0	0	0	42	5	0	0	47
Sub-area 9	0	27	54	69	34	4	0	188
Sub-area11	57	66	31	5	40	6	0	205
Total	101	123	111	142	101	67	11	656

Table 3: Haplotype frequency in RFLP analysis from samples collected in the 1997 JARPN, by sub-area.

Hap.	Sub-area			
	7	8	9	
	June	July	May	June
1	1	26	26	39
2		2		
3	1			1
4				
5		2	1	
6		1		
7				
8				
Total	2	31	27	40

Table 4: Haplotype frequency for all samples in RFLP analysis.

Hap.	Sub-area					Total
	6	7	8	9	11	
1	0	171	41	177	154	543
2	1	4	3	2	9	19
3	7	5	0	5	15	32
4	0	0	0	0	1	1
5	22	3	2	3	19	49
6	0	3	1	1	3	8
7	0	0	0	0	2	2
8	0	0	0	0	2	2
Total	30	186	47	188	205	656

Table 5: Nested statistical test in the RFLP analysis 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	-0.66	100.66	-0.00660	0.6882+/-0.0050
Sub-area 9	-0.53	100.53	-0.00535	0.6338+/-0.0048
Sub-area 11*1	9.49	90.51	0.09490	0.0002+/-0.0001
Sub-area 11*2	-0.34	100.34	-0.00343	0.4460+/-0.0049

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

Table 6: Statistical nested analysis by AMOVA including all the geographical sub-areas (May and June data were included in sub-area 11).

	df	total variance(%)	PHI	P
Among Korea/ eastern side of Japan	1	85.13	FCT: 0.8513	0.0000+/-0.0000
Among areas/ eastern side of Japan	3	0.00	FSC: 0.0003	0.3580+/-0.0052
Within areas	543	14.86	FST: 0.8513	0.0000+/-0.0000

Table 7: Haplotype frequency in each sub-area, by month.

a) Sub-area 7

Hap.	Months						Total
	4	5	6	7	8	9	
1	42	27	23	23	21	35	171
2	0	2	2	0	0	0	4
3	1	0	1	2	0	1	5
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	26	26	22	38	186

b) Sub-area 8

Hap.	Months		Total
	7	8	
1	36	5	41
2	3	0	3
3	0	0	0
4	0	0	0
5	2	0	2
6	1	0	1
7	0	0	0
8	0	0	0
Total	42	5	47

c) Sub-area 9

Hap.	Months					Total
	5	6	7	8	9	
1	26	53	63	32	3	177
2	0	0	2	0	0	2
3	0	1	3	0	1	5
4	0	0	0	0	0	0
5	1	0	1	1	0	3
6	0	0	0	1	0	1
7	0	0	0	0	0	0
8	0	0	0	0	0	0
Total	27	54	69	34	4	188

d) 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

Table 8: Variable sites defining 41 unique haplotypes by sequencing analysis in the North Pacific minke whale. On the right side of this table are the frequencies of the haplotypes in each sub-area.

Hap No.	1111222222222334411288922370002578991306 59070020562348903290812 63	sub-area 6	7	9	Remark RFLP Hap.
1	GAAAAATTACTTCACCGGCCTGTGTGTACA	18			5
2	GAAAAATTACTTCACCGGCCTGTGTGTATA	2			5
3	GAAAA-TTGCTTCACCGACCTGTGCACACA	6			3
4	GAAAA-TTGCTTTACCGACCTGTGCACACA	1			3
5	GAAAAATTACTTCACCGGCCTGTGTGTACG	1			2
6	GAAAA-TTGCTTTACCGACCTGTGTATACG		2	16	1
7	GAAAA-TTGCTTTACCGACCTGTGCACATG		3	9	1
8	GAAAA-TTGCTTCACCGACCTGTGTATACG		3	5	1
9	GAAAA-TTGCTTTACCGACCTGTGCATATG		1	7	1
10	GAAAA-TTGCCTCACCGACCTGCGCACATG		1	7	1
11	GAAAA-TTGCTTCACCGACCTGTGCATGTG		2	5	1
12	GAAAA-TTGCCTTACCGACCTGCGCACATG		3	2	1
13	GAAAA-TTGCTTCGCCGACTTGTGCATATG		2	3	1
14	GAAAA-TTGCTTCACCGACCTGTGCATATG			4	1
15	GAAAA-TTGCCTCACCGACCTGCGCACGTG		1	3	1
16	GAAAA-TTGCTTTACCGACCTGCGCACATG			4	1
17	GAAAA-TTGCTTCACCGACCTGTGCATACG			3	1
18	GAAAAATCGCCTCACCGACCCGTGCACATG		1	2	1
19	GAAAA-TTGCTTTACCGACTTGTGCATATG		1	2	1
20	GAAAA-TTGCTTTGCCGACTTGTGCATATG		1	2	1
21	GAAAA-TTGCTTCACCGACCTGTGCATATA			3	1
22	GAAAA-TTGCTTTACCGACCTGTGTATACA			3	3
23	GAAAAATCGCCTCACCGGTCCGTGCACATG			2	1
24	GAAAAATCGCCTCACCGATCCGCGCACATG		1	1	1
25	GAAAA-TTGCCTCATCGACCTGCGCACATG		2		1
26	GAAAA-TTGCTTTACCGACCTGCGCATATG		1	1	1
27	GAAAA-TTGCCTCACCGACCTGTGCACACG			2	1
28	GAAAA-TTGTTCCACCGACCTATGCATACG			1	1
29	GAAAA-TTGCTTCACCAACCTGCGCATATG		1		1
30	GAAAA-TTGCTCCACCGACCTGTGCACATG			1	1
31	GAAAAATCGCCTCACCGACTCGTGCACATG		1		1
32	GAAAAACCGCCTCACCGACCCGTGCACATG			1	1
33	GAAAAATCGCCTCACCGATCCGTGCACATG		1		1
34	GAAAA-TTGCCTCACCGACCTGCGTATATG			1	1
35	GAAAA-TTGCCTCACCGACCTGCGTACATG			1	1
36	GAAAA-TTGCCTCACCGACTTGCGCACGTG		1		1
37	GAAAA-CTGCTTTACCGACCTGTGCATATG			1	1
38	GAAAA-TTACTTTACCGACCTGCGCATATG			1	2
39	GAAAA-TTGCTTTACTGACCTGTGCACATG			1	1
40	GAAAAATTACTTCACCGGCCTGTATGTACA			1	5
41	GAAAA-TTGCTTCACCGACCTGTGCGCACG		1		1
	Total	28	30	95	

Table 9: Statistical analysis by AMOVA among three geographical sub-areas by sequencing analysis. Above diagonal: probability, below diagonal: FST value.

	sub-area6	sub-area7	sub-area9
sub-area6 (28)		0.000+-0.000	0.000+-0.000
sub-area7 (30)	0.5499		0.063+-0.002
sub-area9 (95)	0.5021	0.0220	

Table 10: Estimates of the nucleon diversity and nucleotide diversity within three sub-areas by sequencing analysis

	Nucleon diversity	Nucleotide diversity
Sub-area 6	0.5529+/-0.0930	0.00463+/-0.00292
Sub-area 7	0.9701+/-0.0154	0.00880+/-0.00499
Sub-area 9	0.9438+/-0.0113	0.00758+/-0.00428

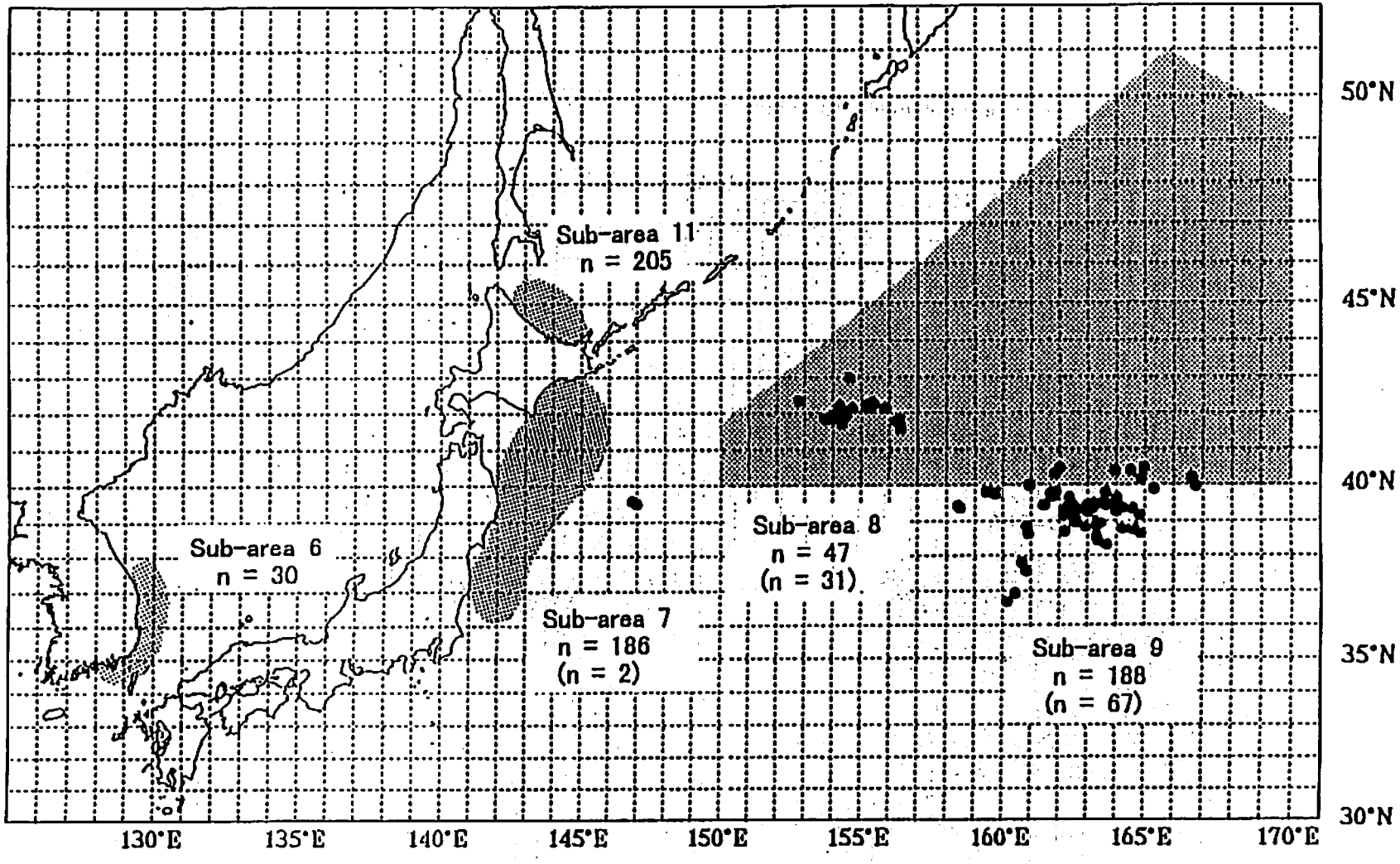

Fig. 1. Geographical localities and sample size examined in the RFLP analysis.
Figures indicate sample size examined in each locality. Closed circles are the geographical position of the samples and in parenthesis are the sample size of samples of the 1997 JARPN survey.

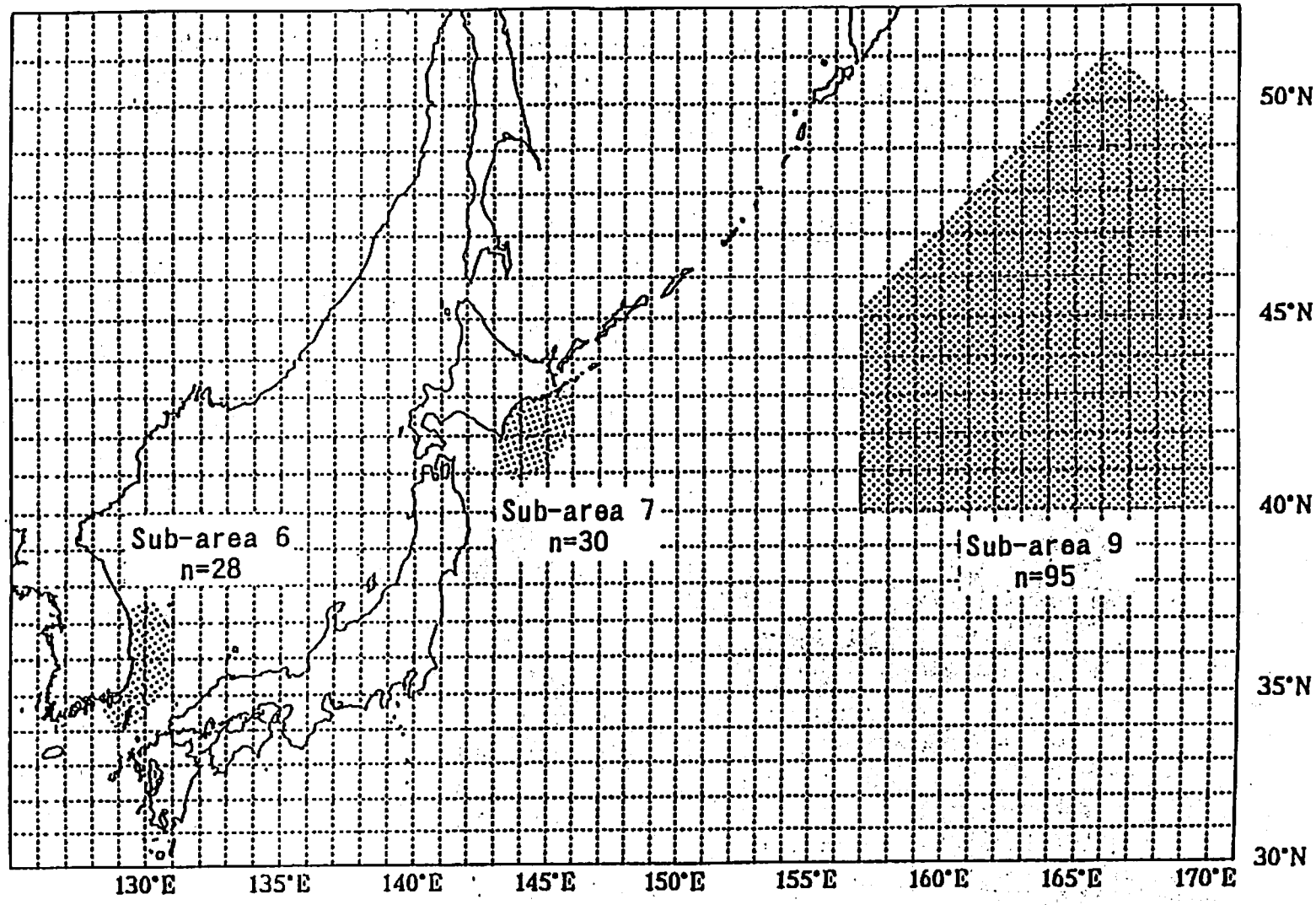

Fig. 2. Geographical localities and sample size examined for sequencing analysis. Figures indicate sample size.

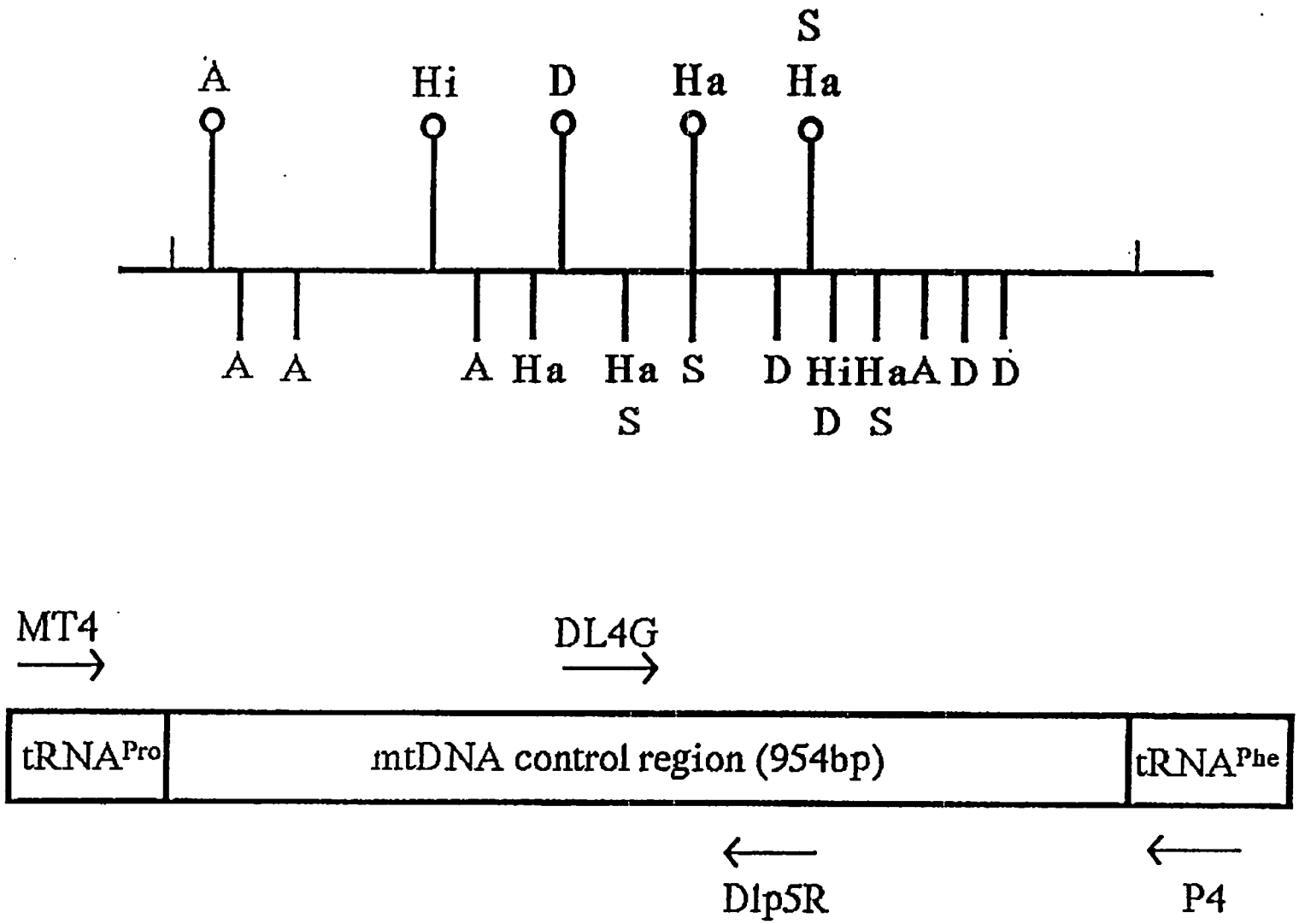

Fig. 3. Map of restriction site of five polymorphic restriction enzymes (above) and PCR primers used (below) within the mtDNA control region. A = *Afa*I, D = *Dde*I, Ha = *Hae*III, Hi = *Hin*fI and S = *Sau*96I. ○ shows the informative site which produces the eight RFLP haplotypes.