An update of the genetic study on stock structure of the Antarctic minke whale based on JARPAII samples

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ABSTRACT

Previous JARPA analyses on stock structure of the Antarctic minke whale based on mtDNA RFLP, microsatellite DNA and morphometric suggested significant differences between whales in the most eastern and western sectors of the research area, and less differentiation in the central sectors. Those results were consistent with the occurrence of at least two stocks and an area of mixing in the central sectors (involving mainly sector VW). The areal and temporal pattern of stocks mixing, which is one of the objectives of JARPAII, is studied in detail in a separated document (SC/F14/J29). In this study genetic samples obtained during surveys of the JARPAII (2005/06-2010/11) were examined using mtDNA control region sequencing and microsatellite DNA to test the previous hypothesis on stock structure derived from JARPA research. A total of 2,278 samples were considered in the mtDNA analysis (IIIE= 491; IVW= 669; IVE= 50; VW= 271; VE= 418 and VIW= 379), and 2,551 in the microsatellite analysis (IIIE= 564; IVW= 734; IVE= 74; VW= 297; VE= 478 and VIW= 404). The hypothesized mixing Area VW was not considered in the analysis. The statistical analysis of heterogeneity followed a step-wise fashion: first, for each annual survey Areas IIIE, IVW and IVE were compared in the western sector, and Areas VE and VIW were compared in the eastern sector. Next the yearly variation in the western and eastern sectors was investigated. Finally a comparison between western and eastern sectors of the JARPAII research area was conducted. This was done for female, males and both sexes combined. For mitochondrial and nuclear markers, significant statistical genetic differences were found between whales from the western and eastern sectors, for females, males and total samples in the case of the mtDNA, and for females and total samples in the case of microsatellites. Furthermore yearly variation was found for females and total samples, mainly in the western sector, in the case of the microsatellites. Therefore genetic results based on JARPAII samples were consistent with the previous hypothesis of at least two stocks in the research area, one in the most western part and the other in the most eastern part, which have been called previously as I (Eastern Indian Ocean) and P (Western South Pacific) stocks for convenience. Furthermore microsatellite analyses suggested substantial yearly variation mainly within the I stock and especially for females. These yearly differences can be explained by the dynamics of the I and P stocks, which mix with each other in different proportions in different years in part of the western sector (see also SC/F14/J29), or by the sporadic intrusion of an unknown third stock occurring in the western part of Area III.

KEYWORDS: ANTARCTIC, FEEDING GROUNDS, GENETICS, ANTARCTIC MINKE WHALE, SCIENTIFIC PERMITS

INTRODUCTION

Results of mtDNA RFLP, microsatellite DNA and morphometric analyses suggested significant differences between whales in the most eastern and western sectors of the JARPA research area, and less differentiation in the central sectors, especially Area VW (Pastene, 2006) (Figure 1). Based on those results, a hypothesis of at least two stocks and an area of mixing in the central sectors (VW) was proposed (IWC, 2008a). The location of the breeding grounds of these two stocks are assumed to be in the eastern Indian Ocean (western side of Australia), and western South Pacific (eastern side of Australia), respectively, based on sighting distribution patterns (Kasamatsu *et al*., 1995) (Figure 2). Based on such assumptions, these stocks are called I (eastern Indian Ocean) and P (western South Pacific) stocks, respectively. The Australian continent seems to act as a natural barrier preventing the gene flow between these two breeding stocks, similar as in the case of other baleen whale species such as the humpback whale.

The longitudinal extent of the mixing area and the yearly pattern of stocks mixing between the I and P stocks, which is one of the objectives of JARPAII, were studied by Kitakado *et al*. (2014) (SC/F14/J29).

New genetic samples were collected from Antarctic minke whales during recent JARPAII surveys. These samples were not used in the analyses that resulted in the stock structure hypothesis mentioned above. In this study we used JARPAII as an 'independent' set of genetic samples to test the pattern of population genetic structure found during the JARPA research, in particular to test whether the whales in the most western and eastern sectors are differentiated genetically. For this purpose we used both the maternal inherited mtDNA control region sequences (this genetic marker is used for the first time in the study of the stock structure of the Antarctic minke whale), and the bi-parental inherited microsatellite DNA.

All the previous mtDNA analyses to investigate stock structure in the Antarctic minke whale based on JARPA samples were based on RFLP. Only a limited number of samples from this species was examined previously using mtDNA control region sequencing in the context of a worldwide phylogenetic analysis of minke whales (Pastene *el al*., 2007), however the utility of such a technique has not been explored in the context of stock structure of this species in the Antarctic based on larger sample sizes. The mtDNA RFLP approach used was informative of the stock structure pattern of the Antarctic minke whale in the feeding grounds (Pastene *et al*., 2006), however the use of direct sequencing has additional advantages: it has a larger resolution, it allows direct comparability between studies, and it enables the use of most powerful analytical methods.

Microsatellite data on the other hand have been used previously based on the profiles of six loci. The number of loci in this study was increased to twelve. Microsatellite DNA data obtained in this study were also analyzed in the context of the recommendations offered during the JARPA review workshop (IWC, 2008a; IWC, 2008b).

MATERIALS AND METHOD

Samples

Genetic samples obtained during the JARPAII surveys in Areas IIIE, IVW, IVE, VW, VE and VIW between 2005/06 and 2010/11 were considered in the genetic analysis (Figure 3). A total of 2, 278 and 2,551 samples were considered in the mtDNA and microsatellite DNA analyses, respectively (Table 1). Samples from the assumed mixing area (Area VW) were not included in the statistical analysis. Genetic information from this sector was examined in detail by Kitakado *et al*. (2014) (SC/F14/J29).

Grouping of samples

The statistical analysis followed a step-wise fashion: first, for each annual survey Areas IIIE, IVW and IVE were compared in the western sector, and Areas VE and VIW were compared in the eastern sector. Next the yearly variation in the western and eastern sectors was investigated. Finally a comparison between western and eastern sectors of the JARPAII research area was conducted. This was done for females, males and both sexes combined.

Laboratory procedures

Genomic DNA was extracted from approximately 0.05g of the outer epidermal layer of the skin tissue using the protocol of Sambrook *et al*. (1989). Extracted DNAs were stored in TE buffer (10mM Tris-HCl, 1mM EDTA, ph8.0).

MtDNA

Sequencing analyses of a 338bp-segment of the control region of mitochondrial DNA (mtDNA) was conducted using the primers MT4 (Arnason *et al*. 1993) and Dlp 5R (*5′*-CCA TCG AGA TGT CTT ATT TAA GGG GAA C-*3′*). Reactions are carried out in 25 uL volumes containing 10-100ng of DNA, 2.5 pmole of each primer, 0.5 units of Ex Taq DNA polymerase (Takara), 2mM of each dNTP, and 10x reaction buffer. 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 are repeated 30 times. The amplification is completed with a final extension step of 10 minutes at 72°C. Subsequent cycle sequencing reactions are performed with 100ng of products generated in the above PCR amplifications using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems). 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 for 10 seconds at 96°C, 20 seconds at 56°C and four minutes at 60°C

are performed. The nucleotide sequence of each cycle sequencing reaction is determined using Applied Biosystems 3500 Genetic Analyzer (Life Technology) under standard conditions. Both strand samples are sequenced in their entirety for all samples.

Microsatellite DNA
Genetic variation at microsatellite loci were analyzed using 12 sets of primers: AC045, AC082, AC087, AC137, CA234, GT129 (Bérubé *et al*., 2005), DlrFCB14 (Buchanan *et al*., 1996), EV1, EV104 (Valsecchi and Amos, 1996), GT023, GT195, and GT211 (Bérubé *et al*., 2000). Although primer sequences followed those of the original authors, an annealing temperature of each of the loci was optimized for the species. PCR amplifications were performed in 15ml reaction mixtures containing 10-100ng of DNA, 5 pmole of each primer, 0.625 units of Ex Taq DNA polymerase (Takara), and 2mM of each dNTP, and 10x reaction buffer containing 20mM MgCl2 (Takara). Amplified products were run on a 6% polyacrylamide denaturating gel using a BaseStation 100 DNA fragment analyzer (Bio-Rad). Although alleles were visualized using Cartographer software specifically designed for the BaseStation, allelic sizes were determined manually in relation to the internal size standard (Genescan 400HD, Life technologies) and the Antarctic minke whale's microsatellites of known size from the 'control' individual that is rerun on each gel.

Statistical analysis
mtDNA
Genetic distances among unique sequences (haplotypes) were estimated using the Kimura's two parameters method (Kimura, 1980). The degree of mtDNA intra-population diversity was estimated using the nucleotide diversity and nucleon diversity (Nei, 1987).

Heterogeneity tests were conducted using the chi-square (Roff and Bentzen, 1989). 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 was observed.

Microsatellite DNA
For data consistency and plausibility, the computer program MICRO-CHECKER (van Oosterhout *et al*., 2004) was used to check for null alleles and reading/typing errors. The number of alleles per locus, allelic richness, and expected heterozygosity per locus was calculated using FSTAT 2.9.3 (Goudet, 1995). Statistical tests for the deviations from expected Hardy-Weinberg genotypic proportions were conducted using GENEPOP 4.0 (Rousset, 2008). Heterogeneity tests among samples were conducted using conventional hypothesis testing procedure. A probability test (or Fisher's exact test) implemented in GENEPOP 4.0 (Rousset, 2008) was used to conduct the heterogeneity tests. Statistical significance was determined using the chi-square value obtained from summing the negative logarithm of p-values over the 12 microsatellite loci (Sokal & Rohlf 1995). F_{ST} value was calculated using FSTAT 2.9.3.

RESULTS
MtDNA
Polymorphic sites and number of haplotypes
The final data set included the first 338 nucleotides of the mtDNA control region. In the total sample of 2,278 whales a total of 498 haplotypes was found. This number was derived from 103 segregating sites (90 transitions and 13 transversions).

mtDNA diversity
Table 2 shows the nucleotide and nucleon diversities for whales in both sectors of the research area. Both indices were high for both groups of whales.

Heterogeneity test
Results of the step wise statistical test are shown in Table 3. No significant differences were found among sectors in each of the surveys in the western and eastern sectors. No significant yearly differences were found in the two sectors. Finally significant differences were found between western and eastern sectors for females, males and both sexes combined. Differences were stronger for females.

Microsatellites
Diversity

Genetic diversity indices are shown in Table 4 for whales in the western and eastern sectors of the research area. The average number of alleles per loci, average allelic richness, and average expected heterozygosity, all over the 12 loci, was high for the both sectors, and the levels of these indexes were quite similar between the two sectors. Both sectors showed evidence of deviation from the expected Hardy-Weinberg genotypic proportions.

Heterogeneity test
Results of the step wise statistical tests are shown in Table 5. No significant differences were found among sectors in each of the surveys in the western and eastern sectors. Yearly differences were found in the two sectors, but mainly in the western sector and for females. Finally significant differences were found between western and eastern sectors for females and both sexes combined with very low F_{ST} values (less than 0.0001), but not for male animals. In the analysis of yearly variation all possible pairwise comparisons showed statistically significant differences in the I stock for both cases (female and both sexes combined), while a significant difference was detected only between 06/07 and 08/09 seasons in the P stock for both sexes combined. Because of quite strong yearly variation especially in the I stock (Table 5), we conducted heterogeneity tests by separating all of the six season samples as single samples to see any trends to the mixing patterns irrespective of the prior stock designations. Although a substantial difference was detected among the samples as expected, no trend was detected (data not shown).

JARPA review recommendations
During the JARPA review workshop some recommendations were made to conduct clustering analyses based on individual genotypes (IWC, 2008a). In response to these recommendations we used Bayesian clustering approach using STRUCTURE (Pritchard *et al*. 2000) to determine the number of genetically distinct populations present in our samples based on information on the individual genotypes. The simulation results detected no clustering in our samples probably due to a very low level of genetic differentiation even between the I and P stocks despite doubling the number of loci used.

DISCUSSION
The main objective of the present study was to test the stock structure hypothesis derived from JARPA analyses by using a set of new genetic samples obtained by JARPAII, and mtDNA control region sequencing and microsatellite DNA.

The genetic results from JARPAII confirmed the previous results from JARPA that the most extreme western and eastern sectors of the research area of JARPA and JARPAII are occupied by different stocks (I and P stocks, respectively). The microsatellite analysis found significant yearly variation mainly in the area occupied by the I stock (western), and especially for females. Yearly variation was not found in the case of the mtDNA despite the fact that some of the tests involved considerable sample sizes e.g. those involving Areas IIIE and IVW. It is possible that the large number of haplotypes represented by single individuals (singleton) decreased the power of the statistical analysis of yearly variation. Despite this the number of samples was large enough for detecting differences between the western and eastern sectors. Further analyses are required to investigate whether or not the absence of yearly differences in the mtDNA analysis was due to lack of statistical power.

An interpretation for the yearly differences in the microsatellite analysis is that both the I and P stocks distribute in part of Area IV in different proportions in different years. This is supported by the significant departure from the Hardy-Weinberg equilibrium found. Also this explanation matches well with the yearly changes in the distribution and mixing of stocks reported by Kitakado *et al* (2014) (SC/F14/J29). An alternative explanation is that the yearly differences are due to the temporal intrusion (and mix) of an additional unknown stock from the contiguous west sector of the research area (e.g. Area IIIW).

The significant yearly differences found by the microsatellite analyses could be due to a Type Error I. This is possible because the large sample size used and the sensitivity of the method (the number of loci was increased to twelve) could result in 'significant' p values which are not necessarily of biological significance. Significant microsatellite differences between the western and eastern sectors are consistent with the results of the mtDNA and therefore the hypothesis of at least two different stocks separated geographically is better supported.

If the explanation that the distribution and mixing of the stocks changes substantially by year is correct, then continuous research of the reasons for such changes will be necessary in the future. Stock distribution can change according to changes in krill distribution which in turn depends on changes in oceanographic conditions in the area.

Same as in the genetic analyses of humpback whales in the feeding grounds, the present genetic study on Antarctic minke whales showed more temporal and geographical differentiation in females than males supporting the view of a stronger fidelity of females to migratory destination than males.

Regarding the work conducted in response to the recommendations from the JARPA review meeting, the number of the microsatellite loci used was doubled from the previous six to current 12. Even with this substantial increase in the loci numbers, however, the level of genetic differentiation among the samples was very low. For instance, the F_{ST} values were less than 0.0001 between the I and P stocks for females and both sexes combined. Because of this low differentiation, the analyses conducted using STRUCTURE were not informative of the stock structure Antarctic minke whales.

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Table 1. Number of samples used in the genetic analysis: A= mtDNA, B= microsatellite DNA

A

	All areas			I stock									Mixing area			P stock					
				IIIE			IVW			IVE			VW			VE			VIW		
	FM	F	M	FM	F	M	FM	F	M	FM	F	M	FM	F	M	FM	F	M	FM	F	M
05/06	562	251	311	34	17	17	377	166	211	37	17	20	112	49	63	2	2	0			
06/07	466	319	147										0	0	0	287	205	82	179	114	65
07/08	535	271	264	224	75	149	212	135	77	13	3	10	86	58	28						
08/09	188	91	97										16	3	13	82	42	40	90	46	44
09/10	370	207	163	233	137	96	80	43	37	0	0	0	57	27	30						
10/11	157	98	59										0	0	0	47	20	27	110	78	32

B

	All areas			I stock									Mixing area			P stock					
				IIIE			IVW			IVE			VW			VE			VIW		
	FM	F	M	FM	F	M	FM	F	M	FM	F	M	FM	F	M	FM	F	M	FM	F	M
05/06	708	311	397	95	46	49	414	179	235	61	29	32	136	55	81						
06/07	502	349	153										0	0	0	320	232	88	182	117	65
07/08	551	278	273	229	79	150	222	138	84	13	3	10	87	58	29						
08/09	226	112	114										16	4	12	111	58	53	99	50	49
09/10	396	228	168	240	142	98	98	59	39	0	0	0	58	27	31						
10/11	170	108	62										0	0	0	47	20	27	123	88	35

Table 2. Mitochondrial DNA diversity indices for Antarctic minke whales from two stocks/sectors

Genetic index	Areas IIIE+IV (I stock)	Areas VE+VIW (P stock)
Nucleon diversity	0.9800	0.9752
Nucleotide diversity	0.0156	0.0148

Table 3. Results of the spatial and temporal statistical analysis of heterogeneity test (mtDNA sequencing). Figures shown are *p*-values.

	I stock			**P stock**		
	05/06	07/08	09/10	06/07	08/09	10/11
F+M						
Among sectors/season	0.4097	0.6488	0.0898	0.3777	0.6960	-
Among seasons/stock	0.3642			0.5057		
Between I and P	0.0001					
Female						
Among sectors/season	0.1246	0.6803	0.2728	0.5067	0.0510	-
Among seasons/stock	0.6301			0.1994		
Between I and P	0.0001					
Male						
Among sectors/season	0.5498	0.8285	0.6050	0.5807	0.8205	-
Among seasons/stock	0.7864			0.1686		
Between I and P	0.0085					

Table 4. Microsatellite diversity indices for Antarctic minke whales from two stocks/sectors

Genetic index	Areas IIIE+IV (I stock)	Areas VE+VIW (P stock)
No. alleles per locus	19.5	19.0
Allelic richness	18.8	19.0
Heterozygosity	0.854	0.858
Hardy-Weinberg	<0.001	0.0042

Table 5. Results of the spatial and temporal statistical analysis of heterogeneity test (microsatellite DNA). Statistical results are for all loci combined. ns= not significant.

	I stock			P stock		
	05/06	07/08	09/10	06/07	08/09	10/11
F+M						
Among sectors/season	0.034	Ns	ns	ns	ns	Ns
Among seasons/stock	0.0000838			0.004		
Between I and P	0.00769 (*Fst*= 0.0002)					
Female						
Among sectors/season	ns	Ns	ns	ns	ns	Ns
Among seasons/stock	0.000625			0.047		
Between I and P	0.0000559 (*Fst*= 0.0005)					
Male						
Among sectors/season	ns	Ns	ns	ns	ns	Ns
Among seasons/stock	Ns			0.00227		
Between I and P	ns					

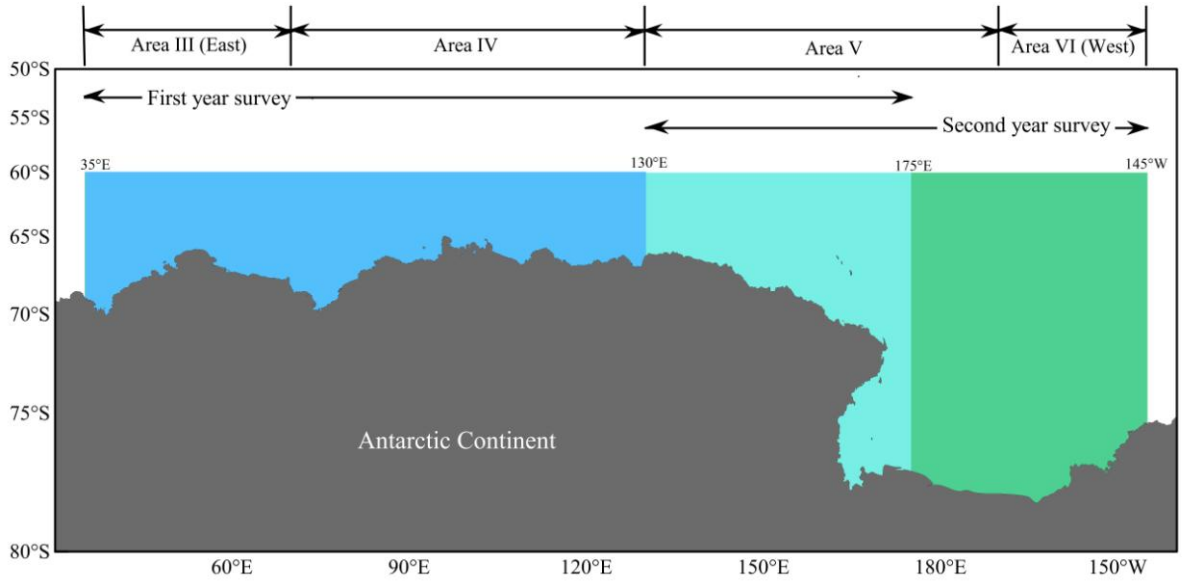

Figure 1. JARPAII research area. Whales in the 'blue' and 'green' sectors showed genetic and morphometric differentiation according to the JARPA research. It is assumed that those two sectors are occupied by different stocks (I and P stocks, respectively). The sector in the middle was proposed as an area of mixing. The longitudinal extension of the area of mixing and the yearly pattern of mixing of stocks were studied by Kitakado *et al*. (2014) (SC/F14/J29). In the present study genetic samples from JARPAII were used to test the hypothesis of genetic differentiation between whales from the extreme sectors. This was done using mtDNA sequences and microsatellite DNA.

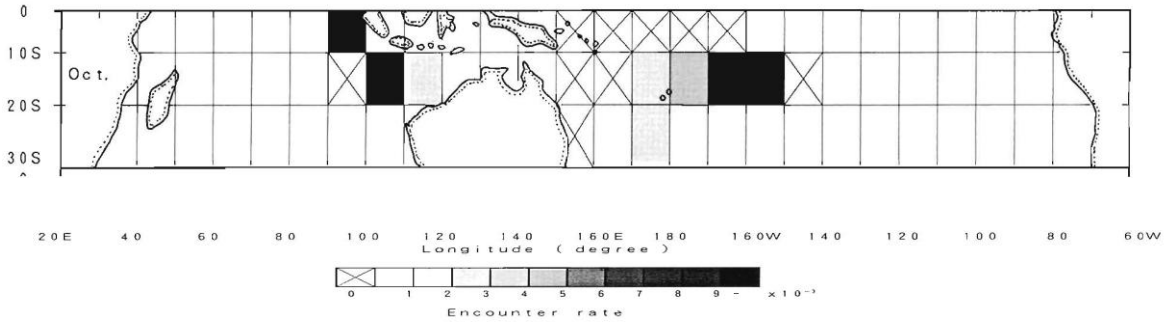

Figure 2: Encounter rates of Antarctic minke whales in 10º squares of latitude and longitude in waters 0º-30ºS in October (from Kasamatsu *et al*., 1995).

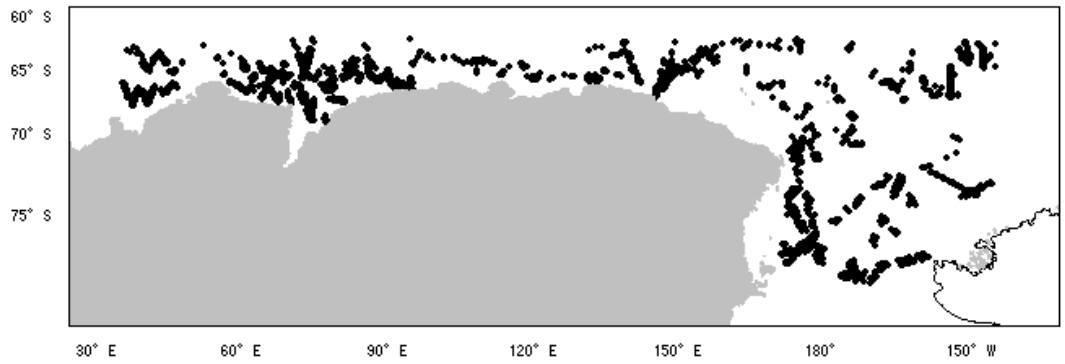

Figure 3. Geographical distribution of the Antarctic minke whales sampled by JARPAII between the 2005/06 and the 2010/11 austral summer season.