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# Genetic analyses on stock identification in the Antarctic humpback and fin whales based on samples collected under the JARPA

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**ABSTRACT**

Biopsy samples from 287 humpback and 23 fin whales obtained during surveys of the Japanese Whale Research Program under Special Permit in the Antarctic (JARPA) were analyzed for mtDNA and microsatellite (humpback whale only) variation. In the case of the humpback whale samples were grouped according the information of stock distribution in the feeding ground derived from the IWC/SC comprehensive assessment of the species. Analysis of mtDNA control region sequences discriminated clearly among Stocks C, D, E, F and G in the feeding ground. However analysis based on six microsatellite loci, while exhibiting some degree of genetic heterogeneity, was unable to discriminate among these stocks. Different degree of fidelity to breeding areas between females and males are suggested to explain such result. Analysis of mtDNA suggests that the historical sector of mixing between Stock D and E at 110-130°E is being occupied in recent years by the D stock. Regarding fin whales, level of mtDNA diversity found is high and similar to that in the Antarctic minke whale. No significant differences were found in the comparison between Areas III+IV and V, corresponding approximately to Indian and western South Pacific Oceans populations, respectively. However this result is attributed to the small number of samples used in the analysis

## INTRODUCTION

JARPA has a non-lethal research component involving sighting and oceanographic surveys, skin biopsy sampling and experiment on photo identification (photo-id). Skin biopsy sampling for genetic analysis and photo-id experiments for individual identification have been conducted on three baleen whale species: the humpback whale (*Megaptera novaeangliae*), the blue whale (*Balaenoptera musculus*) and the right whale (*Eubalaena australis*). In recent surveys, biopsy sampling in the fin whale (*Balaenoptera physalus*) has been conducted. This paper summarizes the genetic analyses conducted under JARPA on humpback and fin whales in the Antarctic.

### Background on Southern Hemisphere humpback whale

There is a considerable amount of information on the pattern of distribution and seasonal movement of humpback whales in the Southern Hemisphere. Most of the information is derived from the analysis of 'Discover' marks and catch distribution conducted in the past. Mackintosh (1965) showed that humpback whales tend to gather into five or six distinct feeding concentrations in the Antarctic during the austral summer season. He denominated these concentrations as Groups I-V (with a Group IIa and IIb). These concentrations correspond roughly to Management Areas I-VI.

Two of the areas of feeding concentrations in the Antarctic are Areas IV (70°-130°E) and V (130°E-170°W), where JARPA surveys are conducted. The geographic boundary between these two Areas was defined considering the distribution of catches and the results of mark-recapture analysis (Omura, 1953; Chittleborough, 1959).

Figure 1 shows the putative breeding grounds, feeding grounds and migratory corridors for Southern Hemisphere humpback whales as suggested by the IWC/SC's comprehensive assessment (IWC, 2001). These were based mainly on information on sighting and catch distribution and mark-recapture analysis. Seven breeding stocks are identified, labeled as A through G in Figure 1.

Genetic analyses in the Southern Hemisphere humpback whale have been based in the maternal-

inherited mtDNA. While mtDNA in breeding areas and migratory corridors demonstrated significant differences between Western Australia (Stock D), Eastern Australia (Stock E) and Colombia (Stock G) (Baker *et al*., 1998), little is known on the genetic population structure in the feeding ground.

Making use of biopsy samples collected from Areas IIIE, IV, V and VIW by the JARPA, we conducted a genetic analysis based on the maternal-inherited mtDNA and bi-parental inherited nuclear DNA (microsatellites) to test for genetic heterogeneity in the feeding ground. Of particular interest was to test the feeding areas proposed in Figure 1 for Stocks C, D, E, F and G.

**Background on Southern Hemisphere fin whale**

Little information is currently available on the stock structure of this species in the Antarctic. As in the case of the blue whale, earlier mark-recapture analysis showed that most whales return to the same part of the Antarctic year after year (Brown, 1954). Subsequent mark-recapture studies conducted by Brown (1962) suggested that the six whaling areas are probably more valid for blue and humpback whales than for fin whales (see also Mackintosh, 1965). The past information suggested there was certain segregation in the feeding ground between certain longitudes in four sectors which lie: South of the Atlantic Ocean, South of the Indian Ocean, South of Western South Pacific Ocean and South of Eastern South Pacific Ocean (Mackintosh, 1965). South of the Indian Ocean correspond approximately to JARPA Areas IIIE and IV and South of Western South Pacific to JARPA Areas VIW and V.

A previous study based on mtDNA used biopsy samples from JARPA to investigate the phylogenetic relationships of fin whales from different oceanic basins (Goto *et al*., 2003). Although one of the clade in the tree included all the fin whales from the Antarctic, such clade also included fin whales from North Pacific and North Atlantic (Figure 2) suggesting little time for fin whales inhabiting different oceans since their divergence to accumulate own unique haplotypes.

No studies based on DNA have been conducted to investigate stock structure of fin whales in the Southern Hemisphere. In this study we conducted a preliminary mtDNA analysis using biopsy samples obtained from JARPA to test for the oceanic division in the Antarctic suggested by Mackintosh (1965).

## MATERIALS AND METHOD

**Samples**

Biopsy samples were obtained along the sighting surveys of the JARPA, on an opportunistic basis. These have been collected using an air gun described in Kasamatsu *et al*. (1991) a more recently using Paxarm. Table 1 shows the number of biopsy samples of humpback and fin whales collected by JARPA, by survey and Area. All biopsy samples are checked for the possibility of re-sampling by using a set of microsatellite. For that reason the number of samples used in the analysis is less than those listed in Table 1. When cow-calf pairs were sampled, only one of them was used in the analysis.

**Grouping of samples**

*Humpback whale*

Samples were grouped primarily according the distribution of stocks in the feeding ground as shown in Figure 1: Stock C (35-55°E, n=34, 9 for mtDNA and microsatellite, respectively), Stock D (80-110°E, n=79, 62), Stock E (130°E-170°W, n=64, 54) and Stock F (170-145°W, n=36, 36). For comparison purpose, a sample from Stock G (n= 11) was used.

Two other additional sectors were defined in the Antarctic: 55°-80°E (n= 29) for which no previous information is available and 110°-130°E (n= 34), which has been regarded as a mixing sector between Stocks D and E stocks (Chittleborough, 1959; Dawbin, 1966).

*Fin whale*

Samples were grouped into two sectors in the Antarctic: 35°-130°E (corresponding to the Indian Ocean group) and 130°E-170°W (corresponding to the western South Pacific group). Sample sizes are small, n= 8 and n= 15, for these two sectors, respectively.

**Biochemical analysis**

Genomic DNA was extracted from approximately 0.05g of the outer epidermal layer of the skin biopsy. For extracting genomic DNA, we used established protocols (Sambrook *et al*, 1989). Laboratory

procedure for mtDNA control region sequencing and microsatellites was described in Pastene *et al*. (2000). Sex determination was made using the method of Abe *et al*. (2001).

In the case of the humpback whale a segment of 323bp of the mtDNA control region was examined. The nuclear DNA analysis involved six microsatellite loci (GATA417, GATA28, GATA98, TAA31, GATA53 and GT23).

In the case of fin whales only mtDNA analysis was conducted. In this case a segment of 298bp of the mtDNA control region was examined.

**Statistical analysis**

*mtDNA*

Genetic distances among haplotypes were estimated using Kimura's two parameters method (Kimura, 1980). The degree of mtDNA diversity within stock or longitudinal sectors was estimated using the nucleotide diversity.

Following recommendations from the IWC/Scientific Committee, heterogeneity tests were conducted using the randomized chi-square test (Roff and Bentzen, 1989) with a total of 10,000 permutations in each test. A P-value smaller than 0.05 was used as a criterion to reject the null hypothesis. Heterogeneity tests were conducted to investigate differences between sexes within and among stocks.

*Microsatellites (humpback whale only)*

All the statistical tests were conducted using the computer program GENEPOP (Raymond and Rousset, 1995). Statistical tests were conducted to investigate differences between sexes within stock, test for the deviation from Hardy-Weinberg genotypic proportion in the stocks and to investigate differences among stocks. Decision of statistically significance on hypothesis testing was made using the Fisher Exact Test obtained from summing the negative logarithm of P-values over the total loci (Fisher, 1950).

**RESULTS**

**Humpback whale**

*mtDNA*

A total of 64 polymorphic sites defined 82 haplotypes in the total sample of 287 whales. Nucleotide diversity for the total sample was high (0.0272). Among the stocks C-G it ranged from 0.0238 to 0.0283 (Table 2).

Table 3 shows the results of the heterogeneity test for sex differences in each stock. No significant differences were found between sexes. Male and female samples were pooled in the subsequent analysis.

Table 4 shows the results of pair-wise statistical comparison among stocks. The total P-value was highly significant (P= 0.0001). Apart from the comparison between C and G, all the comparisons resulted in significant differences.

Table 5a shows the results of the statistical comparison between Stocks C, D and sector 55°-80°E. Results suggest that whales in that particular sector are similar to Stock C and significantly different from Stock D. When the comparison is made at a more fine scale, sector 55°-70°E is similar to Stock C and different from Stock D. Sector 70°-80°E is similar to both stocks.

Table 5b shows the results of the statistical comparison between Stocks D, E and mixed assemblage sector 110°-130°E. Results suggest that whales in that particular sector are similar to Stock D and significantly different from Stock E.

*Microsatellite*

Table 6 shows the number of alleles and expected heterozygosity by locus. The average number of alleles per locus ranged from 7.4 to 12.6. Expected heterozygosity ranged from 0.511 to 0.877.

None of the heterogeneity tests conducted to investigate differences between sexes resulted in significant P-values (Table 7). Male and females samples were pooled in subsequent analyses.

Table 8 shows the results of the test for deviation from Hardy-Weinberg expectation in five stocks. None significant deviation from expectation was observed.

Table 9 shows the results of the heterogeneity test among stocks. The result for all loci and all stocks showed a significant P-value (0.042). Pair-wise comparisons showed little evidence of separation with only one comparison showing a P-value smaller than 0.05 (Stocks E/F).

**Fin whale**
mtDNA
A total of 16 polymorphic sites identified a total of 20 haplotypes in the total sample of 23 whales (Table 10). Nucleotide diversity estimate for the total sample was 0.0121 and no significant differences were found in the comparison between Area IIIE+IV and Area V, due probably to the small sample size (Table 11).

**DISCUSSION**

**Humpback whale**
Analysis of mtDNA provided support for the pattern of distribution of stocks C, D, E, F and G in the Antarctic suggested by Figure 1. This result is important because it suggest consistency between the non-genetic analyses conducted in the past and the mtDNA analysis conducted in this study. These stocks seem to occupy specific longitudinal sectors in the Antarctic. This result contrasts with that obtained using microsatellites. While some degree of heterogeneity was observed for nuclear DNA, discrimination among stocks in the Antarctic feeding ground was much less evident using this marker than in the case of mtDNA. The fact that microsatellites was unable to discriminate clearly among stocks in the Antarctic does not deny the existence the multiple stocks. It is possible that female humpback whale exhibit a strong fidelity to breeding areas than males. Genetic differences among stocks will not be evident at the nuclear DNA if a few reproductive males interchange and breed in different breeding areas.

Because mtDNA discriminated clearly among stocks, this marker was used to investigate Antarctic sectors for which no information was previously available or sector where mixing of stocks was suspected. Results of this analysis suggest that Stock C occupy sector 55°-70°E while sector 70°-80°E is probably occupied by both C and D stocks.

Pattern of mixing between D and E in sector 110°-130°E seems to have changed with time. Early mark-recapture analyses suggested that the sector between 110-130°E was occupied mainly by the E stock (Chittleborough, 1959; Dawbin, 1966). Our mtDNA analysis suggests that this sector is occupied mainly by the D stock. An additional analysis was conducted to investigate change in the proportion of D stock in this sector with time (Table 12). This table shows the contribution of Stock D to this mixing area, by JARPA survey. Although the standard deviations of the estimates are large due to small sample size, proportion of this stock in the mixed area seems to have increased with time. This is consistent with the information that Stock D has increased its abundance in recent years.

The relation between Stocks C, D and sector 55-80°E and the pattern of mixing between Stocks D and E (between 110° and 130°E) should be further investigated using a larger number of samples and analyses should be conducted by year and for each sex. Changes in geographical distribution of the stocks are possible as a response to the increase in abundance. These changes should be investigated in the future.

**Fin whales**
Genetic diversity, as measured by mtDNA, was examined for the first time for fin whales in the Antarctic. Level found is higher and similar to that found for Antarctic minke whale.

Our heterogeneity test comparing Areas IIIE+IV and V (assumed to belong to Indian and western South Pacific Ocean Populations, respectively) provided no evidence of structure. However, this result is not surprising given the small number of samples used in the analysis. Probably this result reflects the low power of the analysis.

Samples sizes available for analysis on stock structure are small and there is a need to collect more samples for this purpose. Experiments of satellite tracking would provide valuable information on

movement of fin whales in the feeding ground as well movement between feeding grounds and lower latitudes.

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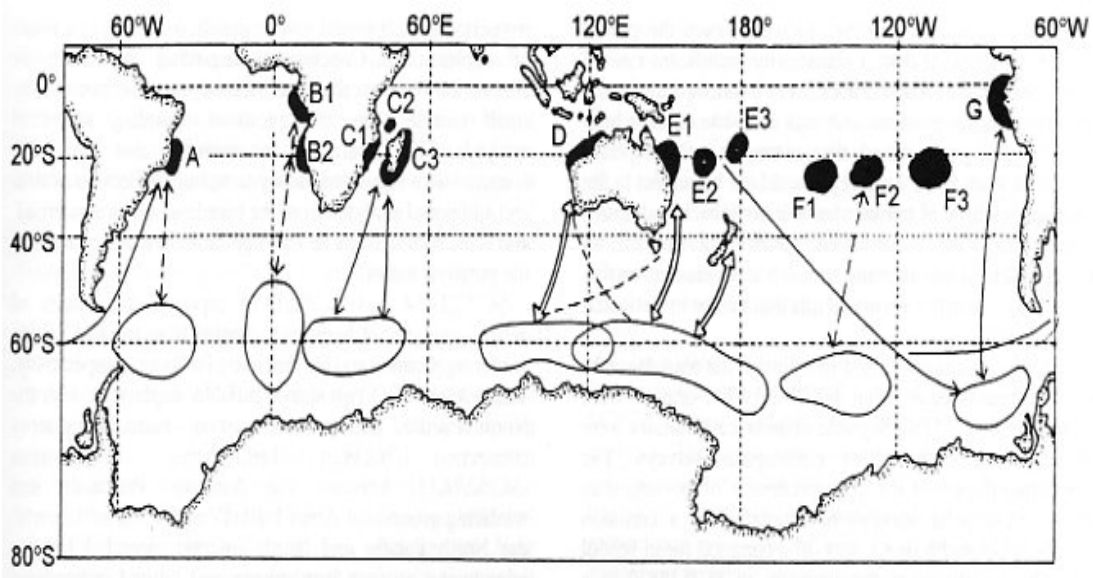

Figure 1: Breeding areas, migratory corridors and feeding areas of southern humpback whale stocks according the IWC/SC comprehensive assessment (after IWC, 2001)

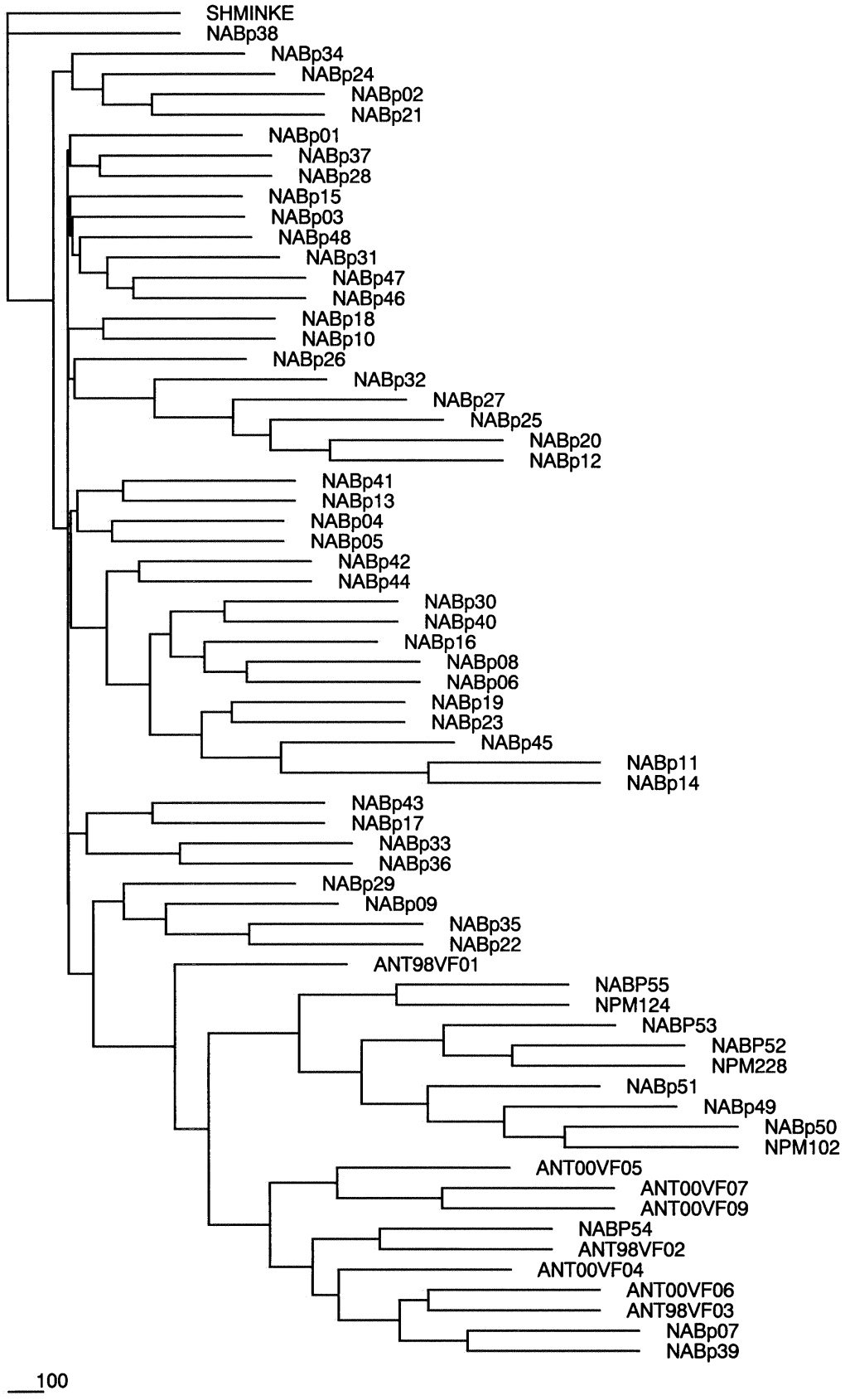


Figure 2: Neighbor-Joining-based tree of mtDNA haplotypes in the fin whale worldwide. Antarctic samples are from the JARPA surveys (After Goto *et al.*, 2003)

Table 1: Summary of biopsy samples of humpback and fin whales taken during JARPA surveys, by IWC Area.

| Area | Humpback whale | Fin whale |
|---|---|---|
| | | |
| IIIE | 56 | 4 |
| IV | 134 | 4 |
| V | 75 | 18 |
| VIW | 38 | 0 |
| TOTAL | 303 | 26 |

Table 2: Estimates of nucleotide diversity in Antarctic humpback whale stocks.

| C | D | E | F | G | Total |
|---|---|---|---|---|---|
| 0.0267 (0.0011) | 0.0268 (0.0007) | 0.0276 (0.0011) | 0.0283 (0.0013) | 0.0238 (0.0040) | 0.0272 (0.0005) |

Table 3: Statistical comparison of mtDNA haplotype frequencies between sexes using randomized chi-square test (P values derived from 10,000 simulations).

| C (F:22 M:12) | D (F:32 M47) | E (F:35 M:29) | F (F:14 M:22) | G (F:7 M:4) |
|---|---|---|---|---|
| 0.9157 | 0.2675 | 0.4466 | 0.9490 | 0.6711 |

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Table 4: Results of statistical comparisons of mtDNA haplotype frequencies among Stocks C, D, E and F in the Antarctic using randomized chi-square test (P values derived from 10,000 simulations). The total P-value was 0.0001. In parenthesis is the sample sizes.

| | C (34) | D (79) | E (64) | F (36) | G (11) |
|---|---|---|---|---|---|
| C | - | **0.0046** | **0.0001** | **0.0004** | 0.0724 |
| D | | - | **0.0001** | **0.0001** | **0.0009** |
| E | | | - | **0.0237** | **0.0024** |
| F | | | | - | **0.0130** |
| G | | | | | - |

Table 5a: Results of statistical comparisons of mtDNA haplotype frequencies between Stock C and sector 55-80°E and between D and sector 55-80°E using randomized chi-square test (P values derived from 10,000 simulations). In parenthesis is the sample size.

| | 55-70E (16) | 70-80E (13) | 55-80E (29) |
|---|---|---|---|
| C (34) | 0.5715 | 0.9576 | 0.9301 |
| D (79) | **0.0034** | 0.1718 | **0.0125** |

Table 5b: Results of statistical comparisons of mtDNA haplotype frequencies between Stock D and 110-130°E sector and between E and 110-130°E sector using randomized chi-square test (P values derived from 10,000 simulations). In parenthesis is the sample size.

| | 110-120E (20) | 120-130E (14) | 110-130E (34) |
|---|---|---|---|
| D (79) | 0.1163 | 0.5814 | 0.4231 |
| E (64) | **0.0480** | **0.0155** | **0.0210** |

Table 6: Number of samples (N), alleles (A) and expected heterozygosity (He) at seven microsatellite loci analyzed in Antarctic humpback whale.

| | Microsatellites | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | GATA417 | | | GATA28 | | | GATA98 | | | TAA31 | | | GATA53 | | | GT23 | | |
| Stock | N | A | He | N | A | He | N | A | He | N | A | He | N | A | He | N | A | He |
| C | 9 | 8 | 0.839 | 9 | 6 | 0.530 | 9 | 6 | 0.641 | 9 | 10 | 0.864 | 9 | 6 | 0.778 | 9 | 7 | 0.808 |
| D | 62 | 12 | 0.903 | 62 | 11 | 0.539 | 62 | 13 | 0.804 | 62 | 18 | 0.884 | 62 | 8 | 0.811 | 62 | 9 | 0.780 |
| E | 54 | 14 | 0.883 | 54 | 11 | 0.630 | 54 | 9 | 0.759 | 54 | 16 | 0.898 | 54 | 12 | 0.804 | 54 | 9 | 0.765 |
| F | 36 | 14 | 0.910 | 36 | 10 | 0.407 | 36 | 8 | 0.714 | 36 | 11 | 0.858 | 36 | 8 | 0.825 | 36 | 7 | 0.768 |
| G | 11 | 9 | 0.851 | 11 | 5 | 0.447 | 11 | 7 | 0.802 | 11 | 8 | 0.851 | 11 | 6 | 0.736 | 11 | 5 | 0.690 |
| All | 172 | 11.4 | 0.877 | 172 | 8.6 | 0.511 | 172 | 8.6 | 0.744 | 172 | 12.6 | 0.871 | 172 | 8.0 | 0.791 | 172 | 7.4 | 0.762 |

Table 7: Results of the heterogeneity test between sexes in five stocks of humpback whale in the Antarctic (figures shown are P-values).

| Locus | C | D | E | F | G |
|---|---|---|---|---|---|
| GATA417 | 0.415 | 0.507 | 0.066 | 0.162 | 0.989 |
| GATA28 | 0.650 | 0.284 | 0.624 | 0.340 | 0.202 |
| GATA98 | 1.000 | 0.073 | 0.437 | 0.635 | 0.625 |
| TAA31 | 0.285 | 0.616 | 0.347 | 0.345 | 0.800 |
| GATA53 | 0.859 | 0.131 | 0.436 | 0.526 | 0.846 |
| GT23 | 0.579 | 0.785 | 0.661 | 0.323 | 0.134 |
| All loci | 0.887 | 0.263 | 0.396 | 0.416 | 0.707 |

Table 8: Results of the test for deviation from Hardy-Weinberg expectation in five humpback whale stocks in the Antarctic (figures shown are P-values).

| | C | D | E | F | G |
|---|---|---|---|---|---|
| GATA417 | 0.912 | 0.460 | 0.564 | 0.253 | 0.969 |
| GATA28 | 1.000 | 0.440 | 0.267 | 0.460 | 0.613 |
| GATA98 | 0.869 | 0.155 | 0.350 | 0.647 | 0.873 |
| TAA31 | 0.082 | 0.685 | 0.273 | 0.261 | 0.963 |
| GATA53 | 0.793 | 0.340 | 0.643 | 0.781 | 0.685 |
| GT23 | 0.992 | 0.816 | 0.368 | 0.984 | 0.053 |
| All loci | 0.919 | 0.594 | 0.497 | 0.754 | 0.783 |

Table 9: Results of the heterogeneity test among humpback whale stocks in the Antarctic (Figures shown are P-values).

| | | Locus | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | GATA417 | GATA28 | GATA98 | TAA31 | GATA53 | GT23 | All loci |
| All stocks | | 0.005 | 0.262 | 0.401 | 0.251 | 0.330 | 0.478 | **0.042** |
| C | D | 0.217 | 0.477 | 0.407 | 0.387 | 0.287 | 0.365 | 0.388 |
| C | E | 0.025 | 0.238 | 0.366 | 0.336 | 0.672 | 0.202 | 0.104 |
| C | F | 0.408 | 0.270 | 0.522 | 0.654 | 0.561 | 0.413 | 0.661 |
| C | G | 0.159 | 0.468 | 0.205 | 0.804 | 0.561 | 0.205 | 0.359 |
| D | E | 0.020 | 0.176 | 0.562 | 0.179 | 0.705 | 0.662 | 0.133 |
| D | F | 0.666 | 0.416 | 0.513 | 0.639 | 0.043 | 0.855 | 0.493 |
| D | G | 0.012 | 0.545 | 0.328 | 0.247 | 0.627 | 0.482 | 0.131 |
| E | F | 0.101 | 0.181 | 0.379 | 0.053 | 0.068 | 0.911 | **0.045** |
| E | G | 0.013 | 0.666 | 0.317 | 0.470 | 0.703 | 0.074 | 0.084 |
| F | G | 0.012 | 0.447 | 0.290 | 0.632 | 0.462 | 0.129 | 0.078 |

Table 10: Variable sites defining 20 mtDNA haplotypes in the Antarctic fin whale. The column on the left is haplotype ID. The number above lists the nucleotide position of the polymorphic sites starting from the 5' end of the mtDNA control region. Haplotypes '2' through '20' are listed with reference to haplotype '1'. A dot indicates an identical nucleotide at the position relative to haplotype '1'.

```
                  10

           1222 222222
  668890015 556888
 6087874144 696347

 1     ACACTTTCTA AGCTTT
 2     ..GT...... G.....
 3     ..G......G ......
 4     .TGT..C... ......
 5     .TGT...T.. ....CC
 6     ..GT....CG ......
 7     ..GT...T.. ....CC
 8     .TGT...T.. ..T...
 9     .TG....... ......
10     .TG....T.. ......
11     ..GTC..... ....C.
12     ..G....T.. .A....
13     ..G....T.. ......
14     ..GT...T.. ......
15     ...T...T.. ......
16     .TG....... .A....
17     .TGT...... ......
18     ..GT...TCG ...C..
19     .TG..CC... .....C
20     -..T...T.. ......
```

Table 11: Estimates of nucleotide diversity in Antarctic fin whale and result of the heterogeneity test comparing Areas IIIE+IV and V (column far right).

| Area IIIE+IV (8) | Area V (15) | Total (23) | Area III+IV/Area V |
|---|---|---|---|
| 0.0133 (0.0025) | 0.0118 (0.0009) | 0.0121 (0.0009) | P=0.8932 |

Table 12: Yearly change in the proportion of humpback whale Stock D in sector 110-130°E, according mtDNA analysis.

| Sector/Survey | Proportion of Stock D (standard deviation) |
|---|---|
| 110-130°E (n=10, 93/94, 95/96) | 0.6015 (0.1865) |
| 110-130°E (n=11, 97/98, 99/00) | 0.6640 (0.2166) |
| 110-130°E (n=13, 01/02, 03/04) | 0.7591 (0.1172) |
| 110-130°E (n=34, total) | 0.7422 (0.0999) |