Populations Differentiation in the Western North Pacific Minke Whale as Revealed by RFLP Analysis of Mitochondrial D-Loop DNA

Mutsuo Goto and Luis A. Pastene

Ecology Section
The Institute of Cetacean Research
4-18 Toyomi-cho, Chuo-ku, Tokyo 104, Japan

ABSTRACT

A restriction length polymorphism analysis (RFLP) of the D-loop region of mitochondrial DNA (mtDNA) was conducted in order to investigate differentiation of populations in the western North Pacific minke whale. A total of 377 minke whales, obtained during former coastal whaling operations in Japan and Korea and during a Japanese special permit catch in offshore areas of the western North Pacific, were examined. The PCR-amplified D-loop region of mtDNA was analyzed with eight kinds of four-base restriction enzymes, discriminating eight mtD-loop DNA haplotypes. We examined mtD-loop DNA diversity among five geographical localities: coastal area of Korea (Area 6), southern part of the Okhotsk Sea (Area 11), eastern Hokkaido (Area 7N), Sanriku (Area 7S) and an offshore area of the western North Pacific (Area 9). Our results suggest a clear mtDNA differentiation between minke whales from Korea and those from the eastern coast of Japan and Okhotsk Sea. Furthermore, coastal and offshore minke whales in the eastern side of Japan were similar genetically. Thus, our results are consistent with the hypothesis that two genetic populations occur in the western North Pacific. A monthly analysis of haplotypes composition in Sanriku (Area 7S) and Okhotsk Sea (Area 11), suggests that samples taken in April in the Okhotsk Sea present a medium composition between those of the Korean and Japanese coastal samples. This suggests some degree of temporal mixing between the two populations in that locality. Our results are consistent with previous morphological and isozyme analysis. Since no significant differences were found among three IWC's areas in the eastern side of Japan (Areas 7, 9 and 11), our mtDNA data do not support the sub-stock scenario of the IWC.

INTRODUCTION

There are morphological and ecological evidences (Ohsumi, 1983) and genetic evidences (Wada, 1984; 1991), supporting the occurrence of two biological populations in coastal waters off Japan, one distributed in the Sea of Japan (J-stock according the IWC' terminology) and the other distributed in the eastern coast of Japan and Okhotsk Sea (O-stock).

As Baker *et al.* (1994) pointed out, the identification of populations and their geographical (and temporal) boundaries are fundamental for management schemes of exploited and protected species. In the case of exploited species, identification of populations and their boundaries is fundamental for estimating abundance, setting catch limits and interpreting catch statistics and

life-history parameters (Baker *et al.*, 1994). With this regard, in preparation for the implementation of the Revised Management Procedure (RMP), a Working Group on North Pacific Minke Whale Management Trials faced the problem of stock identity in the western North Pacific minke whale. Instead to accept the view of two separated populations, which is supported by biological evidences, the group adopted the scenario of three stocks and several sub-stocks around Japan (IWC, 1994).

Attempts to calculate catch limits under the guideline of the RMP have been constrained because disagreement on the evidence needed to accept or not the alternative of the sub-stocks scenario. Under this situation, further information on stock identity in the western North Pacific minke whale, using different approaches, is necessary in order to corroborate or not the sub- stock scenario adopted by the IWC.

Recently, several biochemical genetic approaches have been developed for studying genetic variation in cetacean species. A review on these was conducted by Hoelzel and Dover (1989). The mitochondrial DNA has been recommended for studies of stock identity due to its maternal mode of inheritance and fast evolutionary rate. Within this molecule, the D- loop region is the most variable part, so that by examining the mtD-loop DNA of mitochondrial genome we should expect greater resolution of genetic differences between conspecifics. A previous RFLP analysis of the minke whale mtD-loop DNA was conducted by Hoelzel and Dover (1991), which compared samples from different oceans. We used this approach for conducting an investigation of stock identity in the western North Pacific.

Here we presented the results of a RFLP analysis of the mitochondrial D-loop DNA of minke whales using samples from five localities of the western North Pacific.

MATERIALS AND METHODS

Samples and localities

Minke whales used in this study were caught by the past Korean and Japanese coastal small-type whaling operations during 1982- 1987. Four localities were determined using the geographical position of the samples taken (Fig. 1): Korea (Area 6 of the Working Group on North Pacific Minke Whale Management Trials, involving one individual from Area 5), eastern Hokkaido (Area 7N,), Sanriku (Area 7S) and Okhotsk Sea (Area 11). In addition, we used samples from one offshore locality (Area 9). The latter were obtained during a 1994 Japanese special permit catch. The number of samples used in our mtDNA survey are shown in Table 1, by locality and month.

Tissue samples and DNA extraction

Total DNA (mtDNA+nuclear DNA) was isolated from liver or muscle tissue that had been frozen at -20°C for some few months (case of the samples obtained under Japanese permit catch in 1994) to 12 years (case of commercial samples taken in 1982), by the standard technique (Sambrook *et al.*, 1989). Briefly, 50mg of samples was mixed with 500ul of homogenize solution containing 1% SDS and 1mg proteinase K. The mixture was incubated at 37°C for overnight. After the incubation, crude DNAs was prepared by phenol/chloroform method. The DNAs were dissolved in 500ul TE buffer and stored at -20°C until use.

Amplification of the mtD-loop DNA

The D-loop region of the mitochondrial genome was amplified by using the polymerase chain reaction (PCR). We used the procedure described by Hoelzel (1992). Primers for amplification of about 1,100bp minke whale mtDNA segment involving the D-loop region were designed on the basis of published minke whale (Hori *et al.*, 1994) and sperm whale sequences (Dillon and Wright, 1993): the light-strand Primer-1 (*5'*-CAAGGAAGAAGTATTACACTCCACCA-*3'*) and the heavy-strand Primer-2 (*5'*-CAGAATTGGAATTCATTTTCAGTGTCTTGGTTT-*3'*), that annealed $tRNA^{Pro}$ and $tRNA^{Phe}$, respectively.

RFLP analysis

After amplification of mitochondrial D-loop region, 5ul of the PCR-products was digested with 2 units of the following eight kinds of four-base recognition endonuclease: *Afa*I, *Dde*I, *Hae*III, *Hinf*I, *Mbo*I, *Msp*I, *Sau*96I and *Scr*FI. Restriction fragmental patterns were determined by electrophoresis in a 2.5% agarose gel and stained with 250ng/ml ethidium bromide. The fragments were visualized under short-wave ultraviolet light. The size of each fragment was estimated by comparing their relative mobilities with those fragments of the pHy Marker (Takara Shuzo Co.). Distinctive restriction fragment patterns produced by each enzyme were assigned letters. Individuals were assigned haplotypes consisting of the list 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.

Data analysis

Pairwise genetic distances among eight mitochondrial D-loop haplotypes were calculated using maximum likelihood methods (Nei and Li, 1979; Nei and Tajima, 1983). Genetic distances among localities was estimated using equation 10.21 of Nei (1987). Phylogenetic relationship among localities was described by an UPGM-derived dendrogram and it was based on the genetic distances among them.

Quantification of the geographical mtDNA variation was carried out using the Analysis of Molecular Variance (AMOVA) model of Excoffier *et al.* (1992). This model calculates estimates of variance components from a genetic distance matrix and the PHI-statistic (PHIst) which is the correlation between a random pair of haplotypes drawn from the putative sub-population to a random pair drawn from the total population. The significance of variance components were tested by resampling with 2,000 times replacement. The AMOVA was performed on a standard distance matrix calculated from the number of restriction sites differences between pairs of haplotypes.

Furthermore, mtDNA haplotype frequencies were employed to determine genetic relationship among the localities. Significance of the haplotype frequencies differences among localities were estimated using the randomized chi-square test of independence (Roff and Bentzen, 1989).

RESULT

Mitochondrial DNA variation

Five enzymes were polymorphic: *Afa*I, *Dde*I, HaeIII, *Hinf*I and *Sau*96I while the other three,

*Mbo*I, *Msp*I and *Scr*FI, were monomorphic. A total of six polymorphic sites were detected defining eight unique haplotypes in the 377 minke whale samples. Composite patterns of these haplotypes are shown in Table 2.

Geographical distribution of mtD-loopDNA haplotypes

The geographical distribution of the eight haplotypes in the five areas is shown in Fig. 2. As it can be observed from this figure, haplotype '1' is the predominant haplotype in the coastal localities of Japan (Areas 7N, 7S and 11) and in the offshore sample (Area 9). On the other hand, this haplotype is not represented in 30 samples from coastal areas of Korea (Areas 5 and 6). Instead, the predominant haplotype in this sample is haplotype '5' followed by haplotype '3'. With the exception of the Okhotsk Sea sample (Area 11), these two haplotypes are scarcely represented in the other localities (see Appendix 1 for distribution of haplotypes by locality and month).

Monthly distribution of haplotypes in Areas 11 and 7S.

A monthly examination of haplotype distribution in Area 11 (Okhostk Sea), showed that the predominant haplotypes of the Korean sample (haplotypes '3' and '5') are well intermingle in the April sample but they were scarcely represented in the rest of the months for that locality (Fig. 3A). The results of a chi-square test for temporal heterogeneity in this locality indicates significant differences in haplotype frequencies among months (P=0.0010). Pairwise comparisons results indicate that the only significant differences are those between April and May (P<0.0005), April and June (P=0.0145) and April and Jul+Aug.+Sept. (P= 0.0545). In contrast, haplotypes '3' and '5' are scarcely represented in the monthly samples (including the April sample) of Area 7S (Sanriku) (Fig. 3B). According to the results of the chi-square test, there is not significant differences in haplotypes frequencies among months in this locality (P= 0.3915).

Comparison among areas (April data excluded from Area 11)

According to the results shown above, minke whales distributed in Area 11 in April could be from two different populations, thus we have excluded April data for Area 11 in the comparison among areas. Results of the AMOVA are summarized in Table 3. Of the total molecular variance 82.70% is due to Korea-eastern side of Japan division, which was highly significant, -0.15% (not significant) accounted for the divisions within the eastern side of Japan (e.g. Areas 11, 7 and 9) and 17.45% (highly significant) of the total variance was due to within the areas. These results indicates that Areas 11, 7N, 7S and 9 are closely related among them and highly divergent from minke whales from Korea (Areas 5 and 6).

Table 3 also shows the pairwise comparisons among localities. PHIst values obtained from the comparisons between the areas of the eastern side of Japan with the Korean area are large and highly significant. In contrast when the areas of the eastern side of Japan are compared among them, these values are small and not significant. Similar results were obtained when the haplotype frequencies of the areas were compared (Table 4).

DISCUSSION

In this study, a RFLP analysis of the non-coding portion of mitochondrial genome, the D-loop region, was conducted in the western North Pacific minke whale. The results are consistent with

previous isozyme surveys in the western North Pacific minke whale conducted by Wada (1984; 1991), which found significant differences in allele frequencies at one locus between minke whales from Korea and Japan and intermediate frequencies in the Okhotsk Sea during part of the year. Thus, the results found using mtDNA are supported by previous finding using a nuclear marker. These results are then of some significance.

The results derived from our RFLP analysis of the mitochondrial D-loop DNA support the view that two different populations occur in the western North Pacific, one distributed around the Korean Peninsula and the other distributed in the eastern side of Japan from the Japanese coast to at least 170°E (the most easterly longitude examined). Coastal and offshore minke whales in the eastern side of Japan were genetically homogeneous.

A phylogenetic analysis of the five localities (Fig. 4) further support that view. According to this figure, the four areas in the eastern side of Japan, including the offshore sample from Area 9, are located on a same cluster. Within this, Area 9 was closely related with Area 7. This cluster is independent from that of the Korean sample (Areas 5 and 6). Thus two different biological stocks occur in the western North Pacific, the J-stock and the O-stock, according to IWC's terminology.

Further, we detected that these two populations mix each other in the Okhotsk Sea locality in April. Since this was also detected by a nuclear marker (Wada, 1984; 1991), this suggest the temporal intermingling of two reproductively isolated stocks.

As it was mentioned earlier, a Working Group on North Pacific Minke Whale Management Trials adopted the scenario of several sub-stocks around Japan, as the management units in preparation for the implementation of the RMP in the North Pacific minke whale (IWC, 1994). However, the definition of the units in the eastern side of Japan (e.g. Areas 7, 9 and 11) are not supported by genetic evidences, at least as revealed by isozyme and mtDNA analysis.

The sample size used for Area 9 was small in comparison with that used in the other areas examined. In order to corroborate the results regarding this area, the present analysis should be conducted again when more samples from that area become available. Also the eastern geographical extension of the O-stock should be investigated in the future in order to define longitudinal boundaries for this stock.

ACKNOWLEDGMENTS

Samples from the former coastal whaling operation were available from the National Research Institute of Far Seas Fisheries. We thanks Drs. H. Hatanaka, H. Kato and Mr. T. Kishiro for permit and facilitate the use of these samples. Also Dr. S. Wada, National Research Institute of Fisheries Science, supplied some samples from the Okhostk Sea locality. Samples from the Area 9 were obtained during the 1994 survey of the Japanese Whale Research Program in the North Pacific. Our acknowledgments to the researchers and crew members that participated in that survey. Our gratitude to Mr. S. Itoh, The Institute of Cetacean Research for computer assistance and T. Saito for logistical arrangements at the Marine Station of Ayukawa. Mrs. S. Azumi collaborated in the process of DNA extraction.

REFERENCES

Baker, C.S., Slade, W., Bannister, J.L., Abernethy, R.B., Weinrich, M.T., Lien, J., Urban, J., Corkeron, P., Calambokidis, J., Vasquez, O. and Palumbi, S.R. 1994. Hierarchical structure of mitochondrial DNA gene flow among humpback whales *Megaptera novaengliae*, world-wide. *Molecular Ecology* 3:313-327.

Dillon, M. and Wright, J. M. 1993. Nucleotide sequence of the D-loop region of the Sperm whale(*Physeter macrocephalus*) mitochondrial genome. *Mol. Biol. Evol.* 10(2): 296-305.

Excoffier, L., Smouse, P. E. and Quattro, J. M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 479-491.

Hoelzel, A.R. and G.A. Dover 1989. Molecular techniques for examining genetic variation ad stock identity in cetacean species. *Rep. int. Whal. Commn* (special issue 11):81-120.

Hoelzel, A. R. and Dover, G. A. 1991. Mitochondrial D-loop DNA variation within and between populations of the minke whales (*Balaenoptera acutorostrata*). *Rep. int. Whal. Commn* (special issue 13): 171-81.

Hoelzel, A. R. (Ed.). 1992. *Molecular Genetic Analysis of Populations. A Practical Approach.* Oxford University Press. Oxford, New York, Tokyo. 315pp.

Hori, H., Bessho, Y., Kawabata, R., Watanabe, I., Koga, A. and Pastene, L.A. 1994. World-wide population structure of minke whales deduced from mitochondrial DNA control region sequences. Paper SC/46/SH14 presented to the IWC Scientific Committee, May 1994, (unpublished), 11pp.

International Whaling Commission. 1994. Report of the Scientific Committee. *Rep. int. Whal. Commn* 44: 41-201.

Nei, M. 1987. *Molecular Evolutionary Genetics.* Columbia University Press, New York. x+512pp.

Nei, M. and Li, W.H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci.* USA 76:5269-5273.

Nei, M. and Tajima, F. 1983. Maximum likelihood estimation of the number of nucleotide substitutions from restriction sites data. *Genetics* 105:207-217.

Ohsumi, S. 1983. Minke Whales in the coastal waters of Japan in 1981, with reference to their stock boundary. *Rep. int. Whal. Commn* 33: 365-371.

Roff, D.A. and Bentzen, P. 1989. The statistical analysis of mtDNA polymorphims: chi-square and the problem of small samples. *Mol. Biol. Evol.* 6(5):539-45.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular cloning: A laboratory manual.* 2nd Ed., Cold Spring Harbor Laboratory, NY.

Wada, S. 1984. A note on the gene frequency differences between minke whales from Korean and Japanese coastal waters. *Rep. int. Whal. Commn* 34: 345-347.

Wada, S. 1991. Genetic heterogeneity in the Okhotsk Sea-west Pacific stock of minke whales. Paper SC/43/Mi32 presented to the IWC Scientific Committee, May 1991, (unpublished) 17pp.

Table 1: Number of samples examined for mtD-loop DNA RFLP analysis, by IWC area (see Fig. 1) and month. Samples from Areas 6, 7, 9 are from former coastal whaling operations in Japan and Korea conducted between 1982 and 1987. Samples from Area 9 were obtained during a 1994 Japanese special permit catch.

Areas	Months							Total
	4	5	6	7	8	9	10	
Area 6	0	0	0	0	0	19	11	30
Area11	57	66	31	5	8	6	0	173
Area7N	0	0	9	14	5	23	0	51
Area7S	44	30	15	11	2	0	0	102
Area 9	0	0	0	8	9	4	0	21

Table 2: Composite pattern of eight western North Pacific minke whale mtD-loop DNA haplotypes.

Haplotype	Restriction Enzymes							
	*Afa*I	*Dde*I	*Hae*III	*Hinf*I	*Mbo*I	*Msp*I	*Sau*96I	*ScrF*I
1	C	C	C	C	N	N	C	N
2	B	C	C	C	N	N	C	N
3	C	C	B	C	N	N	C	N
4	C	C	C	C	N	N	D	N
5	B	C	B	C	N	N	C	N
6	C	C	D	C	N	N	D	N
7	C	C	B	D	N	N	C	N
8	C	B	C	C	N	N	C	N

Table 3: Summary of the results of AMOVA. April data for Area 11 were excluded in the analysis. The P value is the probability of a more extreme variance component or PHI statistics than that observed, in comparison to a null distribution of these values on 2,000 random permutations of the data matrix. In analysis A the PHIct and the among Korea/eastern side of Japan variance component involves the permutation of whole areas among these two regions, PHIsc and the among areas within the eastern side of Japan involves the random permutation of individuals among areas within that region, PHIst and the within areas components involves the random permutation of individuals among the five areas.

	df	% total variance	PHI	P
A= nested analysis				
Among Korea/ eastern side of Japan	1	82.70	CT:0.827	**<0.0005**
Among areas/ eastern side of Japan	3	-0.15	SC:-0.009	0.9180
Within areas	315	17.45	ST:0.826	**<0.0005**
B= pairwise comparisons				
A11-A7N			ST:-0.0119	0.9725
A11-A7S			ST:-0.0031	0.5977
A11-A9			ST:-0.0205	0.8946
A11-A6			ST: 0.7933	**0.0000**
A7N-A7S			ST:-0.0029	0.5032
A7N-A9			ST:-0.0308	0.9770
A7N-A6			ST: 0.7889	**0.0000**
A7S-A9			ST:-0.0064	0.5042
A7S-A6			ST: 0.8499	**0.0000**
A9-A6			ST: 0.7707	**0.0000**

Table 4: Results of chi-square tests for heterogeneity in mtD-loop DNA haplotype frequencies among areas. April data for Area 11 were excluded in the analysis. Probabilities calculated from the Monte Carlo approach of Roff and Bentzen (1989).

	Area 6	Area 11	Area 7N	Area 7S	Area 9
Area 6		<0.0005	<0.0005	<0.0005	<0.0005
Area 11			0.9465	0.8120	0.8825
Area 7N				0.4685	0.9100
Area 7S					0.4045

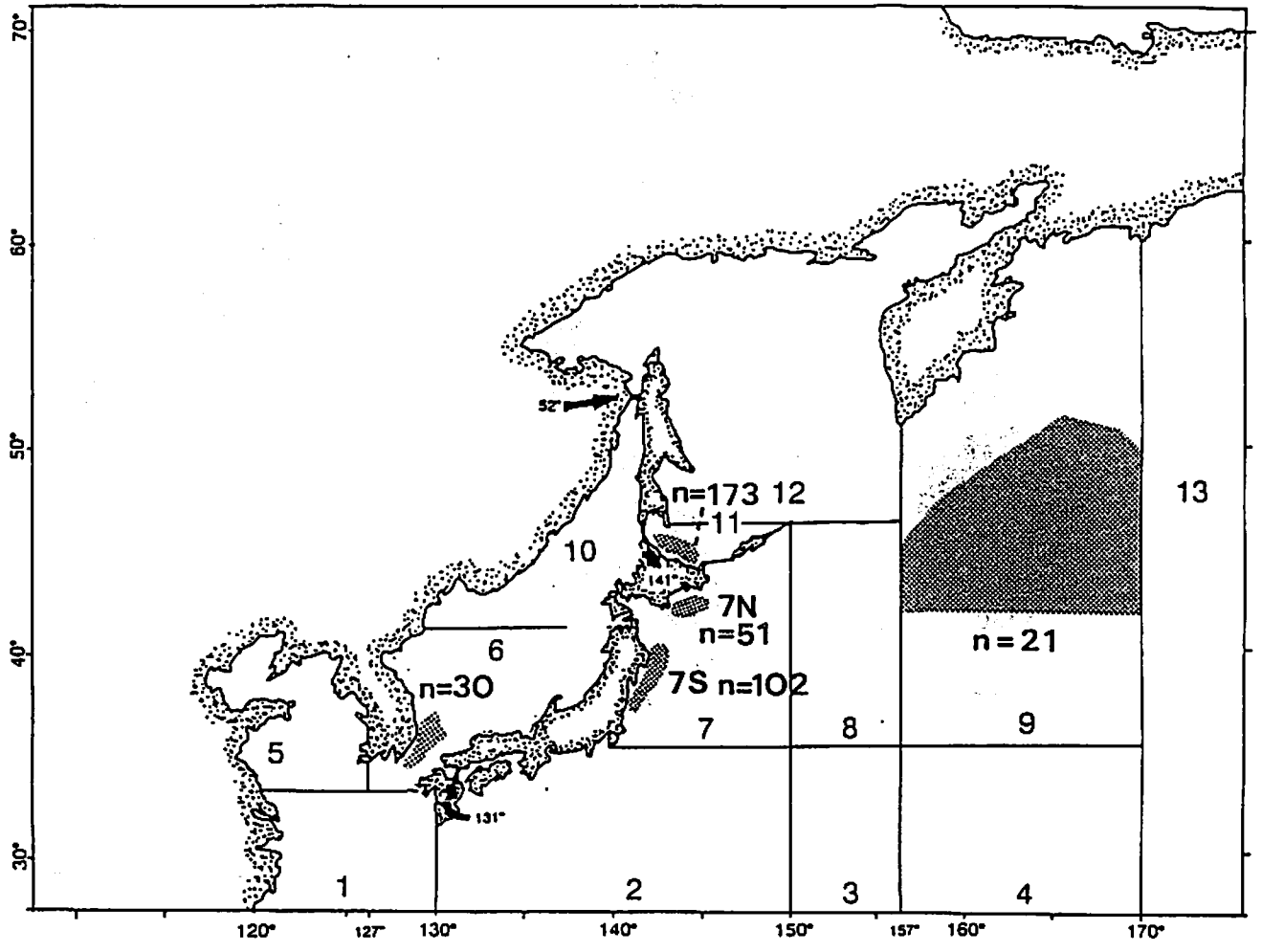

Fig. 1 Areas adopted by the Working Group on North Pacific Minke Whale Management Trials in preparation for implementation of RMP (after IWC, 1994) and geographical localities examined for mitochondrial D-loop DNA variation in the western North Pacific minke whale. Okhotsk Sea (Area 11), Eastern Hokkaido (Area 7N), Sanriku (Area 7S), Offshore Area (Area 9) and Coastal Area of Korea (Area 6).

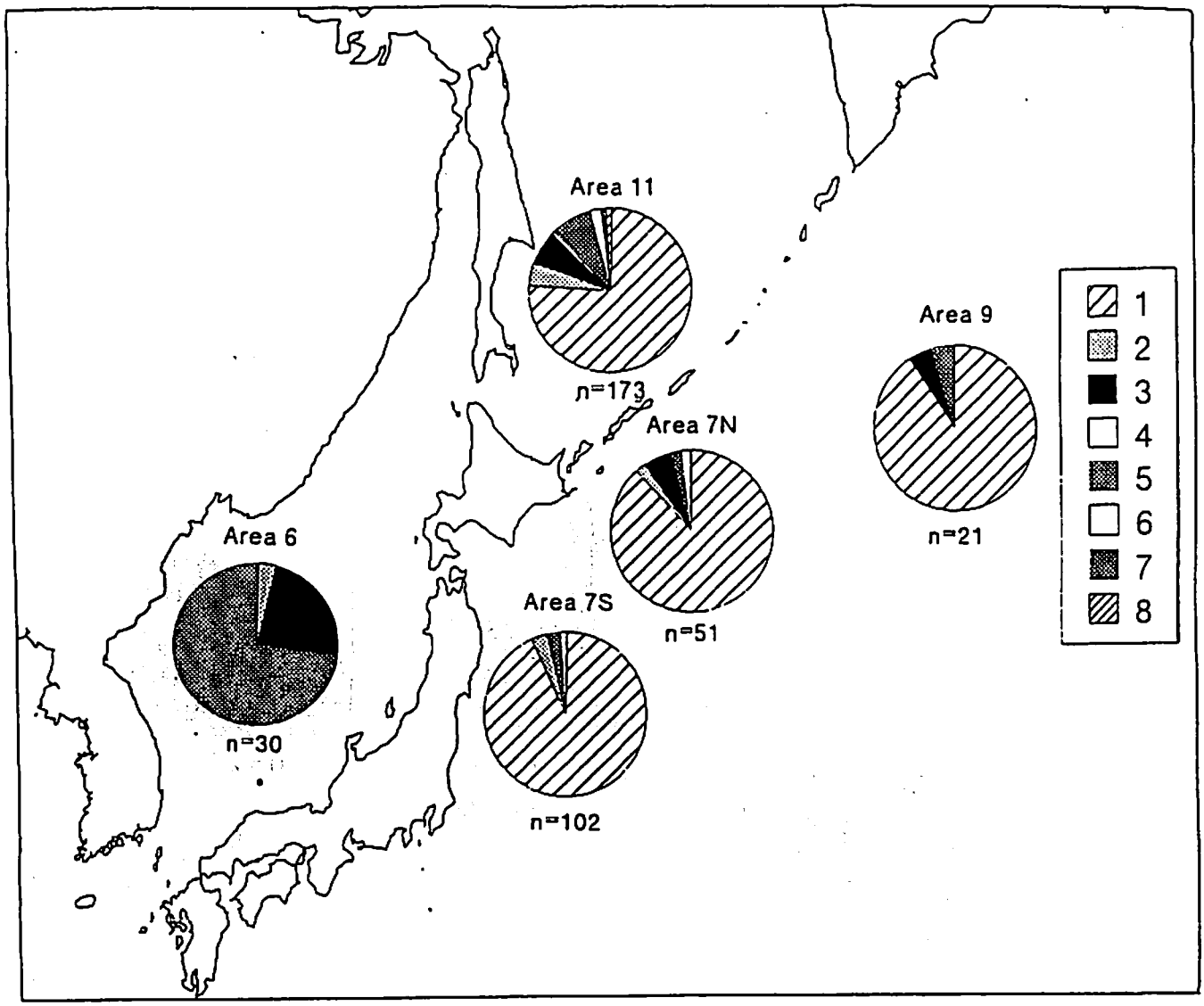

Fig. 2 Geographical distribution of 8 mitochondrial D-loop DNA haplotypes

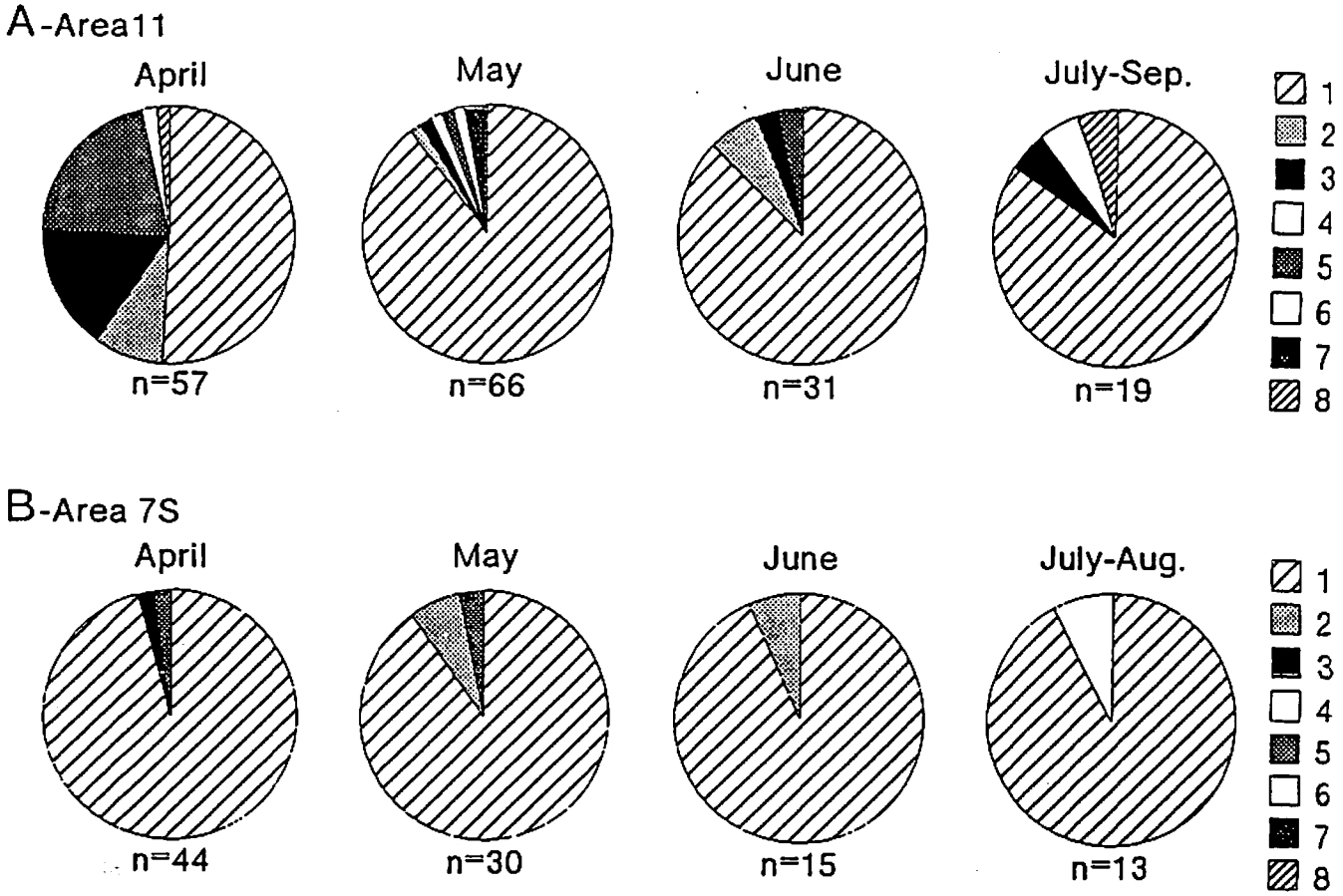

Fig. 3 Monthly frequency of 8 mitochondrial D-loop DNA haplotypes in Area 11 and Area 7S.

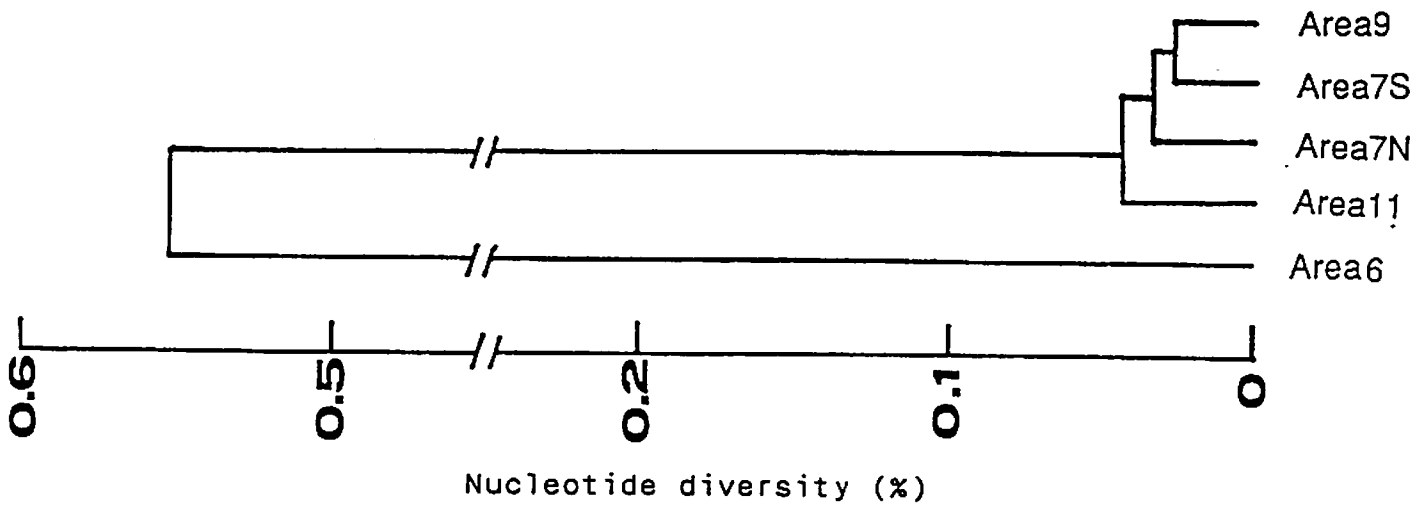

Fig. 4 Phylogenetic relationship among minke whales from five areas, as revealed by UPGM-derived dendrogram based on genetic distance among these localities.

Appendix 1: Distribution of mtD-loop DNA haplotypes by locality and month

Area 6

Haplotype ID	September+October
1	0
2	1
3	7
4	0
5	22
6	0
7	0
8	0
Total	30

Area11

Haplotype ID	April	May	June	July	August	Septem.	Total
1	29	59	27	3	7	6	131
2	5	1	2	0	0	0	8
3	9	1	1	1	0	0	12
4	0	1	0	0	0	0	1
5	12	1	1	0	0	0	14
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	8	6	173

Area7N

Haplotype ID	June	July	August	September	Total
1	8	12	5	20	45
2	1	0	0	0	1
3	0	2	0	1	3
4	0	0	0	0	0
5	0	0	0	1	1
6	0	0	0	1	1
7	0	0	0	0	0
8	0	0	0	0	0
Total	9	14	5	23	51

Area7S

Haplotype ID	April	May	June	July	August	Total
1	42	27	14	10	2	95
2	0	2	1	0	0	3
3	1	0	0	0	0	1
4	0	0	0	0	0	0
5	1	1	0	0	0	2
6	0	0	0	1	0	1
7	0	0	0	0	0	0
8	0	0	0	0	0	0
Total	44	30	15	11	2	102

Area 9

Haplotype ID	July	August	September	Total
1	8	8	3	19
2	0	0	0	0
3	0	0	1	1
4	0	0	0	0
5	0	1	0	1
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
Total	8	9	4	21