A preliminary analysis of mitochondrial DNA in humpback whales (*Megaptera novaeangliae*) from Antarctic Areas IV and V

Luis A. Pastene, Mutsuo Goto, Hideaki Abe and Shigetoshi Nishiwaki

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

ABSTRACT

In this report we present a preliminary analysis of mitochondrial DNA (mtDNA) in humpback whales from Antarctic Areas IV and V. Material used were skin biopsies sampled during two Japanese Whale Research Program Under Special Permit in the Antarctic (JARPA) surveys in these areas. A total of 30 samples (19 in Area IV and 11 in Area V) were analyzed by two methods: a restriction fragment length polymorphism (RFLP) analysis of the mtDNA control region and sequencing of a segment of that region. In the first approach, we used ten different restriction enzymes to examine polymorphism in the PCR-amplified mtDNA control region. A total of nine unique restriction sites defined seven unique haplotypes and the sequence divergence among these haplotypes ranged from 0.0030 and 0.0218. In the second approach, we sequenced a 336bp-long segment of the mtDNA control region. A total of 36 polymorphic sites defined 23 haplotypes and the sequence divergence among haplotypes ranged from 0.0030 and 0.0435. Nucleotide diversity for the total sample was five-fold higher using the sequencing method (0.0025 obtained by RFLP against 0.0127 obtained by sequencing). Statistical analysis using the Analysis of Molecular Variance (AMOVA) and the randomized chi-square test of independence showed no significant differences between humpback whales from Areas IV and V. The combination of the small sample size used in this study and the pattern of geographical distribution of Groups IV and V in the Antarctic (as revealed by previous analysis of mark-recapture data), may explain the absence of significant mtDNA differences between these two groups in the Antarctica.

INTRODUCTION

There is a considerable amount of information on the pattern of distribution and seasonal migratory movement of humpback whales from Groups IV and V. Most of that information is derived from the analysis of 'Discovery'-type marks, which were used in the past. Mackintosh (1942; 1965) showed that humpback whales tend to gather into five or six distinct feeding concentrations in the Antarctic during the austral summer season. Two of the areas within which these concentrations occur are Areas IV (70°-130°E) and V (130°E-170°W). The geographical boundary of these two areas in the Antarctic were defined taken into consideration the distribution of catches and the results of mark-recapture analysis (Omura, 1953; Chittleborough, 1959a).

Mark-recapture analysis demonstrated that whales from Antarctic Area IV in the austral summer, migrate to the western coast of Australia in winter (Chittleborough, 1959a). Recently, Gill and Burton (1995) using photo-identification of individuals based on natural

markings, demonstrated the movement of a humpback whale between Perth, Western Australia and Antarctic Area IV (resighting position: 64°20'S;101°12'E).

On the other hand, Dawbin (1959) and Chittleborough (1959a), examining mark-recapture data, suggested that some whales distributed in summer in Antarctic Area V migrates to the east coast of Australia, while others migrates along the coastline of New Zealand and south-west Pacific islands (Tonga, Cook Islands, Niue, Samoa, Fiji) during the austral winter. Kaufman *et al.* (1990), using photo-identification of individuals, demonstrated the movement of a humpback whale between Antarctic Area V at position 68°46'S;170°52'W and Eastern Australia.

Baker *et al.* (1994; 1995) analyzed mtDNA in humpback whales from the migratory corridor of Western and Eastern Australia as well the wintering ground of Tonga. They presented evidences for mtDNA differentiation between humpback whales from Western and Eastern Australia. On the other hand, there are no previous genetic studies involving humpback whales from the feeding grounds of Antarctic Areas IV and V. Analyses of such samples are necessary in order to investigate the pattern of segregation of Groups IV and V in the feeding grounds during the austral summer season. The IWC Scientific Committee had noted that the analysis of more samples in the Southern Hemisphere, particularly from feeding grounds is necessary before a comprehensive understanding of stock structure could be obtained (IWC, 1995).

Making use of skin biopsies sampled during the JARPA surveys in Antarctic Areas IV and V, we have begun a study of mtDNA in humpback whales from these areas. The mtDNA is recommended for studies of stock identity due to that this molecule is maternally inherited (Hutchinson *et al.*, 1974) and its evolutionary rate is as much as ten times faster than nuclear DNA (Brown *et al.*, 1979). This should allow greater resolution of genetic differences between conspecifics and between closely related species. Sequence changes in animal mitochondrial genomes are of four principle types: sequence rearrangements, additions, deletions and nucleotide substitutions (Hoelzel and Dover, 1989). The substitution rate is not constant along the mtDNA molecule. The D-loop-containing control region has been considered to be the most rapidly evolving part of the molecule. Given these properties, we have chosen the mtDNA control region for examining the genetic differences in the Antarctic humpback whale.

We have conducted both RFLP and sequencing analyses of the mtDNA control region. This was made in order to compare the degree of resolution of these two approaches.

MATERIALS AND METHODS

Samples

Samples used in this study were skin biopsies collected from humpback whales in their feeding grounds of Antarctic Areas IV and V (Fig. 1). Information on sampling is given in Table 1. The skin biopsy samples were collected during the JARPA surveys, using a biopsy dart shooting gun described by Kasamatsu *et al* (1991), with some minor modifications. Samples from Area IV were obtained during the JARPA survey of the 1993/94 austral summer season while those from Area V during the 1994/95 summer season. After sampling, the skin biopsies were stored at -20°C until use. A total of 19 and 11 biopsy samples were analyzed in Areas IV

and V, respectively. Biopsy samples obtained from calf accompanying the mother (one case in Area IV, three cases in Area V), were excluded from the analysis.

Extraction of DNA

Total DNA (nuclear+mitochondrial DNA) was extracted from approximately 0.05g of the outer epidermal layer of the skin biopsy, following the standard protocol described in Sambrook *et al.* (1989). The tissue was homogenized in 500ul of TES buffer. Previous addition of 25ul of Sodium Dodecyl Sulfate (SDS, 20%), 25ul of Proteinase K (PK) (20mg/ml) was added and the homogenate was incubated at 37°C overnight. After the incubation period, the DNA solution was mixed with an equal volume of a 25:24:1 phenol/chloroform/isoamyl alcohol solution, shaken thoroughly and centrifuged to precipitate proteins. Finally, DNA was precipitated by adding 1 ml of 99.5% ethanol and incubating at -70°C for 15min. The extracted DNA was suspended in 500ul of TE buffer.

RFLP analysis

A RFLP analysis of the mtDNA control region was carried out after the amplification of this region by the polymerase chain reaction (PCR). Here we followed the instructions for DNA amplification given by Hoelzel (1992). Primers for amplification of about 1,050bp segment were designed following Hori *et al.* (1994) and Dillon and Wright (1993) (Fig. 2).

PCR products were digested with ten different four-base sequence recognition restriction enzymes: *Afa*I, *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hinf*I, *Mbo*I, *Msp*I, *Sau*96I and *ScrF*I. Restriction fragments produced by each enzyme were separated by submarine electrophoresis in 2.5% agarose gel. After electrophoresis, the gels were stained with ethidium bromide and then photographed using Polaroid 667 film under 312nm UV irradiation. The size of each fragment was estimated by comparing their relative mobilities with those of the PHy marker. Distinctive restriction fragment patterns produced by each enzyme were assigned letters. Individuals were assigned haplotypes consisting of a list of the letters designating the fragment profiles produced by each of the ten restriction enzymes.

Sequencing analysis

We sequenced a 336bp-long segment of the mtDNA D-loop-containing control region. This segment was amplified by PCR using primers designed from Hori *et al.* (1994) and Dillon and Wright (1993) (Fig. 2). The DNA sequences were determined with an automatic sequencer, the Applied Biosystems 377 (ABI 377), following the protocols of the manufacturer. For each sample both strands were sequenced.

Sequences were aligned using the 'Sequence Navigator', a DNA sequence comparison software developed by Applied Biosystems.

Data analysis

In the case of the RFLP analysis, genetic distance between haplotypes was estimated using maximum-likelihood methods (Nei and Li, 1979; Nei and Tajima, 1983). In the case of the sequencing analysis, the Kimura's two parameters method (Kimura, 1980) was used for estimating genetic distances between two sequences.

The degree of mtDNA diversity within each geographical area was estimated using the index of nucleotide diversity (Nei, 1987 pp. 256). The net genetic distances between Area IV and V

was estimated from equation 10.21 of Nei (1987).

A phylogenetic tree of unique sequences was constructed by the neighbor-joining method (Saitou and Nei, 1987), using a minke whale sequence as an outside group (Arnason *et al.*, 1993). The robustness of the phylogenetic relationships was tested using bootstrapping (Felsenstein, 1985).

The geographic differentiation of mtDNA variation was tested using the Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) and the randomized chi-square test of independence (Roff and Bentzen, 1989). In both cases the significance of these statistics (P) was tested using random permutation. In each trial, 2,000 randomizations of the original data sets were made.

RESULTS

RFLP Analysis

Humpback whale mtDNA haplotypes

Apart from *Hha*I, *Hinf*I and *Msp*I, all the other restriction enzymes showed polymorphism. Table 2 shows a matrix of presence or absence of 9 restriction sites defining seven humpback whale mtDNA haplotypes. Sequence divergence among haplotypes ranged from 0.0030 and 0.0218.

Nucleotide diversity

The nucleotide diversity in the total sample (n= 30) was 0.0025. In Antarctic Areas IV (n= 19) and V (n= 11) it was 0.0028 and 0.0020, respectively. The net genetic distance between these two areas was 0.0025.

Geographical distribution of haplotypes

Table 2 shows the distribution of seven haplotypes in Areas IV and V. Haplotypes '1' and '2' were the predominant haplotypes and they were present in both areas in similar proportions. Haplotypes '3', '4' and '5' occurred only in humpback whales from Area IV while haplotypes '6' and '7' were specific for Area V.

The statistical comparison between Antarctic Areas IV and V showed no significant differences between these areas by both chi-square (chi-square raw data= 7.854, P= 0.228) and AMOVA (PHIst= 0.015, P= 0.297).

Sequencing analysis

Nucleotide sequences

A 336bp-long segment of the mtDNA D-loop-containing control region was sequenced in 18 humpback whales from Area IV and 10 from Area V. A total of 36 substitutions (35 transitions; 1 transversion) defined 23 unique sequences (haplotypes) in 28 humpback whales (Table 3). Sequence divergence among haplotypes ranged from 0.0030 and 0.0435.

Phylogeny of haplotypes

Fig. 3 shows the phylogenetic relationships among 23 mtDNA unique sequences (haplotypes) described by the neighbor-joining method. In some cases, the mtDNA relationship between haplotypes is well correlated with the geographical distribution of individuals. For example,

haplotypes '22' and '23' are located in an independent cluster, which is supported by a high bootstrap value, is related to individuals distributed in Area V. Also haplotypes '5' and '7' conform an independent cluster (with high bootstrap value), which is related to individuals distributed in Area IV. Other clusters, however, are conformed by haplotypes of individuals distributed in both Antarctic Areas IV and V.

Nucleotide diversity

The nucleotide diversity in the whole sample (n= 28) was 0.0127. In Areas IV (n= 18) and V (n= 10) it was 0.0123 and 0.0138, respectively. The net genetic distance between areas was 0.0128.

Geographical distribution of haplotypes

Table 3 shows the distribution of 23 haplotypes in Areas IV and V. Haplotype '10' was the only haplotype shared by both management areas. All the other haplotypes (most of which were represented by a single individual) were specific to either Area IV or Area V.

The statistical comparison between Areas IV and V showed no significant differences between these two areas using chi-square (chi-square raw data= 25.096, P= 0.174) and AMOVA (PHIst= 0.008, P= 0.2964).

DISCUSSION

In this report we have presented the results of a preliminary mtDNA analysis in humpback whales distributed in the Antarctic feeding grounds of Areas IV and V during the austral summer season. For carrying out such analysis, we have used skin biopsies sampled systematically in these areas as part of a non-lethal component of the JARPA surveys.

Two different methods were employed for examining the mtDNA control region, a RFLP analysis of the whole control region and a sequencing analysis of a part (a 336bp-long segment) of that region. A comparison of the level of resolution between these two approaches is summarized in Table 4. Nucleotide diversity for the total sample was five-fold higher using the sequencing method (0.0025 obtained by RFLP against 0.0127 obtained by sequencing). It should be pointed out that our RFLP analysis involved the whole mtDNA control region while the sequencing analysis involved only a portion of that region. If sequencing is conducted for a larger segment of the control region, resolution of the sequencing approach should be higher . It should be mentioned that RFLP analyses of the mtDNA control region is being used to examine stock identity in the western North Pacific minke (Goto and Pastene, 1996) and Bryde's whale (Pastene *et al.*, 1996). In both cases three localities were compared, but at the resolution level of the RFLP analyses, no significant differences among these localities have been found. Based on the data summarized in Table 4, we recommended that, in addition to the RFLP approach, sequencing be also employed in those studies.

Using both approaches, the values of nucleotide diversity found within Areas IV and V are similar to the net genetic distance found between these areas. Furthermore from Fig. 3 it is observed that, in general, there is not a good correlation between geographical distribution of haplotypes and phylogenetic relationship among them. In addition, statistical comparisons between areas using AMOVA and chi-square, showed no significant differences among

Antarctic Areas IV and V. Thus no geographical structure of the mtDNA variation could be demonstrated for the Antarctic humpback whales from these areas.

No significant differences between areas can be the result of low power of the analysis due to the small sample size used. Another explanation can be found in the results of previous analyses of mark-recapture data. Dawbin (1966) indicated that humpback whales tend to disperse most widely in cold waters, with some overlap between groups that started from widely separated breeding areas. Also he indicated that there is a marked tendency for humpback whales from one breeding area to return regularly to the same breeding area from season to season, but that a few whales do change breeding areas between seasons. Despite this fact, it seems that the effect of interchange between breeding areas is negligible given the marked mtDNA differentiation between humpback whales from Western and Eastern Australia (Baker *et al.*, 1994; 1995).

Chittleborough (1959b) analyzed humpback whales captured during February 1959 in Antarctic Areas IV and V and on the basis of sex ratio, maturity and body length information, in addition to the information derived from mark-recapture data analysis, concluded that humpback whales taken between 110°E and 130°E (eastern part of Area IV), was composed of a mixture of the Groups IV and V. Fig. 4 reproduce Fig. 2 of Dawbin (1966), in which he summarize the distribution and seasonal migratory movement of humpback whales from Groups IV and V, as demonstrated by mark-recapture data. In Group IV, there are 40 whales marked in Antarctic Area IV, mainly in the western part, and recovered in Western Australia and three marked in Western Australia and recovered in Antarctic Area IV. Nine whales were marked and recovered in low latitude. The geographical distribution range of this group in the Antarctic extend approximately from 70°E and 115°E . With regard Group V, there are 10 whales marked in the Antarctic and recovered in Eastern Australia and 9 marked in Eastern Australia and recovered in the Antarctic Area V. A total of 37 whales were marked and recovered in low latitudes. The range of geographical distribution of Group V in the Antarctic extend from 110°E and 170°E (Fig. 4). Near longitude 130°E, there is one case of a whale marked in the Antarctic and recovered in Western Australia. Thus, according to this information there is an overlap in the Antarctic boundary between humpback whales from Groups IV and V. Also it should be noted that the boundaries of Groups IV and V (Fig. 4) do not correspond to the actual boundaries of Areas IV and V.

As it can be observed from Fig. 1, most of the biopsy samples obtained during the JARPA survey of 1993/94, are from the eastern part of Area IV, in where mark-recapture data indicate overlap in the distribution of Groups IV and V. This is another plausible explanation for the absence of significant mtDNA differences found in our study. Thus the combination of the small sample size and the pattern of distribution of Groups IV and V in the Antarctic may explain the absence of significant differences between Areas IV and V. Further analysis including more samples, specially from the western part of Area IV, are necessary to corroborate the hypothesis established above. Also a direct comparison between humpback whales from Western and Eastern Australia with those of Antarctic Areas IV and V is desirable in order to understand the population genetic structure of the humpback whale of Groups IV and V across its seasonal migratory range.

ACKNOWLEDGMENTS

Skin biopsy samples of humpback whales were obtained during the JARPA surveys of 1993/94 and 1994/95 in Areas IV and V, respectively. We would like to acknowledge the researchers and crew members that participated in these surveys. We thanks T. Kimura, Faculty of Science, Nagoya University, for his assistance in the data analysis and Per Palsboll, Department of Population Biology, University of Copenhagen, for useful comments and suggestions.

REFERENCES

Arnason, U., Gullberg, A. and Widegren, B. 1991. The complete nucleotide sequence of the mitochondrial DNA of the fin whale, Balaenoptera physalus. *J. Mol. Evol.* 33:556-558.

Arnason, U., Gullberg, A. and Widegren, B. 1993. Cetacean mitochondrial DNA control region: sequences of all extant baleen whales and two sperm whale species. *Mol. Biol. Evol.* 10:960-70.

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.

Baker, C.S., Florez-Gonzales, L., Rosenbaum, H.C. and Bannister, J. 1995. Molecular Genetic identification of sex and stock structure among humpback whales of the Southern Hemisphere. Paper SC/47/SH1 presented to the IWC Scientific Committee, May 1995 (unpublished). 25pp.

Brown, W.M., George, M. and Wilson, A.C. 1979. Rapid evolution of mitochondrial DNA. *Proc. Natl Acad. Sci.* USA 76 (1): 967-971.

Chittleborough, R.G. 1959a. Australian marking of humpback whales. *Norsk Hvalfangsttid* 48: 47-55.

Chittleborough, R.G. 1959b. Intermingling of two populations of humpback whales. *Norsk Hvalfangsttid* 48: 510-521.

Dawbin, W.H. 1959. New Zealand and South Pacific whale marking and recoveries to the end of 1958. *Norsk Hvalfangsttid* 48: 213-238.

Dawbin, W.H. 1966. The seasonal migratory cycle of humpback whales. *In* Norris, K.S. (ed.). Whales, dolphins and porpoises. University of California Press, Berkerly. pp. 145-171.

Dillon, M.C. 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* 137:479-491.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783-791.

Gill, P.C. and Burton, C.L.K. 1995. Photographic resight of a humpback whale between Western Australia and Antarctic Area IV. *Marine Mammal Science* 11(1):96-100.

Goto, M. and Pastene, L.A. 1996. Population genetic structure in the western North Pacific minke whale examined by two independent RFLP analyses of mitochondrial DNA. Paper SC/48/NP5 presented at this meeting.

Hoelzel, A.R. (ed.). 1992. *Molecular Genetic Analysis of Populations. A practical approach.* Oxford University Press. Oxford, New York, Tokyo. 315pp.

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

Hori, H., Bessho, Y., Kawabata, R., Watanabe, I., Koga, A. and Pastene, L. 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.

Hutchinson, C.A., Newbold, J.E., Potter, S.S. and Edgell, M.H. 1974. Maternal inheritance of mammalian mitochondrial DNA. *Nature* 251:536-537.

International Whaling Commission.1995. Report of the Scientific Committee. *Rep. int. Whal. Commn* 45:53-221.

Kasamatsu, F., Iwata, S. and Nishiwaki, S. 1991. Development of biopsy skin sampling system for fast swimming whales in pelagic waters. *Rep. int. Whal. Commn* 41:555-557.

Kaufman, G.D., Osmond, M.G., Ward, A.J. and Forestell, P.H. 1990. Photographic documentation of the migratory movement of a humpback whale (*Megaptera novaeangliae*) between east Australia and Antarctic Area V. *Rep. int. Whal. Commn* (special issue 12):265-267.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111-120.

Mackintosh, N.A. 1942. The southern stocks of whalebone whales. *Disc. Rep.* 22:197-300.

Mackintosh, N.A. 1965. *The stocks of whales.* Fishing News (Books) Ltd., London. 232pp.

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.

Omura, H. 1953. Biological study on humpback whales in the Antarctic whaling areas IV and V. *Scientific Reports of the Whales Research Institute*, Tokyo 8:81-102.

Pastene, L.A., Goto, M., Itoh, S., Wada, S. and Kato, H. 1996. Intra and interoceanic patterns of mitochondrial DNA variation in the Bryde's whale *Balaenoptera edeni*. Paper SC/48/NP15 presented at this meeting.

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

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual.* Second Edition. Cold Spring Harbor Laboratory, New York.

Table 1: Ancillary information of the humpback whales from Areas IV and V sampled for skin biopsies.

Individual ID	School size	Estimated body length	Sampling date	Sampling position
93IV-001	1	13.1m	93/12/07	63°00'S;123°34'E
93IV-002	1	11.4m	93/12/18	61°18'S;115°15'E
93IV-003	2	12.2m	94/01/13	60°43'S;114°35'E
93IV-004	2	14.3m	94/01/17	61°31'S;108°10'E
93IV-005*	"	13.1m	"	"
93IV-006	1	11.6m	94/01/18	60°26'S;106°57'E
93IV-007	2	11.0m	94/01/19	61°17'S;101°39'E
93IV-008*	"	11.6m	"	"
93IV-009	4	11.0m	94/01/20	60°51'S;100°50'E
93IV-010*	"	11.6m	"	"
93IV-011	4	13.1m	94/01/20	61°20'S;101°45'E
93IV-012	2	12.2m	94/01/24	64°20'S;92°51'E
93IV-014	2	14.3m	94/02/12	66°54'S;70°07'E
93IV-015	2	12.2m	94/03/09	64°44'S;115°41'E
93IV-016*	"	13.1m	"	"
93IV-017	2	13.1m	94/03/10	65°16'S;120°28'E
93IV-018	2	12.8m	94/03/10	65°18'S;120°50'E
93IV-019	2	12.8m	94/03/12	65°34'S;120°20'E
93IV-020*	"	12.2m	"	"
94V-050	2	11.0m	94/12/08	65°06'S;176°42'W
94V-051	1	10.4m	94/12/16	62°01'S;169°38'E
94V-052	2	11.6m	94/12/29	64°09'S;138°33'E
94V-053*	"	13.1m	"	"
94V-054	3	11.6m	95/01/15	63°07'S;153°38'E
94V-055*	"	12.8m	"	"
94V-056*	"	11.3m	"	"
94V-057	2	12.2m	95/01/25	64°52'S;173°55'E
94V-059	2	13.4m	95/01/30	65°48'S;177°21'W
94V-061	2	13.1m	95/02/02	65°32'S;172°14'W
94V-062	2	13.1m	95/02/13	65°40'S;170°26'W

* = same school as individual above

Table 2: Table of presence or absence of nine restriction sites defining seven mtDNA haplotypes in the Antarctic humpback whale. In the composite patterns of haplotypes, letters sequences from left to right refer to the digestion profiles for the restriction enzymes *Afa*I, *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hinf*I, *Mbo*I, *Msp*I, *Sau96*I and *ScrF*I. The distribution of these haplotypes in Areas IV and V is also shown.

Hap. ID	Composite Pattern	Site Matrix	Area IV	Area V
1-	BAAAAAAAAA	1 0 1 0 0 0 0 1 1	**8** (93IV-003,004,006, 009,011,014, 015,018)	**6** (94V-050,052, 053,055,056,059)
2-	AAAAAAAAAA	1 1 1 0 0 0 0 1 1	**4** (93IV-001,005,007, 008)	**3** (94V-054,061, 062)
3-	AABAAAAAAA	1 1 1 1 0 0 0 1 1	**2** (93IV-010,017)	**0**
4-	ABBAAAAAAA	1 1 0 1 0 0 0 1 1	**4** (93IV-002,012,019, 020)	**0**
5-	CAAAAABACB	0 1 1 0 0 1 0 0 0	**1** (93IV-016)	**0**
6-	AABBAAAABA	1 1 1 1 1 0 1 1 1	**0**	**1** (94V-057)
7-	AAAAAAAACA	1 1 1 0 0 0 0 0 1	**0**	**1** (94V-051)
			19	11

Table 3: Variable sites defining 23 Antarctic humpback whale unique sequences in the mtDNA control region. Position 10 of the humpback whale sequence correspond to position 15,053 in the fin whale mtDNA (Arnason *et al.*, 1991).

Haplotype	1111111111122222222222223 1255677889012334666894444556666667781 0679407569186456127360567373456893 46	AREA IV	AREA V
1	TGGTTCTTTTCGTAGTTCCTTTGTCATGTTCTTGTC	93IV-001	
2	..................T.C...C....C...C...	93IV-002	
3	........C.AA..AC.T.....C......T..A.T	93IV-003	
4	........C.A...AC.T.....C..C..........	93IV-004	
5	....C..C.....G...TT...........T..A..	93IV-005	
6	.......C..A....C.......C..C..........	93IV-006	
7	....C..C.....G...TT.....T.....T..A..	93IV-007,008	
8	........C.A...AC.T....AC.............	93IV-009	
9	.....................................C...	93IV-010,017	
10	........C.A...AC.T.....C.............	93IV-011	94V-055,056
11	...................C...C....C...C...	93IV-012,019	
12	........C.A....C.............CT....T	93IV-014	
13	....C....C..C..C.TT....C......T..AC.	93IV-015	
14	.A.............C.T..CC..T..A.C......	93IV-016	
15	....C....C..C..C.TT..............A..	93IV-018	
16	........C.T...AC.T.....C.............		94V-050
17	.A..C.......C..C.TT...........T..AC.		94V-051
18	........C.AA..ACCT.....C......T..A..		94V-052
19	...C..C...A....C.......C......T.....		94V-053
20	C.......C..........................		94V-054
21	......................C..C.CC..C...		94V-057
22	..A..T....T.C....T.......G...CTC.A..		94V-059
23	..A..T......C....T.......G...CTC.A..		94V-061
		18	10

Table 4: Comparison of the range of sequence divergence among haplotypes, nucleotide diversity within and between areas, estimated by RFLP analysis of mtDNA control region and sequencing analysis of a 336bp-long segment of the mtDNA control region.

	RFLP	**Sequencing**
Number of base substitutions	9	36
Range seq. div. among haplot.	0.0030 - 0.0218	0.0030 - 0.0435
Nucleotide diversity (total sample)	0.0025	0.0127
Nucleotide diversity (Area IV)	0.0028	0.0123
Nucleotide diversity (Area V)	0.0020	0.0138
Net. genetic distance AreaIV-AreaV	0.0025	0.0128

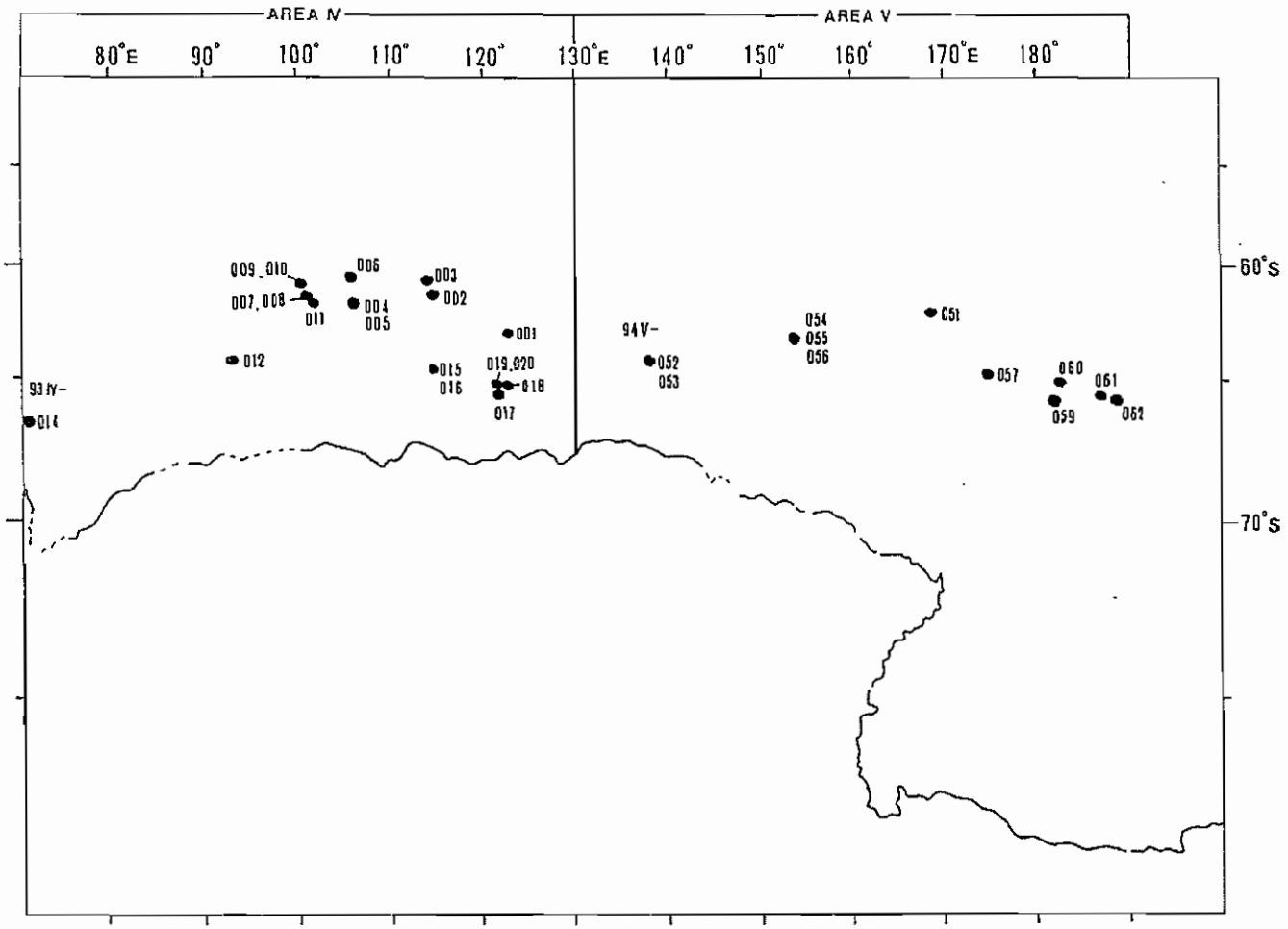
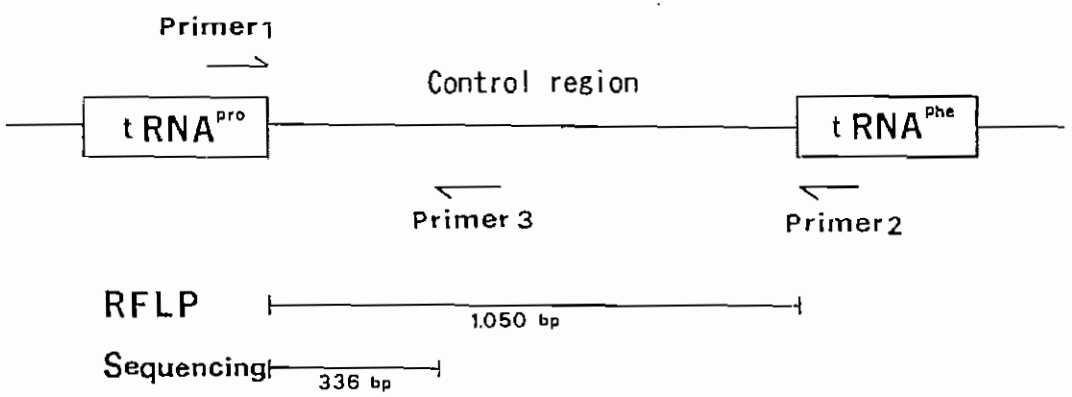


Fig. 1: Geographical distribution of humpback whales from Antarctic Areas IV and V analyzed in this study. Skin biopsies obtained from those whales were sampled during the JARPA surveys of the 1993/94 (Area IV) and 1994/95 (Area V) austral summer seasons. Individual ID as shown in Tables 1, 2 and 3.



Primer 1	5'-CAAGGAAGAAGTATTACACTCCACCA-3'
Primer 2	5'-CAGAATTGGAATTCATTTTCAGTGTCTTGCTTT-3'
Primer 3	5'-GAAGAGGGATCCCTGCCAAGCGG-3'

Fig. 2: Genetic map of mitochondrial DNA control region analyzed in this study. RFLP analysis involved the whole mtDNA control region (approximately 1,050bp) while the sequencing analysis, a 336bp-long segment of the control region. Primers used for PCR amplification are also shown.

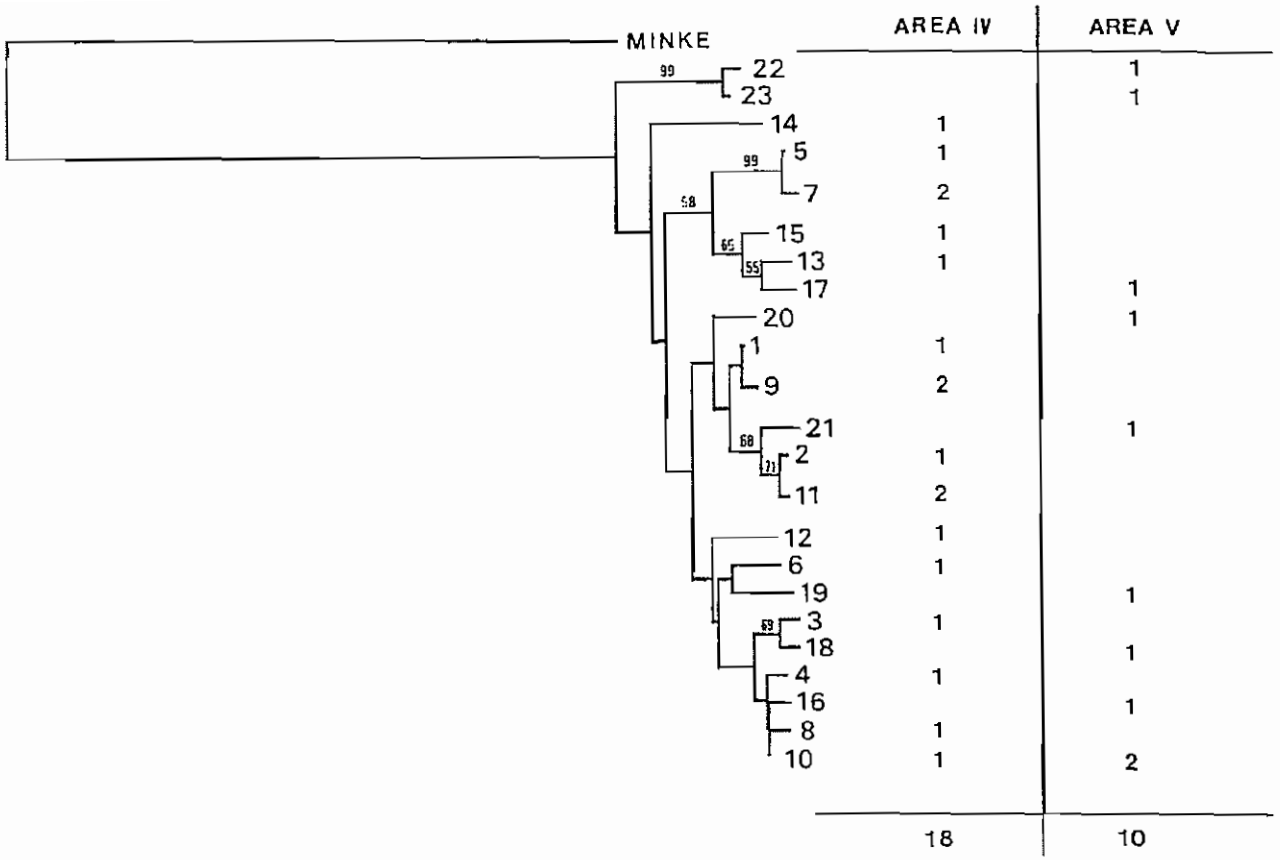


Fig. 3: Phylogenetic relationship among 23 unique mtDNA control sequences in Antarctic humpback whales from Areas IV and V. The tree was constructed by the neighbor-joining method and rooted with the homologous sequence of the minke whale (Arnason *et al.* 1993). Number at nodes show agreement in a consensus of 400 bootstrap simulations (percentage of cases). Only the bootstrap estimates over 50% are shown.

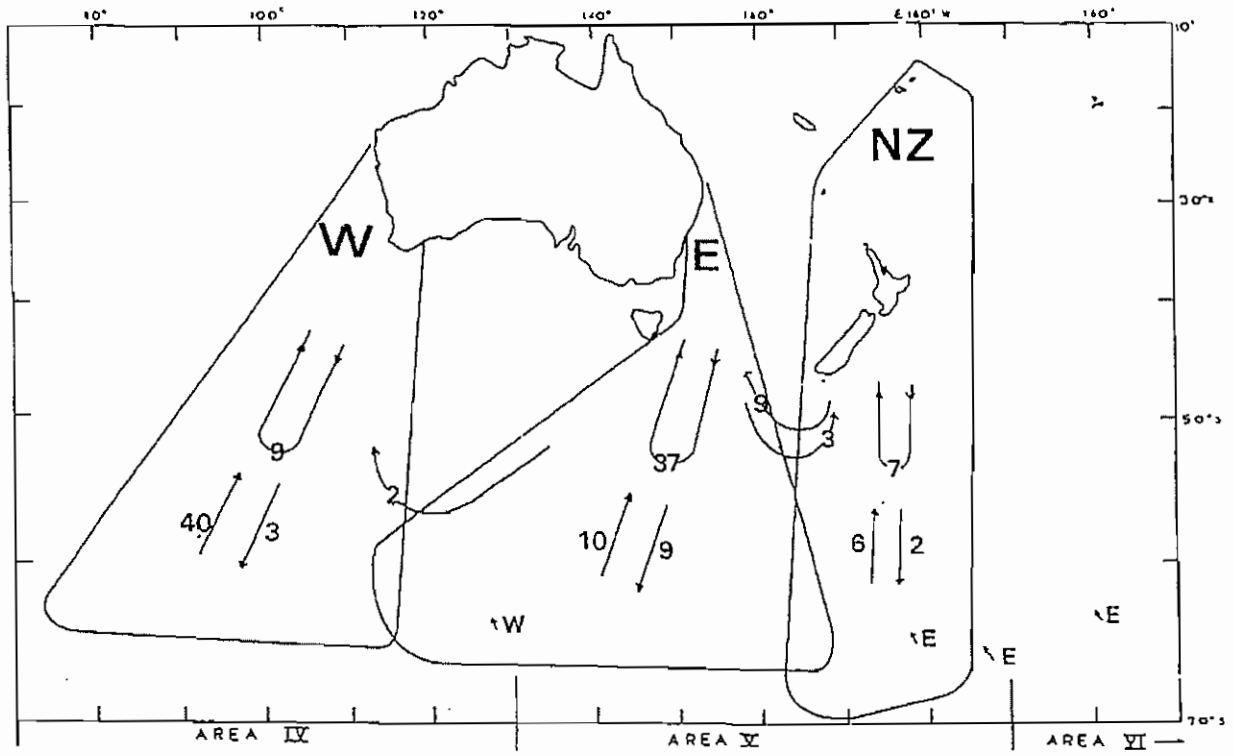


Fig. 4: A modified version of Fig. 2 of Dawbin (1966) showing the movement of humpback whales between low-latitude localities and the Antarctic Areas IV and V, and between low-latitude localities. Information is based in the results of the analysis of mark-recapture data between 70°E and 150°W. W= Western Australian group, E= Eastern Australian group, NZ= New Zealand group. The geographical range of the three groups were defined by the positions of whales marked in the Antarctic and recovered in low-latitude localities (arrows pointing north), the position of whales marked in low-latitude localities and recovered in the Antarctic (arrows pointing south), the position of whales marked and recovered in the same low-latitude locality, in one or more season later (looped arrows within each locality). Interchange between low-latitude localities is shown by curved arrows crossing boundaries between W, E and NZ (see more details in Dawbin, 1966). Note that the boundaries in the Antarctic do not correspond with the actual boundaries of Areas IV and V and that there is an overlap at the boundaries between groups.