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What do we know about the stock structure of Antarctic minke whales in the Indo-Pacific region of the Antarctic? A brief review of methodologies and research outputs

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ABSTRACT

This paper presents a brief review of the studies on stock structure of the Antarctic minke whale conducted under the Japanese Whale Research Program under Special Permit in the Antarctic (JARPA, austral summer seasons 1987/88–2004/05) and the Second Phase of JARPA (JARPAII, 2005/06–2013/14), and summarizes the main research findings. The stock structure of the Antarctic minke whale was examined using genetic and non-genetic approaches. Both research programs were conducted in the International Whaling Commission (IWC) Management Areas IIIE (35°–70°E), IV (70°–130°E), V (130°E–170°W) and VIW (170°–145°W). Both genetics, *i.e.*, RFLP of the whole mitochondrial DNA (mtDNA), sequencing of mtDNA control region, microsatellite DNA, and non-genetic, *i.e.*, morphometric and mean body length at physical maturity analyses were used. These studies provided evidence for the occurrence of at least two stocks in the research area, which were called 'eastern Indian Ocean stock' (I-stock) and 'western South Pacific stock' (P-stock), which overlap geographically in the central part of the research area. The results of a modelling approach incorporating genetic and morphometric data suggested that the two stocks have a soft boundary (or area of mixing) mainly in the western part of Area V (130°–165°E), which changes by year and sex.

INTRODUCTION

The Antarctic minke whale *Balaenoptera bonaerensis* (Figure 1), like all the other Southern Hemisphere baleen whales species apart from the Bryde's whale (*B. edeni*), was managed by the International Whaling Commission (IWC) on the basis of six geographical 'Areas' (Figure 2). The IWC established these Areas from the 1974/75 austral summer season, based mainly upon information from Mackintosh (1942; 1966) on distribution of catches of blue, fin and humpback whales (see review by Donovan, 1991). These Areas were used by the IWC for the implementation of the New Management Procedure (NMP) on baleen whale species.

However, biological evidence for the particular boundaries are weak, especially for those species such as the Antarctic minke whale, whose data were not considered when the original management Areas were established. In this regard, important questions were formulated originally by Hoelzel and Dover (1989): 'Are the Antarctic minke whales found in two geographically distinct management Areas from two different genetic stocks?' or 'Are individuals from more than one genetic stock present in a particular management Area? If so, what level of interchange may have occurred between different genetic stocks?' Several approaches were used in the past to identify genetic stocks of this species in the Antarctic feeding grounds and to determine to what extent genetic stocks and IWC management Areas coincide.

Studies on stock structure of the Antarctic minke whale

Figure 1. Antarctic minke whale (*Balaenoptera bonaerensis*).

started at the end of the decade of the 1970's, and results of genetic and non-genetic analyses were revised by the IWC Scientific Committee (IWC SC) during the comprehensive assessment (CA) of the species in 1990. All the analyses presented at the CA were based on samples and data from commercial pelagic whaling in the Antarctic. The genetic studies were based mainly on allozyme at that time, although studies based on mitochondrial and nuclear DNA were also conducted, most of the analyses involved small sample sizes from only Areas IV and V. Non-genetic studies revised in the 1990's CA involved morphology, catch and sighting distribution pattern, analysis of Discovery marks and ecological markers. Results from the different approaches failed to unambiguously identify any isolated stock in the Antarctic (IWC, 1991).

Studies on stock structure under the Japanese Whale Research Program under Special Permit in the Antarctic (JARPA) started after the CA. It was considered that JARPA samples were more useful for studies on stock structure than the commercial samples, given the wider geographical coverage of the surveys and that samples were taken along track-lines in a random mode design. Studies on stock structure of Antarctic minke whale continued under the second phase of JARPA (JARPAII).

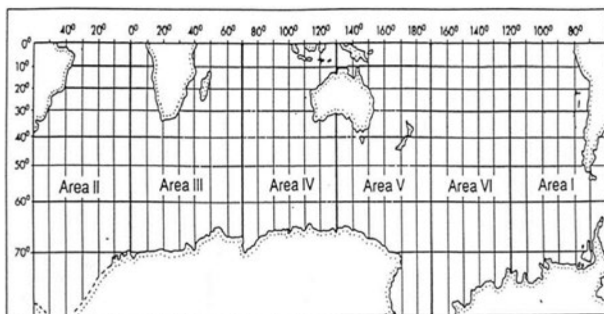


Figure 2. IWC Antarctic Areas for the management of baleen whale species (except Bryde's whale) (Donovan, 1991).

This paper reviews the studies on stock structure conducted under the JARPA and JARPAII, emphasizing the technical aspects and main research outputs.

SAMPLING AND ANALYTICAL APPROACHES

JARPA

JARPA was conducted during the austral summer seasons 1987/88–2004/05 and the research area was composed of Areas IIIE (35°–70°E), IV (70°–130°E), V (130°E–170°W) and VIW (170°–145°W) (Figure 2). The main analytical procedure used in the genetics and non-genetic studies was hypothesis testing under the null hypothesis of panmixia.

Genetic approaches

Mitochondrial DNA (mtDNA)

In JARPA, restriction fragment length polymorphisms (RFLP; Box 1.1) was applied for the whole mtDNA (Pastene *et al.*, 1993; 1996). The mtDNA is an extra-chromosomal genome in the cell mitochondria and is inherited only from the mother. This marker is widely used in constructing intra- and related species phylogenies and inferring evolutionary history.

The mtDNA extracted from a total of 6,256 Antarctic minke whales was digested with six-base sequence recognition endonucleases (*Acc*I, *Ban*I, *Eco*RV, *Hinc*II, *Hpa*I and *Ssp*I). Restriction fragments were separated by submarine electrophoresis in 1% agarose gels. After electrophoresis, the gels were stained with ethidium bromide and were

Box 1.1 Restriction Fragment Length Polymorphisms

This method can use for total DNA, mitochondrial DNA or specific sequences first amplified from total DNA by PCR. The DNA is digested with restriction endonucleases, which are called 'restriction enzymes', to generate a series of DNA fragments. These restriction enzymes only cut DNA at specific sequences, which yields a consistent set of fragments that can be separated according to the size by agarose gel electrophoresis (Figure 1.1). The RFLP pattern is different between individuals which have different haplotypes because mutations destroy or generate new cutting sites, which enable to measure genetic variations.

Haplotype frequency data for the mitochondrial analyses

Haplotype	Population 1	Population 2
A	1	2
B	1	1

Figure 1.1 Measurement of genetic variations by RFLP analysis
Restriction sites shown by arrows from single restriction enzyme (*Hae III* recognizes sequence of 'GGCC' and cuts the site into 'GG' and 'CC') are mapped in five individuals from two populations, and the resulting pattern of restriction fragments on an agarose gel is shown in the right-hand diagram. The right-bottom of the diagram shows a fundamental data for the stock structure analyses generated by RFLP analysis.

This box was written based on Beebee and Rowe (2008) and Lowe *et al.* (2004)

photographed using Polaroid film under an UV irradiation. Distinctive restriction fragment patterns produced by each enzyme were assigned letters. Individuals were assigned haplotypes consisting of a list of the letters designating the fragment profiles produced by each of the six restriction enzymes. Then, the composite haplotype for each individual comprises a string of six letters.

Haplotype frequencies were employed to determine genetic relationships between the samples of the designed strata. Genetic relationships were first quantified using the chi-square statistics for heterogeneity of mtDNA haplotype frequencies (Roff and Bentzen, 1989). The level of significance obtained by this method is referred to in this paper as the *P*-value. A *P*-value smaller than 0.05 was used as a criterion to reject the null hypothesis of panmixia. Additionally, the quantification of the geographical differentiation of mtDNA was carried out using the analysis of molecular variance (AMOVA) of Excoffier *et al.* (1992).

More detailed descriptions of laboratory and analytical procedures were provided in Pastene *et al.* (1993; 1996).

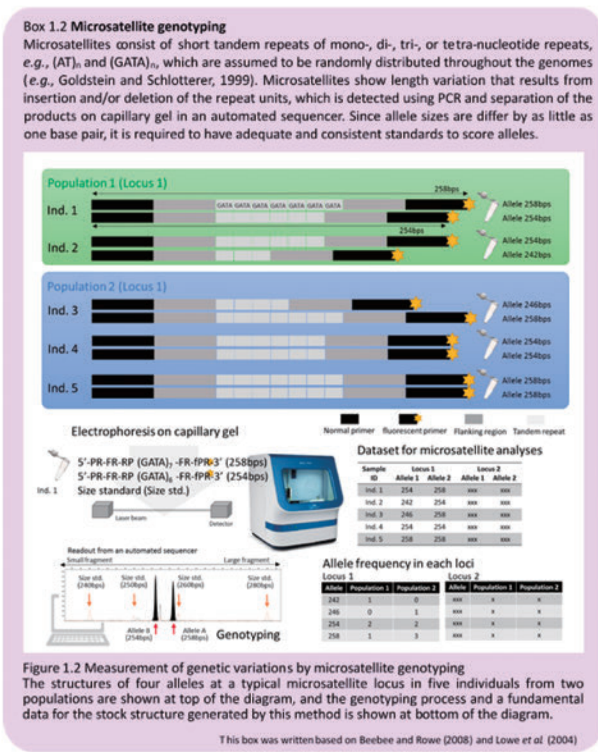
Microsatellite DNA

In addition to the mitochondrial RFLP analysis, microsatellite analyses (Box 1.2) was also applied on JARPA samples. Microsatellites are codominant and become a popular marker for many aspects of molecular ecology, in particular for intraspecific studies, because of its high mutation rate and polymorphisms compared to other markers.

Microsatellite polymorphisms were examined at six loci in samples from a total of 6,260 minke whales: EV1, EV104 (Valsecchi and Amos, 1996), GT023, GT211, GT4195 (Bérubé *et al.*, 2000), and DlrFCB14 (Buchanan *et al.*, 1996). Polymerase chain reaction (PCR) amplifications at these loci were performed, which were run with an internal size standard (GENESCAN400HD, Applied Biosystems Japan) using BaseStation100 DNA fragment analyzer. Allelic sizes were determined manually in relation to the internal size standard and Antarctic minke whale DNA of known size that were re-run on each gel.

The allele frequencies at the six microsatellite loci were calculated and the departure from expected Hardy-Weinberg genotypic proportions was tested at each locus as well as overall loci. The number of alleles per locus, allelic richness and heterozygosity were also computed. A conventional hypothesis testing procedure was performed using a heterogeneity test in allele frequencies. The probability test or Fisher's exact test with the Markov chain method was used for the heterogeneity tests among minke whales in the designed strata. Statistical significance of the heterogeneity tests 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).

More detailed descriptions of laboratory and analytical procedures were provided in Pastene *et al.* (2006).



Box 1.2 **Microsatellite genotyping**

Microsatellites consist of short tandem repeats of mono-, di-, tri-, or tetra-nucleotide repeats, *e.g.*, $(AT)_n$ and $(GATA)_n$, which are assumed to be randomly distributed throughout the genomes (*e.g.*, Goldstein and Schlotterer, 1999). Microsatellites show length variation that results from insertion and/or deletion of the repeat units, which is detected using PCR and separation of the products on capillary gel in an automated sequencer. Since allele sizes are differ by as little as one base pair, it is required to have adequate and consistent standards to score alleles.

Figure 1.2 Measurement of genetic variations by microsatellite genotyping
The structures of four alleles at a typical microsatellite locus in five individuals from two populations are shown at top of the diagram, and the genotyping process and a fundamental data for the stock structure generated by this method is shown at bottom of the diagram.

This box was written based on Beebee and Rowe (2008) and Lowe *et al.* (2004)

Non-genetic approaches

Morphometric analyses

The relationship between body length and longitudinal strata for ten body measurements of Antarctic minke whale was investigated using ANCOVA. A cluster analysis was also applied for an estimated average length of measurements to examine the degree of relationships among whales in six longitudinal strata. A more detailed description of the analytical procedure was provided in Hakamada (2006).

Mean body length at physical maturity

Mean body length at physical maturity was compared among whales in Areas IIIE, IV, V and VIW for males and females. Physically matured individuals were defined as those with epiphysis fusion occurring in the 6th thoracic vertebrae. The t-test and ANOVA were used for testing differences in mean body length among strata of the JARPA survey. A more detailed description of the analytical procedure was provided in Bando *et al.* (2006).

JARPAII

JARPAII was conducted during the austral summer seasons 2005/06–2013/14 in the same research area covered by JARPA. The analyses on stock structure were refined in two ways: by using additional genetic markers and by applying new analytical approaches.

Genetic approaches

Mitochondrial DNA control region sequencing

In JARPAII, sequencing analysis (Box 1.3) was performed instead of the mitochondrial RFLP used in JARPA, which provides higher resolution.

A 338 bp-segment of the mtDNA control region was sequenced for a total of 2,278 samples using the primers MT4 (Arnason *et al.*, 1993) and Dlp 5R or P2 developed by the ICR. PCR and subsequent cycle sequencing reaction for each sample were performed following the manufacture's protocol. The nucleotide sequence of each cycle sequencing product was determined using Applied Biosystems 3500 Genetic Analyzer (Life Technology) under standard conditions. Both strand samples were sequenced in their entirety for all samples.

The statistical approaches used for the RFLP analyses in JARPA (hypothesis testing) were also adopted for the mitochondrial control region sequence data analyses. In addition to this, constructing haplotype network and mismatch distribution analysis were conducted.

More detailed descriptions of laboratory and analytical procedures were provided in Pastene and Goto (2016).

Microsatellite DNA

Genetic variation was analyzed at 12 microsatellite loci (instead of the six used in JARPA) in a total of 2,551 samples: 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). Laboratory protocols were the same as in JARPA.

The statistical approaches used for microsatellite analyses in JARPA were adopted for the JARPAII microsatellite analyses. In addition to this, the Bayesian clustering approach using STRUCTURE (Pritchard *et al.*, 2000) was used to determine the number of genetically distinct populations present in the samples based on information on the individual genotypes.

More detailed descriptions of laboratory and analytical procedures were provided in Pastene and Goto (2016).

Modelling approach

Schweder *et al.* (2011) developed an integrated approach for estimating longitudinal segregation of two stocks using different sources of data: morphometric, microsatellite and mtDNA data. Under this approach, areas of overlap between stocks were allowed to vary by year and sex. A joint likelihood function was defined for the estimation of mixing proportions and statistical tests without assuming any baseline populations. The approach was originally applied to the JARPA data (Schweder *et al.*, 2011) and subsequently to JARPA and JARPAII data. More detailed analytical procedure was described in Kitakado *et al.* (2014).

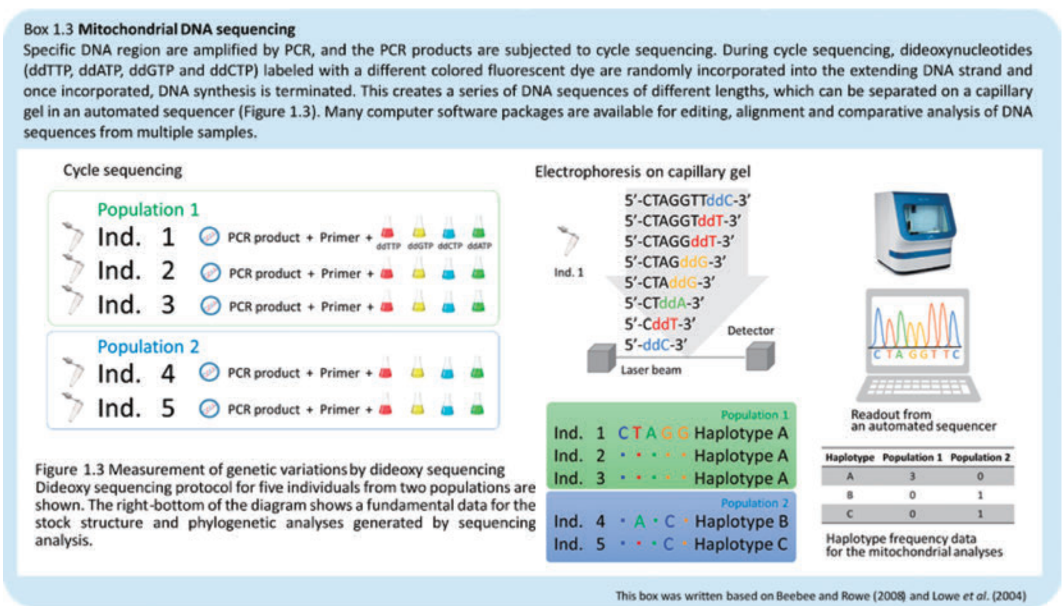


Figure 1.3 Measurement of genetic variations by dideoxy sequencing
Dideoxy sequencing protocol for five individuals from two populations are shown. The right-bottom of the diagram shows a fundamental data for the stock structure and phylogenetic analyses generated by sequencing analysis.

SUMMARY OF RESULTS

JARPA

A summary of the results of the analyses on stock structure under the JARPA is shown in Table 1.

Taking all results together, Pastene (2006) suggested that whales in the eastern part of Area III and western part of Area VI were more differentiated than they were to whales in the Areas IV and V. Therefore, the author concluded that the single stock scenario cannot be applied to Antarctic minke whales in the feeding grounds of Areas IIIE–VIW, and proposed the occurrence of at least two genetic stocks in the research area. The author also suggested that this observation is probably related to the breeding areas in the eastern Indian Ocean and western South Pacific proposed by Kasamatsu *et al.* (1995). The following names were proposed for these stocks by Pastene (2006): 'Eastern Indian Ocean Stock' (I-stock) and 'Western South Pacific Ocean Stock' (P-stock).

While the microsatellite and morphometric analyses were unable to identify any boundary between them, the mtDNA RFLP analyses suggested that the western part of Area V was more related to the I-stock than the P-stock, and a boundary in the sector 150°–160°E was proposed. This was consistent with the results of mark-recapture that showed movement of whales through 130°E (division between Areas IV and V). No western boundary of the I-stock and eastern boundary of the P-stock were proposed.

JARPAII

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

Results of the heterogeneity test for both markers showed significant genetic differences between whales in two sectors, the western (35°–130°E) and eastern (165°E–145°W) research area, confirming that different

Table 1
Summary of the results on stock structure in JARPA.

Analytical approaches	Sex	Period of sampling	Pattern of geographical variation	References
mtDNA	F+M	1987/88–2004/2005	IIIE=IVW=IVE VIW=VE IIIE, IVW, IVE≠VE, VIW Possible boundary in the sector 150°–160°E	Pastene *et al.* (2006)
Microsatellites	F	1989/90–2003/2004	IVWN, IVWS≠VE, VIW Some degree of difference in haplotype frequency between Areas IIIE and IVW	Pastene *et al.* (2006)
	M	1989/90–2004/2005	No significant difference in haplotype frequency	
	F+M	1989/90–2004/2005	IVW≠ VE	
MBLM	F	1987/88–2004/2005	IIIE=IVW=IVE=VW VIW=VE IIIE, IVW, IVE, VW≠VE, VIW	Bando *et al.* (2006)
	M	1987/88–2004/2005	VEN≠VES IIIE=IVW=IVE=VW VIW=VEN, VES IIIE, IVW, IVE, VW≠VEN, VES, VIW	
Morphometrics	F	1987/88–2004/2005	IIIE=IVW=IVE=VW VIW=VE IIIE, IVW, IVE, VW≠VE, VIW	Hakamada (2006)
	M	1987/88–2004/2005	IIIE=IVW=IVE VIW=VE IIIE, IVW, IVE≠VW, VE, VIW	

MBLM indicates mean body length of physically matured whales. A sign '=' means that the statistical test found no significant difference at $\alpha=0.05$

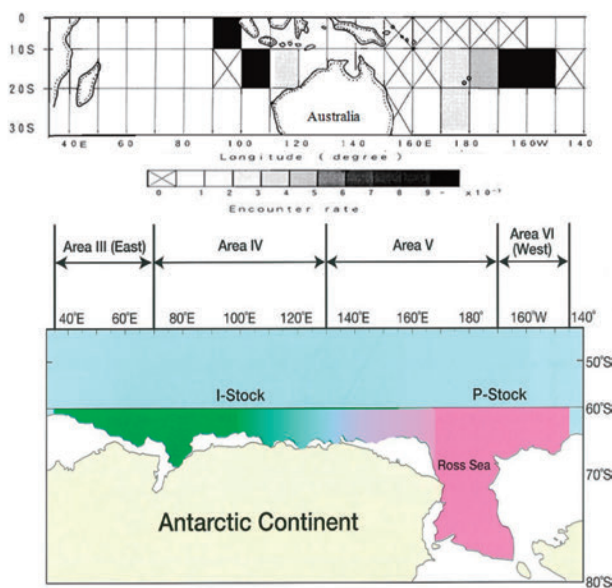


Figure 3. Hypothesis on stock structure of the Antarctic minke whale. The upper figure shows the encounter rates of Antarctic minke whales in 10° squares of latitude and longitude in waters 0–30°S during October (Kasamatsu *et al.*, 1995). The high sighting densities in the eastern Indian Ocean and western South Pacific could correspond to breeding grounds of this species. At least two stocks (I- and P-stocks) occur in the research area of JARPA and JARPAII, which mix through a transition area. The transition area and the mixing rate appears to change by year and sex (Pastene and Goto, 2016).

stocks inhabit the Indian and Pacific sectors of the Antarctic (I- and P-stocks) (Pastene and Goto, 2016). On the other hand, the STRUCTURE analysis for the microsatellites did not show genetic structuring of this species. This observation is probably due to a very low level of genetic differentiation. Microsatellite DNA analyses also showed more dispersal in males than females, and also some degree of annual variation.

The results of the modelling approach confirmed the occurrence of at least two stocks (I- and P-stocks) in the JARPA/JARPAII research area, which could be related to the suggested breeding areas in the eastern Indian Ocean and western South Pacific Ocean (Figure 3). Furthermore, the results indicated that the spatial distribution of the two stocks has a soft boundary (or area of mixing) mainly in Area VW (130°–165°E) (Figure 3), which changes by year. Results also suggested possible sex differences in the pattern of distribution of the two stocks (Kitakado *et al.*, 2014).

CONCLUSION

In conclusion, stock structure analyses for the Antarctic minke whale conducted under the JARPA and JARPAII program indicated that the structure of Antarctic minke whale in Areas IIIE–VIW is more complex than originally thought: there are at least two stocks (I- and P-stocks), which overlap geographically in a wide area located mainly at 130°–165°E, which changes with year and sex.

Pastene and Goto (2016) postulated that Antarctic minke whales originating from breeding grounds in the western South Pacific (P-stock) have some degree of fidelity to the krill concentration associated with the Ross Sea Gyre (120°W–180°) ('home' sector). On the other hand, whales originating in the Indian Ocean (I-stock) have some fidelity to the krill concentration associated with the gyre located between 90°E and 120°E ('home' sector). They also postulated that both stocks could expand longitudinally (depending on the particular oceanographic conditions in a given year) and interact in the middle sectors and that therefore any boundary (or proportion of the populations in areas with mixing) in the Antarctic should be considered 'soft' probably changing annually according to changes in the oceanographic conditions.

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