Genetic characteristics and population structure of humpback whales in the Antarctic feeding ground as revealed by mitochondrial DNA control region sequencing and microsatellite analyses

LUIS A. PASTENE[1], MUTSUO GOTO[1], SHIGETOSHI NISHIWAKI[1], HIDETOSHI YOSHIDA[2] and NAOHISA KANDA[1]

[1]*The Institute of Cetacean Research, Toyomi-cho 4-5, Chuo-ku, Tokyo 104-0055, Japan*
[2]*National Research Institute of Far Seas Fisheries, Orido 5-7-1, Shimizu, Shizuoka 424-8633, Japan*

ABSTRACT

Biopsy samples from 411 humpback whales obtained during surveys of the Japanese Whale Research Program under Special Permit (JARPA) and International Decade for Cetacean Research/Southern Ocean Whale and Ecosystem Research (IDCR/SOWER) were analyzed in order to describe their genetic population structure in the Antarctic feeding ground. Samples were obtained from International Whaling Commission (IWC) designated management Areas III (n= 81), IV (n= 172), V (n= 97) and VI (n= 61), and were examined for i) sex determination, ii) the sequence variation of the first 334bp nucleotides of the mtDNA control region and iii) genetic variation at the genotypes of six microsatellite loci. Duplicated samples were excluded from the analysis. The level of genetic diversity in the Antarctic was high for both genomes: the nucleotide diversity at the mtDNA was estimated at 0.0263 and the mean expected heterozygosity at the nuclear loci at 0.7820 for the total samples. In general results based on both mtDNA and microsatellites were similar and they suggest population structure of humpback whales in the Antarctic feeding grounds. These genetic results are consistent with the previous view based on non-genetic data that Areas III, IV, V and VI are occupied by different populations. The most plausible pattern of structure in the Southern Hemisphere therefore is multiple breeding and feeding grounds with some extent of site fidelity. Marked differences were found between whales in Areas IV and V for both mtDNA and microsatellites, and the same pattern was found for both sexes. Results of the other pair-wise comparisons among Areas showed more subdivisions in females than in males. An explanation for this result is that the difference is due to the lower sample sizes for males in these comparisons. The possibility of intermingle of populations in bordering sectors can not be discarded yet and a comprehensive analysis that involves genetic data from low and high latitude is recommended to solve this issue.

KEYWORDS: HUMPBACK WHALE, ANTARCTIC, FEEDING GROUNDS, GENETICS

INTRODUCTION

Humpback whales, *Megaptera novaeangliae,* are found worldwide in all major oceans. Like other Balaenopterid species, humpback whales migrate between summer feeding grounds in mid- and high latitudinal waters and winter breeding grounds in tropical or subtropical waters. Animals occur primarily in coastal and continental shelf waters.

Three oceanic populations of the humpback whale occur in the North Atlantic, North Pacific and Southern Hemisphere, and different patterns of population structure have been reported within each major oceanic population. In the western North Atlantic, for example, whales congregate to mate and calve in a single breeding area of the West Indies during the winter. In summer they congregate in several high latitude feeding areas. Genetic studies suggest limited exchange among the summer feeding areas, and mixing in the winter breeding area (Palsboll *et al.*, 1997a). The pattern of structure here is single breeding grounds and multiple feeding grounds. In the North Pacific the situation is much more complex. Whales show a strong

fidelity to winter breeding grounds in Costa Rica, Mexico, Hawaii, Southeast Asia, and wide, but philopatric, dispersal to feeding grounds in temperate and high latitudinal waters. Some movements of animals between low latitudinal breeding areas have been reported (Darling and McSweeney, 1985; Darling and Mori, 1993). Genetic studies have shown significant differences between whales distributed in central and eastern North Pacific (Baker *et al.*, 1998a). The pattern of structure here is single breeding grounds and multiple feeding grounds but the case of multiple breeding grounds using a single feeding ground has also been suggested. These phenomena seem to be related with differences in topography among ocean basins and as a consequence, the breeding grounds in the North Atlantic are few, those in the North Pacific are larger in number and those in the Southern Hemisphere are many.

Regarding the Southern Hemisphere, Mackintosh (1965) showed that humpback whales tend to gather into five or six distinct feeding concentrations in the Antarctic during the austral summer season. These feeding concentrations were denominated as Groups I-V (with a Group IIa and IIb) corresponding roughly to IWC Management Areas I-VI. The Groups most documented are Groups IV and V. Omura (1953) examined the distribution of humpback whale in the feeding grounds of Areas IV and V based on catch data. Based on catch information he suggested that two populations occur in these Areas with a boundary around 130°-142°E. He did not discard the possibility of intermingling between these two populations in the feeding ground. He also examined the pattern of distribution by month and suggested that for the month where more data were available (November-March) the boundary between these two populations changed from 120°-130°E in November to eastside of 140°E in December and to 120°-140°E in January. Dawbin (1966) summarize the distribution and seasonal migratory movement of humpback whales from Groups IV and V, as demonstrated by mark-recapture data (Discovery-type marks). Whales from Group IV move mainly between Antarctic Area IV and Western Australia while whales from Group V move between Antarctic Area V and Eastern Australia and along the coast of New Zealand and southwest Pacific islands. Interchange of a few individuals between Groups IV and V was reported. Dawbin (1966) also reported that the boundary of Groups IV and V in the Antarctic do not correspond to the actual boundary between Areas IV and V and that some whales marked in Area VI were recovered in eastern Australia.

These migratory corridors between low latitudinal breeding grounds and high latitudinal feeding grounds have been confirmed by analysis of photo-identified whales. Gill and Burton (1995) reported the movement of a photo-identified whale between Western Australia and Antarctic Area IV and Kaufman *et al.* (1993) that between Eastern Australia and the boundary between Areas V and VI. More recently the matches of three individual humpback whales between Eastern Australia and Antarctic Area V, were reported (Rock *et al.*, in press).

Genetic analyses based on mitochondrial DNA (mtDNA) and samples from low latitude waters (Baker *et al.*, 1998b) showed significant genetic differences between Western Australia, Eastern Australia and Colombia humpback whales, supporting the previous view that different populations occur in those regions (corresponding to Groups IV, V and I mentioned above). Little is known, however, on the genetic diversity and population structure of humpback whales in the Antarctic feeding grounds. The present paper addresses this issue by examining samples obtained in Areas IV and V and their adjacent Areas III and VI. As noted above, previous non-genetics studies had suggested that these Areas are occupied by different populations. Here the hypothesis of different populations occurring in these Areas is tested using genetic data. The analysis is also conducted to investigate the current status of genetic variability of humpback whales.

The analysis is based on the maternal inherited mtDNA and bi-parental inherited microsatellites. Further the analysis is conducted for total samples as well for each sex separately. Previous studies based on mark-recapture suggested a high fidelity of animals to feeding areas (identified approximately by the boundaries of the current Areas) and particular breeding areas. Therefore genetic differences among Areas are expected. There is the possibility, however, of a sex-differentiated pattern of dispersal in the feeding ground. This could be investigated by the genetic analysis conducted separately for males and females. Finally if male-mediated gene flow exist (e.g. some males move and mate females from

different breeding grounds), then we will expect marked genetic differences among Areas for mtDNA and weak genetic differences for microsatellites.

MATERIALS AND METHODS

Samples

Skin biopsy samples were obtained from free-ranging whales along the sighting surveys of the JARPA (Japanese Whale Research Program under Special Permit in the Antarctic) and IDCR/SOWER (International Decade for Cetacean Researeh/Southern Ocean Whale and Ecosystem Research) surveys in Areas IIIE, IV, V and VI (Figure 1), on an opportunistic basis. Biopsy samples in JARPA were collected using an air gun described in Kasamatsu *et al.* (1991) and more recently using a Paxarm system. Biopsy samples in IDCR/SOWER were collected using several methods including Paxarm system and crossbows. At the laboratory all biopsy samples were checked for the possibility of re-sampling (two or more samples taken from a same individual) by comparing the genotype profiles produced by a set of six microsatellites.

Re-sampling rates were higher for IDCR/SOWER than for JARPA samples. For the former it varied from 0% in Area V to 15.0% in Area VI (sample size in Area V was only seven). In the case of JARPA it varied from 4.3% in Area VI to 10.0% in Area III. Taylor and Chivers (1997) demonstrated by simulations that if duplicated samples are not recognized, results of genetic analyses on population structure will be biased. Therefore in the present study duplicates were excluded from all analyses.

When mother/calf pairs were sampled, only the genetic information of the mother was used for the analysis (nine cases in Area V).

Table 1 shows the actual number of samples used in the analysis by IWC management Area and sex, and Figure 1 shows the geographical distribution of samples in the feeding grounds of Areas IIIE, IV, V and VI, by sex. A total of 411 samples are used as follow: Area IIIE: JARPA 50, SOWER 31; Area IV: JARPA 126, SOWER: 46; Area V JARPA 90, SOWER 7; Area VI: JARPA 44, SOWER 17.

Molecular genetic analysis

Extraction of DNA

Genomic DNA was extracted from approximately 0.05g of the outer epidermal layer of the skin biopsy, by standard phenol/chloroform extractions (Sambrook *et al.*, 1989). Extracted DNAs were stored in TE buffer (10mM Tris-HCl, 1mM EDTA, ph8.0).

Sex determination

Sex of the whales sampled was determined following the method of Abe *et al.* (2001), which uses a set of oligonucleotide primers to detect the presence/absence of the SRY gene from the Y chromosome with a microsatellite locus (GATA 417) as an internal control.

Mitochondrial DNA control region sequencing

The first 334 nucleotides at the 5' end of the mitochondrial control region were amplified by the polymerase chain reaction (PCR, Mullis and Faloona 1987). The oligo-nucleotides employed in the PCR amplification were MT4 (Arnason *et al.* 1993) and P2R (5'-GAA GAG GGA TCC CTG CCA AGC GG-3'). Reactions were carried out in 50 uL volumes containing 100 mM KCl, 20 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT; 0.5% Tween 20, 0.5% Nonidet P-40, 200 uM dNTPs, 2.5 pM of each oligo-nucleotide and one unit of *Taq* DNA polymerase. After an initial denaturation step at 95° C for 5 minutes, a PCR amplification cycle of 30 seconds at 94°C, followed by 30 seconds at 50°C and 30 seconds at 72°C was repeated 30 times. The amplification was completed with a final extension step of 10 minutes at 72°C. Subsequent cycle sequencing reactions were performed with 100ng of products generated in the above PCR amplifications using the Prism™ dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.). The oligo-nucleotides used to prime the cycle sequencing reaction were the same as employed in the initial PCR amplification listed above. A total of 25 cycles with 10 seconds at 96°C, 20 seconds at 56°C and four minutes at 60°C were performed. The nucleotide sequence of each cycle sequencing reaction was determined by electrophoresis through a 5% Long Ranger™ (FMC, Inc.) denaturing polyacrylamide matrix on a DNA Prism™ 377 DNA

Sequencer (Applied Biosystems, Inc.) under standard conditions. Both strand samples were sequenced in their entirety for all samples.

Microsatellites
Six microsatellite loci were used in this study: four tetranucleotide motifs (GATA417, GATA28, GATA98, GATA53) (Palsboll *et al.* 1997b), one trinucleotide motifs (TAA31) (Palsboll *et al.* 1997b), and one dinucleotide motifs (GT23) (Bérubé *et al.* 2000). PCR amplifications were performed in 15ul reaction mixtures containing 10 to 100ng of DNA, 5pmol of each primer, 0.625 units of *Ex Taq* DNA polymerase (Takara Shuzo), 2mM of each dNTP, and 10x reaction buffer containing 20mM $MgCl_2$ (Takara Shuzo). Amplified products with internal size standard (GENESCAN400HD, Applied Biosystems Japan) were run on a 6% polyacrylamide denaturing gel (Long Ranger) using BaseStation100 DNA fragment analyzer (BioRad). Although alleles were visualized using Cartographer software speeifically designed for the BaseStation, allelic sizes were determined manually in relation to the internal size standard and humpback whale's DNA of known size that were rerun on each gel.

Grouping of the samples
The samples were grouped according to the boundaries of IWC Management Areas III (0°-70°E), IV (70°-130°E), V (130°E-170°W) and VI (170°-120°W). With regard Area III, biopsy samples were available only for the eastern part of this Area (34°-70°E) (Figure 1).

For the analysis samples collected in different austral summer seasons were pooled under the assumptions that the patterns of seasonal movement are the same for a given breeding population in different years and that lateral movement on feeding grounds and pattern of mixing are similar between years. These assumptions are discussed later.

Data analysis
mtDNA
Genetic distances among unique sequences (haplotypes) were estimated using the Kimura's two parameters method (Kimura, 1980). The degree of mtDNA diversity within each Area was estimated using the nucleotide diversity (Nei and Li, 1979), and the nucleon diversity.

Heterogeneity tests among Areas were conducted as described in Hudson *et al.* (1992), using the chi-square and the Kst* statistics. The level of statistical significance was estimated from 10,000 Monte Carlo simulations as the proportion of simulations in which a similar or more extreme value of chi-square or Kst* was observed. Tests were conducted for all samples combined as well for males and females separately. When multiple tests were conducted, the Rice (1989) correction for multiple tests was performed.

Microsatellites
Allele frequencies, the number of alleles per locus, allelic richness, heterozygosity at the six microsatellite loci and Fst values were computed using FSTAT 2.9.3 (Goudet, 1995). The program GENEPOP 3.2 (Raymond and Rousset, 1995) was used to test for deviations from expected Hardy-Weinberg genotypic proportions at each of the loci.

Heterogeneity tests among Areas were conducted using the probability test (or Fisher's exact test) with Markov chain method implemented in the GENEPOP. Tests were conducted for all samples combined as well for males and females separately. Statistical significance was determined using the chi-square value obtained from summing the negative logarithm of P-values over the six microsatellite loci (Sokal and Rohlf, 1995). When multiple tests were conducted, the Rice (1989) correction for multiple tests was performed.

The Bayesian clustering approach was conducted using STRUCTURE version 2.0 (Pritchard *et al.*, 2000) to determine the number of genetically distinct populations present in the samples. Three independent runs for each value of K between 1 (panmictie) and 5 (number of samples used in this study) with no prior information (i.e. only genetic information was considered) were conducted. All of the simulations were based on a burn-in period of 50,000 iterations and runs of 500,000 iterations. The ancestry model used for the simulation was the admixture model, which assumes individuals may have mixed ancestry. The allele frequency model used

was the correlated allele frequencies model, which assumes frequencies in the different populations are likely to be similar due to migration or shared ancestry.

RESULTS

Sex ratio

The male proportion by Area was as follow: Areas III: 38.3%, IV: 52.9%, V: 47.4% and VI: 59.0%.

Genetic variability

mtDNA

A segment of 334bp of the mtDNA control region was sequenced for all samples. A total of 65 variable sites defined a total of 98 unique sequences (haplotypes) in the sample of 411 humpback whales. Level of mtDNA diversity based on nucleotide and nucleon diversity is shown in Table 2. Both indices showed a high and similar degree of mtDNA diversity through the Areas. The nucleotide diversity at mtDNA for the total samples was estimated at 0.0263.

Microsatellites

All microsatellite loci were polymorphic in all the samples (Table 3). Five of the six loci had more than 10 alleles with an overall average of 15.3 over the all samples. The number of alleles per locus per Area varied between 10.7 and 12.7 with smaller differences in allelic richness values. Average expected heterozygosity was 0.782 over the Areas and ranged from 0.767 to 0.799 per Area. No evidence of significant deviation from expected Hardy-Weinberg expeeted genotypic proportions was observed in each of the Areas for over all loci tests. The P-value smaller than 0.05 observed in the Areas V and VI samples at GATA417 is most likely due to a chance effect.

Genetic divergence

mtDNA

The results of the heterogeneity test are shown in Table 4. The chi-square test resulted more sensitive to detect differences than the Kst* test. Below is a summary of the results for the former test.

Males

Statistically significant difference was detected across all the loci for the overall Areas. Two of the six pair-wise comparisons showed statistically significant differences after correction for multiple tests (Areas III/IV and Areas IV/V) (Table 4).

Females

Statistically significant difference was detected across all the loci for the overall Areas. Four of the six pair-wise comparisons showed statistically significant differences after correction for multiple tests (Areas III/IV, Areas III/V, Areas III/VI and Areas IV/V) (Table 4).

Total

Statistically significant difference was detcctcd across all the loci for the overall Areas. Five of the six pair-wise comparisons showcd statistically significant differences after correction for multiple tests. The only exception was Arcas V/VI (Table 4).

Kst* values indicated low level of genetic divergence (Table 4).

Microsatellites

Males

Statistically significant difference was detected across the loci for the overall Areas (Table 5). Only one of the six pair-wise comparisons showed statistically significant differences after corrections for the multiple tests (Areas IV/V).

Females

Statistically significant differencc was detected across the loci for the overall Areas (Table 5). Three of the six pair-wise comparisons showed statistically significant differences after corrections for the multiple tests (Areas III/IV, Areas III/V and Areas IV/V).

Total

Statistically highly significant difference was detected across the loci for the overall areas (Table 5). Four of the six pair-wise comparisons showed statistically significant differences after corrections for the multiple tests. The only exceptions were Areas III/VI and Areas V/VI.

Overall the Fst values indicated low level of genetic divergence. The overall Fst value for males was 0.008, for females 0.004 and for all samples combined 0.005. Ninety-five % confidence interval for the overall Fst indicated it was significantly different from zero.

In contrast to the heterogeneity tests and Fst shown above, the Bayesian clustering analysis conducted without information on geographie sampling origins presented the highest likelihood probability at K = 1 (Table 6).

DISCUSSION

Genetic variability

Level of genetic variability found for the Antarctic feeding grounds was high, for both mtDNA and microsatellites, and it was similar through the Areas. This is consistent with previous results obtained for the humpback whale worldwide for both markers, mtDNA (Baker *et al.*, 1993; Palsboll *et al.*, 1995) and microsatellites (Valsecchi *et al.*, 1997).

Pattern of stock structure

In general results based on both mtDNA and microsatellites were similar and they suggest population structure of humpback whales in the Antarctic feeding grounds. Genetic differentiation among Areas based on both markers suggests a strong fidelity of humpback whales to migratory destinations. In particular marked differences were found between whales in Areas IV and V for both mtDNA and microsatellites, and the same pattern was found for both sexes. The pattern of structure in the Southern Hemisphere seems to be multiple breeding grounds and multiple feeding grounds as opposed to the pattern found in the North Pacific and North Atlantic.

Although the samples used in the present study were taken on an opportunistic basis along JARPA and IDCR/SOWER surveys, sampling had covered most of the longitude sectors of Areas IIIE, IV, V and VI. The exceptions are a seetor in Area VI for males and the western part of Area V for which samples of both sexes were limited (Figure 1). In general these samples were useful to respond the question of whether population structure exists in those Areas.

The mtDNA analysis showed a marked degree of genetic heterogeneity suggesting population structure of the humpback whales in the Antarctic feeding grounds. By considering the kind of grouping of the samples in the analyses (by longitudinal Areas), these populations appear to be structured longitudinally. It should be noted that despite the marked mtDNA heterogeneity detected, the level of genetic divergence found (as suggested by Kst*) was low. Likewise, the microsatellite analysis detected that the individuals in the samples came from genetically different populations of humpback whales that appeared to be distributed longitudinally. Fst was low but significant, supporting the existence of multiple populations. STRUCTURE, however, failed to detect the evidence of population differentiation among the samples. Considering the low level of genetic differentiations (Fst), this could have been caused by the low power of the analysis due to the low number of microsatellite loci used.

Comparison of results between sexes

The analyses were conducted for both sexes separately in order to check the possibility a sex-differentiated pattern of dispersal in the feeding ground. The mtDNA analysis showed a higher degree of differentiation among Areas for females (four of six pair-wise comparisons were significant statistically) than males (two of six pair-wise comparisons resulted in significant differences). Same as in the mtDNA analysis, the microsatellite results showed more subdivisions in females than in males, three and one signifieant differences in six pair-wise comparisons, respectively. In the eomparison between Areas IV and V results for both sexes were the same for both genetic markers. These results suggest the possibility that males move wider than females in the Antarctic feeding grounds. However, the alternative explanation that

differences are just due to the lower sample size available for males in these comparisons is preferred because the apparent differences between sexes are not consistent with populations structured longitudinally in the feeding ground.

Yearly variation in the pattern of structure

As noted earlier for the analysis samples collected in different austral summer seasons were pooled under the assumptions that the patterns of seasonal movement are the same for a given breeding population in different years and that lateral movement on feeding grounds and pattern of mixing are similar between years. The assumptions ean be examined by comparing the mtDNA composition of humpback whales taken in different years in a same Area. A preliminary analysis of yearly variation was conducted for Area IV, the Area supported by the larger sample size. In this Area JARPA samples from six different years were involved. Both chi-square (P= 0.6950) and Kst* (0.431) tests failed to find signifieant genetic differences among years suggesting that whales from the same populations come to this Area in each year. Similar analyses are desirable for the other Areas.

In general these genetic results support the previous view based on non-genetic information that the Areas are occupied by different populations during the feeding season. In the absence of geographieal barriers, prey distribution, which depends on the oceanographic conditions, eould play a role in separating these populations in the feeding grounds. Our analysis based on samples from the feeding grounds ean not reject yet the possibility that different populations mix to each other in some particular longitudinal sectors. A more accurate longitudinal delineation of populations in the Antarctic feeding grounds and the identification of intermingle sectors can be aeeomplished by a comprehensive analysis that eombines DNA data available from low latitude breeding grounds and DNA data from high latitude feeding grounds.

ACKNOWLEDGEMENTS

We thank all the researehers and crew members of JARPA and IDCR/SOWER surveys involved in the sampling of the biopsy samples used in this study.

REFERENCES

Abe, H., Goto, M., Pastene, L.A., Dewa, K. and Naito, E. 2001. Practical use of multiplex fluoreseent PCR for cetaeean sex identification. *Marine Mammal Science* 17(3): 657-664.

Arnason, U., Gullberg, A. and Widegren, B. 1993. Cetacean mitochondrial DNA control region: sequences of all extant baleen whales and two sperm whale species. *Journal of Molecular Biology and Evolution* 70:960-970.

Baker, C.S., Perry, A., Bannister, J.L., Weinrich, M.T., Abernethy, R.B., Calambokidis, J., Lien, J., Lambertsen, R.H., Urbán, J., Vásquez, O., Clapham, P.J., Alling, A., O'Brien, S.J and Palumbi, S.R. 1993. Abundant mitochondrial DNA variation and world-wide population structure in humpback whales. *Proceedings Natural Academic Science* 90: 8239-8243.

Baker, C.S., Medrano-González, L., Calambokidis, J., Perry, A., Pichler, F., Rosenbaum, H., Straley, J.M., Urbán-Ramírez, J., Yamaguchi, M. and Von Ziegesar, O. 1998a. Population strueture of nuclear and mitochondrial DNA variation among humpback whales in the North Pacific. *Molecular Ecology* 7: 695-707.

Baker, C.S., Flores-Gonzalez, L., Abernethy, B., Rosenbaum, H.C., Slade, R.W., Capella, J. and Bannister, J.L. 1998b. Mitoehondrial DNA variation and maternal gene flow among humpback whales of the Southern Hemisphere. *Marine Mammal Science* 14: (4): 721-737.

Bérubé, M, Jorgensen, H., Mcewing, R., and Palsboll, P.J. 2000. Polymorphic di-nucleotide mierosatellite loci isolated from the humpback whale, *Megaptera novaeangliae*. *Molecular Ecology* 9: 2181-2183.

Darling, J.D. and MeSweeney, D.J. 1985. Observations on the migrations of North Pacific humpback whales (*Megaptera novaeangliae*). *Canadian Journal of Zoology* 63: 308-314.

Darling, J. and Mori, K. 1993. Recent observations of humpback whale (*Megaptera novaeangliae*) in Japanese waters off Ogasawara and Okinawa. *Canadian Journal of Zoology* 71: 325-333.

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

Gill, P. and Burton, C.L.K. 1995. Photographic re-sight of a humpback whale between western Australia and Antarctic Area IV. *Marine Mammal Science* 11: 96-100.

Goudet, J. 1995. FSTAT, version 1.2: a computer program to calculate F-statistics. *J. Hered.* 86: 485-486.

Hudson, R.R., Boos, D.D. and Kaplan, N.L. 1992. A statistical test for detecting geographic subdivision. *Molecular Biology and Evolution* 9:138-151.

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., Smultea, M.A. and Forestell, P.H. 1987. Use of lateral body pigmentation patterns for photographic identification of East Australian (Area V) humpback whales. *Cetus* 7: 5-1.

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

Mullis, K.B. and Faloona, F. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology* 155:335-350.

Nei, M. and Li, W.H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Science, USA 76:5269-5273.

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

Palsboll, P.J., P.J. Clapham, D.K. Mattila, F. Larsen, R. Sears, H.R. Siegismund, J. Sigurjonsson. O. Vásquez & P. Arctander. 1995. Distribution of mtDNA haplotypes in North Atlantic humpback whales: The influence of behavior on population structure. *Marine Ecology Progress Series* 116: 1-10.

Palsboll, P.J., J. Allen, M. Bérubé, P.J. Clapham, T.P. Feddersen, P. Hammond, H. Jorgensen, S. Katona, A.H. Larsen, F. Larsen, J. Lien, D.K. Mattila, J. Sigurjónsson, R. Sears, T. Smith, R. Sponer, P. Stevick & N. Oien. 1997a. Genetic tagging of humpback whales. Nature London 388: 767-769.

Palsboll, P.J., Bérubé, M., Larsen, A.H., and Jorgensen, H. 1997b. Primers for the amplication of tri-and tetramer microsatellite loci in baleen whales. *Molecular Ecology* 6: 893-895.

Pritchard, J.K., Stephens, M. and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945-959.

Raymond, M. and Rousset, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism . *J. Heredity* 86:248-249.

Rice, W.R. 1989. Analyzing tables of statistical tests. *Evolution* 43: 223-225.

Rock, J., Pastene, L.A., Kaufman, G., Forestell, P., Matsuoka, K. and Allen, J. (in press). A note on East Australia Group V stock humpback whale movement between feeding and breeding areas based on photo-identification. *Journal of.Cetacean.Research and.Management* (in press).

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

Sokal, R.R., and Rohlf, F.J. 1995. *Biometry: the Principles of Statistics in Biological Research*. Freeman and Company, New York.

Taylor, B.L. and Chivers, S.J. 1997. Anonymous alleles can bias results. The Society for Marine Mammology (Special Publication 3): 341-346.

Valsecchi, E., PalsbØll, P.J., Hale, P., Glockner-Ferrari, D.A., Ferrari, M., Clapham, P., Larsen, F., Mattila, D., Sears, R., Sigurjonsson, J., Brown, M., Corkeron, P. and Amos. B. 1997. Microsatelitte genetic distance between oceanic population of the humpback whale (*Megaptera novaeangliae*). *Molecular Biology Evolution* 14: 355-362.

Table 1: Number of samples of the humpback whale used in the present genetic study, by management Area and sex.

Area IIIE			Area IV			Area V			Area VI		
M	F	T	M	F	T	M	F	T	M	F	T
31	50	81	91	81	172	46	51	97	36	25	61

Table 2: Nucleotide diversity (upper figure) and nucleon diversity (lower figures) of humpback whales in the Antarctic feeding grounds, by management Area (n= number of individuals).

Area III (n= 81)	Area IV (n= 172)	Area V (n= 97)	Area VI (n= 61)
0.0246 (SE:0.0008)	0.0262 (SE:0.0006)	0.0274 (SE: 0.0008)	0.0268 (SE: 0.0009)
0.9688	0.9701	0.9712	0.9670

Table 3: Number of alleles (A), allelic richness (AR), expected heterozygosity (He), and test result for expected Hardy-Weinberg genotypie proportion (HW) (p-value) at six microsatellite loei for humpbaek whales in the Antarctic feeding grounds, by management Area (n= number of individuals)

All samples	Area III (n=81)				Area IV (n=172)				Area V (n=97)				Area VI (n=61)				All (n=411)		
	A	AR	He	HW	A	AR	He	HW	A	AR	He	HW	A	AR	He	HW	A	AR	He
GATA417	14	13.4	0.910	0.9880	12	12.0	0.906	0.5219	17	15.5	0.901	0.0254	14	14.0	0.917	0.0340	19	13.6	0.908
GATA28	10	9.7	0.477	0.8042	11	10.1	0.541	0.9245	12	11.6	0.632	0.5244	11	11.0	0.462	0.5894	14	11.4	0.528
GATA98	9	8.9	0.727	0.0909	13	11.1	0.794	0.1982	10	9.6	0.793	0.3135	8	8.0	0.732	0.6225	13	10.2	0.762
TAA31	14	13.2	0.874	0.4767	19	14.5	0.888	0.7771	15	14.4	0.906	0.7323	13	13.0	0.871	0.6365	24	14.5	0.885
GATA53	9	8.7	0.820	0.5894	11	9.4	0.830	0.6396	13	11.1	0.803	0.6028	10	10.0	0.837	0.9861	13	9.8	0.822
GT23	8	7.9	0.793	0.1704	9	8.3	0.797	0.1104	9	8.6	0.759	0.7344	9	9.0	0.802	0.7133	9	8.3	0.788
Avg.	10.7	10.3	0.767	0.5005	12.5	10.9	0.793	0.5722	12.7	11.8	0.799	0.3537	10.8	10.8	0.770	0.5829	15.3	11.3	0.782

Table 4: Results of the heterogeneity test comparing mtDNA haplotype frequencies of humpback whales, among Antarctic management Areas (sample sizes in parenthesis). An asterisk indicates those comparisons that resulted in significant statistically differences after Bonferroni corrections.

Males	Chi-square P-value	Kst*	Kst* P-value
III/IV (31, 91)	0.0040*	-0.0009	0.5830
III/V (31, 46)	0.0310	-0.0001	0.4470
III/VI (31, 36)	0.0300	0.0034	0.2180
IV/V (91, 46)	0.0000*	0.0049	0.0360
IV/VI (91, 36)	0.0500	0.0021	0.1890
V/VI (46, 36)	0.1570	-0.0002	0.4520

Females	Chi-square P-value	Kst*	Kst* P-value
III/IV (50, 81)	0.0000*	0.0058	0.0400
III/V (50, 51)	0.0000*	0.0083	0.0190
III/VI (50, 25)	0.0030*	0.0028	0.2280
IV/V (81, 51)	0.0000*	0.0049	0.0600
IV/VI (81, 25)	0.2910	-0.0027	0.8070
V/VI (51, 25)	0.7980	-0.0030	0.7380

Males+Females	Chi-square P-value	Kst*	Kst* P-value
III/IV (81, 172)	0.0000*	0.0019	0.0920
III/V (81, 97)	0.0000*	0.0071	0.0040*
III/VI (81, 61)	0.0000*	0.0065	0.0190
IV/V (172, 97)	0.0000*	0.0046	0.0040*
IV/VI (172, 61)	0.0020*	0.0012	0.1670
V/VI (97, 61)	0.0590	0.0008	0.2800

Table 5: Results of the heterogeneity test comparing allele frequencies of six microsatellite loci of humpback whales, among Antarctic management Areas (sample sizes in parenthesis). An asterisk indicates those comparisons that resulted in significant statistically differences after Bonferroni corrections.

Males	Six loci
All Areas	0.00369
III/IV (31, 91)	0.43737
III/V (31, 46)	0.04608
III/VI (31, 36)	0.23073
IV/V (91, 46)	0.00075*
IV/VI (91, 36)	0.02619
V/VI (46, 36)	0.01843

Females	Six loci
All Areas	0.00002
III/IV (50, 81)	0.01129*
III/V (50, 51)	0.00647*
III/VI (50, 25)	0.15472
IV/V (81, 51)	0.00621*
IV/VI (81, 25)	0.19756
V/VI (51, 25)	0.36140

Males+Females	Six loci
All Areas	High. Sig.
III/IV (81, 172)	0.00471*
III/V (81, 97)	0.00000*
III/VI (81, 61)	0.21129
IV/V (172, 97)	0.00000*
IV/VI (172, 61)	0.00427*
V/VI (97, 61)	0.09334

Table 6: Estimated posterior probability of number of populations (K) for the pooled humpback whale samples (n = 411) analyzed using six microsatellite loci and Program STRUCTURE.

K	Log P (x/k)	Probability
1	-9723.0	~1.0
2	-10081.0	~0.0
3	-11180.4	~0.0
4	-11575.1	~0.0
5	-11951.2	~0.0

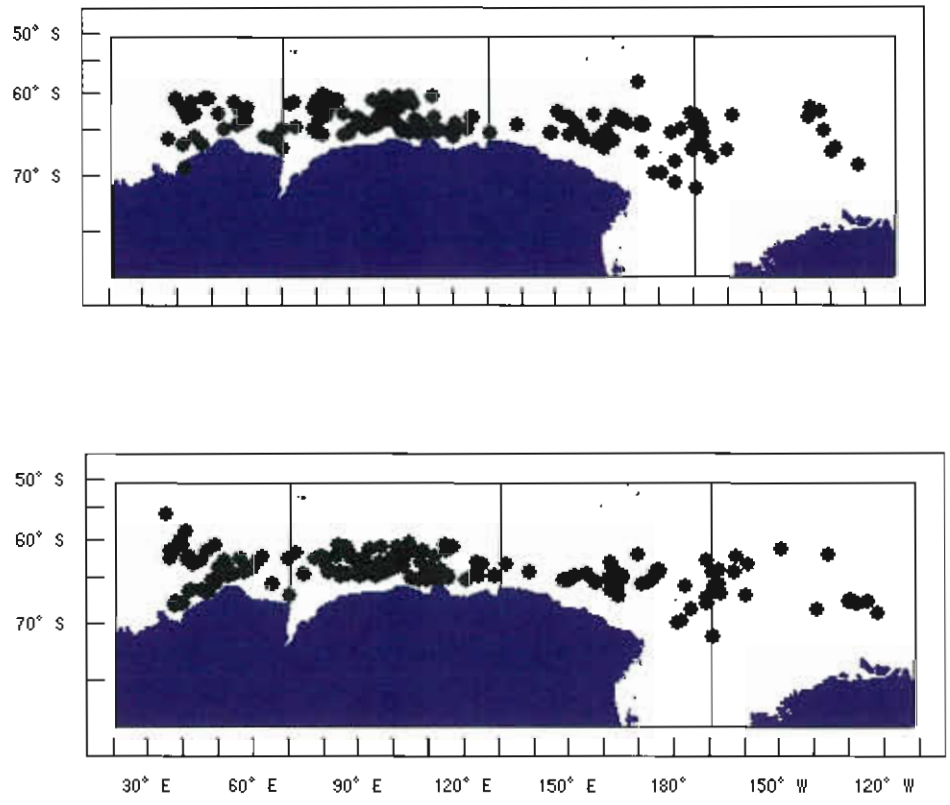

Figure 1: Geographical distribution of humpback whales examined in this study (upper figure: males; lower figure: female).