An update of the genetic study on stock structure of the Antarctic minke whale based on JARPAII samples (SC/65b/J28Rev)

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ABSTRACT

This paper is a revised version of paper SC/F14/J28, which took into consideration some recommendations from the JARPAII Review Workshop. The additional analyses conducted in respond to the recommendations did not change the main conclusions of SC/F14/J28. 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.

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 denaturing 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, as well as their F_{IS} values were calculated 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. In the case of the Areas IIIE+IV the significant departure from the Hardy-Weinberg equilibrium are due to the loci EV1 and CA234. Locus GT129 also presented a low p-value but it was not significant at the 5% level. The F_{IS} values for these loci were small figures, positive in the case of the latter two loci (which could indicate a homozygote excess) but negative in the case of locus EV1. In the case of Areas VE+VIW the departure was due to three loci, EV1, GT195 and CA234. Again the F_{IS} values were small figures, negative for locus EV1 and positive for the latter two loci.

Heterogeneity test
Results of the step wise statistical tests are shown in Table 5-7. 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. For female+male samples combined, the differences are due to the loci DlrFB14, GT23 and AC137. In the case of female samples, the differences are due mainly to loci DlrFB14, GT23 and AC045.

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.

In response to the recommendations from the JARPAII review workshop, Tables 4 (Hady-Weinberg and F_{IS} analyses) and 5-7 (heterogeneity test for microsatellites) were updated.

As noted above in the case of the Areas IIIE+IV the significant departure from the Hardy-Weinberg equilibrium are due to the loci EV1 and CA234. Locus GT129 also presented a low p-value but it was not significant at the 5% level. The F_{IS} values for these loci were small figures, positive in the case of the latter two loci (which could indicate a homozygote excess) but negative in the case of locus EV1. In the case of Areas VE+VIW the departure was due to three loci, EV1, GT195 and CA234. Again the F_{IS} values were small figures, negative for locus EV1 and positive for the latter two loci. The conclusion here is that the analysis of F_{IS} did not show any trend of pattern which could assist the interpretation of the significant deviation from the Hardy-Weinberg equilibrium found in both stocks.

The new information shown in Tables 5-7 clarified which of the loci were informative of the genetic differences found between the I and P stocks samples.

REFERENCES
Arnason, U., A. Gullberg, and B. Widegren. 1993. Cetacean mitochondrial DNA control region: sequences of all extant baleen whales and two sperm whale species. *J. Mol. Biol Evol*. 70: 960-970.
Bérubé, M., Jørgensen, H., Mcewing, R., and Palsbøll, P.J. 2000. Polymorphic di-nucleotide microsatellite loci isolated from the humpback whale, *Megaptera novaeanglliae. Mol. Ecol.* 9: 2181-2183.
Bérubé, M., Rew, M.B., Skaug, H., Jørgensen, H., Robbins, J., Best, P., Sears, R., and Palsbøll, P.J. 2005. Polymorphic microsatellite loci isolated from humpback whale, *Megaptera novaeanglliae* and fin whale, *balaenoptera physalus. Cons. Genet.* 6: 631-636.
Buchanan, F.C., Friesen, M.K., Littlejohn, R.P., and Clayton, J.A. 1996. Microsatellites from beluga whale *Delphinapterus leucas. Mol. Ecol.* 5: 571-575.
Goudet, J. 1995. FSTAT, version 1.2: a computer program to calculate F-statistics. *J. Hered.* 86: 485-486.

International Whaling Commission. 2008a. Report of the Intersessional Workshop to Review Data and Results from Special Permit Research on Minke Whales in the Antarctic, Tokyo, 4-8 December 2006. *J. Cetacean Res. Manage*. 10 (Suppl.):411-445.

International Whaling Commission. 2008b. Report of the Standing Working Group on Special Permits. Annex O. *J. Cetacean Res. Manage*. 10 (Suppl.): 341-350.

Kasamatsu, F., Nishiwaki, S. and Ishikawa, H. 1995. Breeding areas and southbound migrations of southern minke whales *Balaenoptera acutorostrata*. *Mar. Ecol. Prog. Ser*. 119:1-10.

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.

Kitakado, T., Schweder, T., Kanda, N., Pastene, L.A. and Walloe, L. 2014. Dynamic population segregation by genetics and morphometrics in Antarctic minke whales. Doc. SC/F14/J29 (this meeting).

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

Pastene, L.A., Goto M. and Kanda, N. 2006. Genetic analysis on stock structure in the Antarctic minke whales from the JARPA research area based on mitochondrial DNA and microsatellites. Paper SC/D06/J9 presented to the JARPA Review Meeting, December 2006 (unpublished). 22pp.

Pastene, L.A., Goto, M., Kanda, N., Zerbini, A.N., Kerem, D., Watanabe, K., Bessho, Y., Hasegawa, M., Nielsen, R., Larsen, F. and Palsbøll, P.J. 2007. Radiation and speciation of pelagic organisms during periods of global warming: the case of the common minke whale, *Balaenoptera acutorostrata*. *Molecular Ecology* 16: 1481-1495.

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

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

Rousset, F. 2008. Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Moc. Ecol. Resources* 8: 103-106.

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

Valsecchi, E., and Amos, W. 1996. Microsatellite markers for the study of cetacean populations. *Mol. Ecol.* 5: 151-156.

van Oosterhout, C., Hutchinson, W.F., Wills, D.P., and Shipley, P. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* 4:535-538.

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 richiness	18.8			19.0	
Heterozygosity	0.854			0.858	
Hardy-Weinberg / Fis					
EV1	0.0414	-0.0077		0.049	-0.0118
EV104	0.3651	-0.0052		0.2633	0.0045
GT211	0.3568	0.0277		0.1174	0.0247
DlrFB14	0.7559	-0.0088		0.2482	-0.0091
GT195	0.5711	-0.0052		0.0307	0.0052
GT23	0.5893	0.0055		0.8393	0.0136
AC045	0.2246	0.0249		0.1383	0.0266
AC082	0.8994	0.0302		0.5291	0.0265
AC087	0.1931	0.0036		0.6077	0.0059
AC137	0.2285	0.028		0.5547	0.0178
CA234	0	0.0725		0.0008	0.0748
GT129	0.0975	0.034		0.5046	0.0387
All	**<0.001**			0.004	

Table 5. Results of the spatial and temporal statistical analysis of heterogeneity test for male and female samples combined (microsatellite DNA) (left: p-value; right: Fst). .

	I stock						P stock					
	05/06		07/08		09/10		06/07		08/09		10/11	
F+M												
Among sectors within same season	0.034	0.0009	0.378	0.0006	0.31	0.0005	0.059	0.0003	0.328	0.0001	0.691	-0.0008
EV1	0.901	-0.0007	0.612	0.0007	0.039	0.0039	0.870	-0.0006	0.195	0.0039	0.381	-0.0022
EV104	0.949	-0.0019	0.060	0.0026	0.257	0.0006	0.344	0.0002	0.131	0.0013	0.925	-0.0039
GT211	0.422	0.0014	0.903	-0.0010	0.278	0.0011	0.284	-0.0001	0.275	-0.0001	0.757	-0.0004
DlrFB14	0.197	0.0092	0.800	-0.0021	0.770	0.0027	0.328	0.0008	0.151	0.0054	0.046	0.0334
GT195	0.059	0.0043	0.861	-0.0012	0.764	-0.0009	0.110	-0.0001	0.425	-0.0007	0.917	-0.0075
GT23	0.100	0.0014	0.843	-0.0006	0.045	-0.0007	0.194	0.0015	0.441	-0.0024	0.865	-0.0056
AC045	0.113	0.0025	0.153	0.0019	0.301	0.0019	0.489	-0.0001	0.953	-0.0043	0.238	0.0017
AC082	0.039	-0.0004	0.685	0.0002	0.648	-0.0005	0.357	0.0005	0.124	0.0010	0.822	-0.0046
AC087	0.634	-0.0011	0.054	0.0017	0.890	-0.0020	0.366	-0.0003	0.459	0.0021	0.770	0.0012
AC137	0.054	0.0013	0.334	0.0000	0.443	-0.0006	0.059	0.0010	0.283	0.0031	0.215	0.0012
CA234	0.131	-0.0006	0.122	0.0027	0.828	-0.0010	0.189	0.0001	0.688	-0.0030	0.138	0.0007
GT129	0.625	-0.0003	0.658	0.0006	0.309	0.0025	0.042	0.0007	0.835	-0.0027	0.992	-0.0083
Among seasons within a stock			0.0008	0.0001					0.004	0.0004		
EV1			0.917	-0.0002					0.478	-0.0001		
EV104			0.033	0.0011					0.447	0.0006		
GT211			0.216	-0.0003					0.021	0.0004		
DlrFB14			0.269	-0.0010					0.313	0.0013		
GT195			0.007	0.0011					0.192	0.0004		
GT23			0.205	0.0003					0.124	0.0002		
AC045			0.096	0.0003					0.903	-0.0008		
AC082			0.238	-0.0002					0.094	0.0009		
AC087			0.537	-0.0001					0.078	0.0005		
AC137			0.000	0.0002					0.011	0.0019		
CA234			0.046	-0.0002					0.386	-0.0005		
GT129			0.388	-0.0001					0.089	0.0004		
Between I and P					0.00769	0.0002						
EV1					0.227	-0.0001						
EV104					0.546	0.0000						
GT211					0.618	0.0001						
DlrFB14					0.001	0.0036						
GT195					0.411	0.0003						
GT23					0.017	0.0002						
AC045					0.250	0.0002						
AC082					0.242	-0.0002						
AC087					0.574	-0.0001						
AC137					0.041	0.0001						
CA234					0.441	-0.0001						
GT129					0.715	-0.0001						

Table 6. . Results of the spatial and temporal statistical analysis of heterogeneity test for females (microsatellite DNA) (left: p-value; right: Fst).

	I stock						P stock					
	05/06		07/08		09/10		06/07		08/09		10/11	
Female												
Among sectors within same season	0.214	0.0016	0.47	-0.0001	0.445	-0.0006	0.399	-0.0003	0.155	0.0021	0.922	-0.0022
EV1	0.890	-0.0047	0.802	-0.0031	0.224	0.0021	0.435	0.0010	0.004	0.0243	0.672	-0.0108
EV104	0.919	-0.0032	0.008	0.0061	0.115	0.0018	0.719	-0.0012	0.134	0.0095	0.926	-0.0103
GT211	0.411	0.0007	0.468	0.0006	0.634	-0.0005	0.111	0.0018	0.460	-0.0019	0.986	-0.0136
DlrFB14	0.342	0.0109	0.851	0.0011	0.856	-0.0014	0.468	0.0031	0.450	0.0029	0.060	0.0961
GT195	0.527	0.0036	0.942	-0.0039	0.358	-0.0027	0.561	-0.0032	0.854	-0.0034	0.764	-0.0048
GT23	0.479	-0.0003	0.733	-0.0005	0.086	-0.0024	0.148	0.0024	0.194	0.0033	0.587	-0.0050
AC045	0.257	0.0022	0.151	-0.0008	0.167	0.0027	0.311	0.0008	0.818	-0.0051	0.788	-0.0060
AC082	0.130	0.0000	0.868	-0.0015	0.541	-0.0004	0.522	-0.0005	0.231	0.0006	0.999	-0.0185
AC087	0.238	0.0099	0.137	0.0025	0.801	-0.0008	0.522	-0.0016	0.586	0.0036	0.407	0.0040
AC137	0.011	0.0026	0.685	-0.0012	0.232	-0.0004	0.565	-0.0014	0.925	-0.0018	0.222	0.0051
CA234	0.216	0.0044	0.662	0.0004	0.892	-0.0032	0.342	-0.0025	0.294	-0.0041	0.483	-0.0029
GT129	0.814	-0.0021	0.480	-0.0008	0.822	-0.0023	0.158	-0.0007	0.376	-0.0004	1.000	-0.0190
Among seasons within a stock			0.0006	0.0005					0.047	0.0002		
EV1			0.559	0.0004					0.441	0.0002		
EV104			0.203	0.0015					0.845	-0.0007		
GT211			0.233	0.0000					0.266	-0.0009		
DlrFB14			0.457	-0.0004					0.232	0.0033		
GT195			0.338	0.0011					0.013	0.0032		
GT23			0.359	0.0004					0.221	0.0004		
AC045			0.001	0.0028					0.754	-0.0014		
AC082			0.509	-0.0008					0.184	-0.0004		
AC087			0.292	-0.0001					0.164	0.0011		
AC137			0.003	0.0002					0.147	0.0014		
CA234			0.015	0.0006					0.188	-0.0012		
GT129			0.331	0.0002					0.265	-0.0008		
Between I and P					0.0000559	0.0005						
EV1					0.128	0.0001						
EV104					0.693	-0.0002						
GT211					0.903	-0.0003						
DlrFB14					0.005	0.0047						
GT195					0.275	0.0005						
GT23					0.001	0.0006						
AC045					0.016	0.0008						
AC082					0.194	-0.0002						
AC087					0.079	0.0004						
AC137					0.090	0.0002						
CA234					0.184	0.0007						
GT129					0.220	0.0003						

Table 7. Results of the spatial and temporal statistical analysis of heterogeneity test for males (microsatellite DNA) (left: p-value; right: Fst)

|	I stock						P stock					
	05/06		07/08		09/10		06/07		08/09		10/11	
Male												
Among sectors within same season	0.375	0.0010	0.319	0.0004	0.431	0.0021	0.620	0.0007	0.295	-0.0031	0.394	-0.0018
EV1	0.514	0.0023	0.839	-0.0025	0.366	0.0036	0.309	0.0023	0.663	-0.0068	0.352	0.0030
EV104	0.622	0.0003	0.029	0.0055	0.148	0.0035	0.761	-0.0030	0.050	0.0051	0.324	-0.0017
GT211	0.557	0.0032	0.972	-0.0032	0.216	0.0025	0.700	-0.0028	0.086	0.0052	0.489	0.0057
DlrFB14	0.126	0.0156	0.807	-0.0076	0.830	0.0001	0.836	-0.0045	0.149	0.0008	0.289	-0.0189
GT195	0.047	0.0035	0.674	0.0003	0.223	0.0082	0.152	0.0046	0.364	-0.0019	0.600	-0.0090
GT23	0.138	0.0014	0.623	-0.0007	0.080	0.0013	0.212	0.0016	0.929	-0.0114	0.985	-0.0161
AC045	0.322	0.0019	0.139	0.0050	0.811	-0.0014	0.732	-0.0027	0.976	-0.0108	0.092	0.0093
AC082	0.345	-0.0021	0.311	0.0007	0.789	-0.0022	0.536	-0.0011	0.568	-0.0038	0.399	-0.0014
AC087	0.355	0.0019	0.661	-0.0033	0.876	0.0004	0.659	-0.0023	0.748	-0.0062	0.798	-0.0094
AC137	0.650	-0.0014	0.261	0.0014	0.795	-0.0031	0.296	0.0026	0.066	0.0048	0.220	0.0052
CA234	0.764	-0.0038	0.119	0.0018	0.713	-0.0011	0.251	0.0028	0.397	-0.0030	0.065	0.0108
GT129	0.974	-0.0027	0.225	0.0025	0.085	0.0131	0.274	0.0089	0.787	-0.0084	0.835	-0.0091
Among seasons within a stock			0.355	-0.0002					0.0023	0.0010		
EV1			0.923	-0.0011					0.521	-0.0010		
EV104			0.584	-0.0001					0.779	0.0001		
GT211			0.506	-0.0008					0.122	0.0007		
DlrFB14			0.287	0.0022					0.097	-0.0041		
GT195			0.023	0.0004					0.672	-0.0011		
GT23			0.332	0.0008					0.038	0.0031		
AC045			0.649	-0.0011					0.741	-0.0010		
AC082			0.330	-0.0004					0.087	0.0043		
AC087			0.758	-0.0005					0.025	0.0048		
AC137			0.359	-0.0002					0.011	0.0029		
CA234			0.180	-0.0005					0.813	-0.0021		
GT129			0.374	-0.0004					0.018	0.0028		
Between I and P					0.434	0.0000						
EV1					0.946	-0.0007						
EV104					0.529	0.0003						
GT211					0.043	0.0011						
DlrFB14					0.378	0.0012						
GT195					0.460	0.0001						
GT23					0.981	-0.0006						
AC045					0.983	-0.0005						
AC082					0.112	0.0010						
AC087					0.992	-0.0005						
AC137					0.126	0.0001						
CA234					0.112	-0.0003						
GT129					0.846	-0.0006						
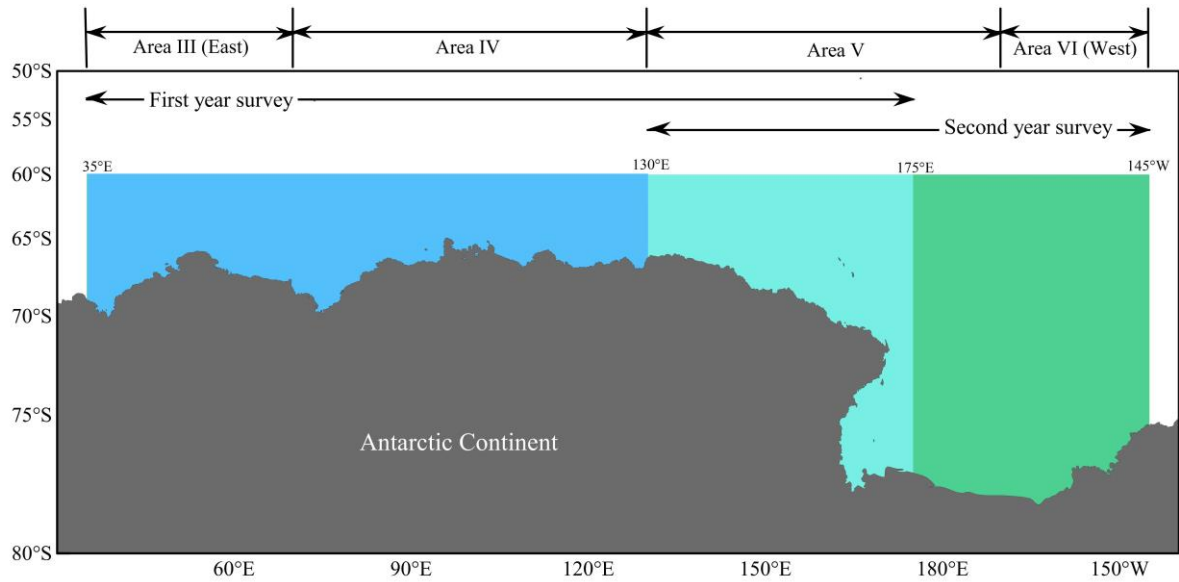
Figure 1. JARPAII research area. Whales in the 'blue' and 'green' sectors showed genetic and morphometric differentiation according to the JARPA research. It is assumed that those two sectors are occupied by different stocks (I and P stocks, respectively). The sector in the middle was proposed as an 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.
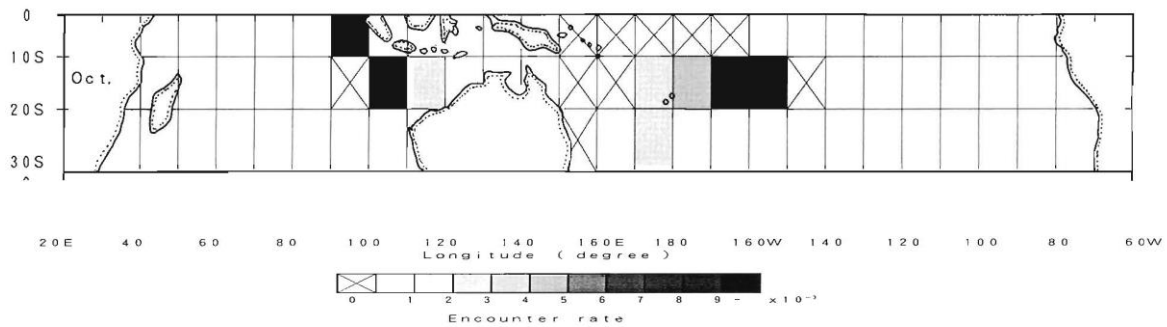

Figure 2: Encounter rates of Antarctic minke whales in 10º squares of latitude and longitude in waters 0º-30ºS in October (from Kasamatsu *et al*., 1995).
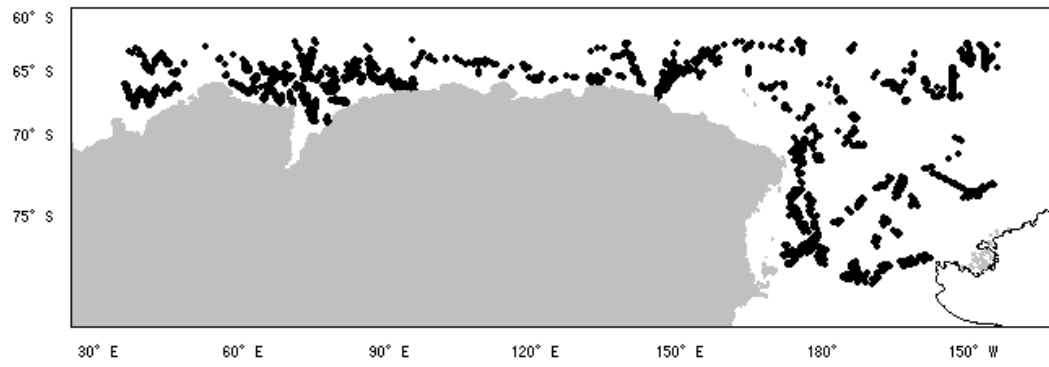
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Figure 3. Geographical distribution of the Antarctic minke whales sampled by JARPAII between the 2005/06 and the 2010/11 austral summer season.