An update of the mitochondrial DNA RFLP analysis in the Antarctic minke whales from Areas III and IV

Luis A. Pastene, Mutsuo Goto and Naohisa Kanda

The Institute of Cetacean Research, 4-18 Toyomi-cho, Chuo-ku, Tokyo 104-0055, Japan

ABSTRACT

A restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) in the Antarctic minke whale from Areas IIIE and IV was conducted using samples from the 1987/88-1999/00 JARPA surveys. Samples were divided following the same criteria used in the previous analyses in these Areas. They were divided into three longitudinal sectors: Area III Eastern (35°-70°E), Area IV Western (70°-100°E) and Area IV Eastern (100°-130°E) and two temporal periods (Early and Late). A total of 2,274 samples was examined in five longitudinal/temporal groups as follow: IIIEE, n=260; IVWE, n=410; IVWL, n=650; IVEE, n=233 and IVEL, n=721. As out-group, we used a 'core sample' from Area V (n=1,443). Quantification of the mtDNA differentiation among groups was carried out using the Analysis of Molecular Variance (AMOVA). Both haplotype (Fst) and sequence (PHIst) statistics were used. Remarkable yearly variation was found for group IVWE, in where the source of mtDNA heterogeneity was attributed mainly to year 1989/90. No yearly variation was found among three surveys in Area IIIEE (95/96, 97/98 and 99/00). A comparison with the 'core sample' from Area V showed significant differences in three cases, all involving Area IV West: IVWE 89/90, IVWE 97/98 and IVWL total. Differences were found between groups IVWE 89/90 and IVWE 97/98. Data combined for the most recent surveys (95/96, 97/98 and 99/00) showed that group IIIEE was similar to adjacent group IVWE and they were similar to the 'core sample' from Area V but different from group IVWE 89/90 ('western stock'). Among the most feasible reasons explaining the fail to detect the 'western stock' in recent surveys are the following: a) remarkable yearly variation in the distribution of stocks in Areas IVW and IIIE and b) a more limited geographical coverage in recent surveys regarding the survey in 1989/90.

KEYWORDS: ANTARCTIC MINKE WHALE, STOCK IDENTITY, GENETICS, MANAGEMENT

INTRODUCTION

One of the main research objectives of the JARPA (the Japanese Whale Research under Special Permit in the Antarctic) is the estimation of biological parameters required for the stock management. Such estimations are being conducted preliminary on the basis of the IWC management Areas IV and V on the assumption that each Area is occupied by a different genetic stock.

The accuracy of the estimation of parameters such as the natural mortality, however, has faced a challenging issue to solve. In the discussion conducted during the 1994 SC meeting on the preliminary results of the estimation of this parameter, it was noted that the accuracy in the estimation stem largely from the stock identity questions and seasonal variations in the migration patterns for different age groups (IWC, 1995).

Studies on stock identity in the Antarctic minke whale have been based mainly on mitochondrial DNA (mtDNA) RFLP analysis. Results of these analyses showed a considerable degree of genetic heterogeneity in Areas IV and V but the pattern of heterogeneity is not correlated with the actual geographical boundaries of these Areas. These results suggest that the stock structure of the Antarctic minke whale could be more complex than it was originally hypothesized. It could be determined by a combination of factors such as longitudinal sector, temporal (between and within surveys) and distance from the ice-edge (Pastene *et al.*,

1996a; Goto *et al.*, 1998). Tables 1 and 2 shows a summary of the mtDNA analysis as presented to the JARPA review meeting in 1997 (Pastene *et al.*, 1997).

While no substantial mtDNA heterogeneity has been observed in Areas V and VI (Pastene and Goto, 2000), a more complex situation is observed in Areas IV and III where considerable inter and intra-year variability has been observed, especially in Area IV. We further examined these Areas by using the total samples available from the JARPA surveys for the period 1987/88-1999/00.

MATERIALS AND METHODS

Samples

Samples of the Antarctic minke whale from Areas IV and IIIE were available from the 1987/88-1999/00 JARPA surveys. For each year and sex, samples were grouped into Area III Eastern Early (group IIIEE, n=260), Area IV Western Early (group IVWE, n=410), Area IV Western Late (group IVWL, n=650), Area IV Eastern Early (group IVEE, n=233) and Area IV Eastern Late (group IVEL, n=721). Thus a total of 2,274 samples was examined in this study. Area III Eastern Late was not considered for the analysis because the small sample size (Table 2). 'Early' refers to whales sampled in December and first half of January. 'Late' refers to whales sampled in the second half of January, February and March. For comparison we used a 'core' sample from Area V (total samples from that Area excepting group VWE, n=1,443, Pastene and Goto, 2000).

Figs. 1 to 7 shows the geographical distribution of each longitudinal/temporal group for each JARPA survey, by sex, including information on the position of the ice-edge.

RFLP analysis

Crude mtDNA extracted from liver tissues was digested with six polymorphic restriction enzymes, same as in the previous studies (Pastene *et al.*, 1996a; Pastene and Goto, 2000): *Acc*I, *Ban*I, *Eco*RV, *Hinc*II, *Hpa*I and SspI. All the procedures for DNA extraction and digestion were the same as in the previous study.

Statistical analysis

The geographical/temporal differentiation of mtDNA was quantified using the Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) as implemented in the computer program AMOVA ver. 1.55. The statistics of primary interest are the haplotype (Fst) and sequence (PHIst), both of which were used. The significance of the observed variance values was tested using a modification of a matrix permutation procedure available in the computer program. All test of statistical significance were based on 10,000 random permutation of the original data sets. The level of significance obtained using this procedure is referred in this paper as the P value.

Samples were grouped into longitudinal/temporal/year groups. First we tested for yearly variation in each of the longitudinal/temporal groups. If no significant yearly differences were found, then samples from different years were pooled in the subsequent analyses.

The pooled longitudinal/temporal groups or longitudinal/temporal/year groups (in the cases where substantial yearly variation was found), were compared with a 'core' sample from Area V (n=1,443). This figure includes all the samples available from Area V with the exception of group VWE (n=208). This group was somewhat different from other samples from Areas V and VI using the Fst statistics and then we excluded them from the analysis (see Pastene and Goto, 2000).

RESULTS

mtDNA haplotypes

Characterization of the mtDNA RFLP haplotypes was documented in a previous paper (Pastene *et al.*, 1996a). By using a set of six polymorphic restriction enzymes, a total of 153 haplotypes were discriminated in the Antarctic minke whale.

Yearly variation in the longitudinal/temporal groups

Results of the statistical analysis of yearly variation in the longitudinal/temporal groups are shown in Table 3, for both Fst and PHIst statistics. The only evidence for yearly variation is given for group IVWE where the P value for the Fst was just over the 5% critical level (0.053) and the P value for the PHIst resulted below this level (0.017).

Pairwise yearly comparison for group IVWE (data not shown) resulted in several P values below 5% level. However, we were unable to allocate the source of mtDNA heterogeneity to any particular year so the comparison of group IVWE with the core sample from Area V was made for each year separately.

Comparison with a 'core' sample from Area V

Pastene and Goto (2000) examined all the JARPA samples available from Areas V and VIW. The results of their mtDNA RFLP analysis revealed limited genetic heterogeneity both temporal and geographic. The only possible source of mtDNA heterogeneity was attributed to whales from Area V West Early (n=208), which was differentiated from other groups in Areas V and VIW (by the Fst) as well from the 'western' sample from Area IV Western Early (by both Fst and PHIst).

The 'core' sample was constructed by using all the samples from Area V excepting those from group VWE. The resulting sample size for this 'core' sample was 1,443.

Those groups in Area III and IV, which showed no significant yearly variation were pooled and compared with the 'core' sample. In the case of group IVWE, which showed substantial yearly variation, the comparison with the 'core' sample was made for each year separately.

Table 4 shows the results of the comparisons between groups in Areas III and IV with the 'core' sample, for both Fst and PHIst statistics. We found three cases where comparisons resulted in low P values for both statistics, all these groups were from Area IV West: group IVWL (P value for Fst=0.066; P value for PHIst=0.026); group IVWE 1989/90 (P value for Fst=0.012; P value for PHIst=0.002) and group IVWE 1997/98 (P value for Fst=0.051; P value for PHIst=0.024).

DISCUSION

As in previous analyses we found remarkable mtDNA heterogeneity in Area IV, mainly in the western sector. Such heterogeneity was observed in the comparison between years but also in the comparison among groups within a year. This result suggest that the dynamic of stocks migrating from lower latitudes into such a sector in the Antarctic could change not only among years but also within a year. If such hypothesis is correct, then the delineation of boundaries in the Antarctic could be a challenging issue and perhaps we should consider 'dynamic' rather than 'fix' boundaries in the Antarctic.

So far the only substantial source of mtDNA heterogeneity found in the analysis of JARPA surveys has been attributed to whales from the western part of Area IV sampled early in the survey of 1989/90 (Pastene *et al.*, 1996a). Preliminary we call this sample the 'western stock'. In a subsequent analysis, which used commercial samples from Area III from a 1978 operation (Pastene *et al.*, 1996b), we found preliminary support for the hypothesis that the 'western stock' could be distributed in adjacent Area III, although the statistical evidence was weak. However the analysis of more recent samples from Area III (available from three JARPA surveys conducted in 1995/96, 1997/98 and 1999/00) derived in ambiguous results and no evidence for such hypothesis has been found. Contrary to our expectation in general these samples were similar to the 'core' sample from Area V and eastern part of Area IV. A summary of the analysis involving Area IIIEE is as follow:

Area IIIEE total (three years)=Area IVWE (three years)='core' sample≠'western stock'

Area IIIEE 95/96=Area IVWE 95/96='core'sample≠'western stock'

Area IIIEE 97/98='core' sample≠Area IVWE 97/98≠'western stock

Area IIIEE 99/00=Area IVWE 99/00='core' sample≠'western stock

The sample combined for the three years showed that Area IIIEE was similar to adjacent Area IVWE. Each of these two was similar to the 'core' sample but different from the 'western stock'. A similar pattern was found when the analysis is made by year and this is the case for 1995/96 and 1999'00. It should be noted here, however, that in the case of 1999/00 Area IIIEE was just marginal significant different (P values between 5 and 10%) from the 'western stock' and that Area IVWE was not distinguished from the 'western stock' probably due to small sample size. At this stage we do not have a reasonable explanation for the situation in 1997/98 in which the sample from Area IVWE was different from both 'core' sample and 'western stock' and also from the adjacent Area IIIEE.

By using samples from JARPA surveys in Area IIIE we have been unable to find additional evidence for the occurrence of the 'western stock' in that Area. Several possible explanations for such situation are offered below:

a) Hypothesis derived from the analysis of Pastene *et al.* (1996a) could not be correct.

This explanation could be correct in case the genetic and/or the statistical methods used were flawed. The genetic technique used in the analysis of Pastene *et al.* (1996a), from which the evidence for a 'western stock' emerged, is an accepted and widely used technique. Furthermore the analysis was conducted using larger sample size and a well-established program, AMOVA, which allows a bootstrap type of significance testing. The grouping of the samples, however, was arbitrary because no previous information on the stock structure exists for the research area, particularly from the lower latitudes. Hypothesis testing will be more powerful if the grouping of samples has been made following previous and independent information on stock structure.

One of the possible problems for the application of the statistical method is the high diversity of haplotypes. Furthermore there is a large number of unique or very low frequency haplotypes in our data set. Bootstrapping and other re-sampling/randomization approaches give misleading results when applied to such data sets because single observations provide poor estimates of character frequencies. It should be noted, however, that the heterogeneity observed in the analysis of Pastene *et al.* (1996a) was attributed to only one single source, e.g. Area IV western early. Such group showed higher PHIst values when compared with other groups, all of them showing P values below 5% or 1% significance level. Then a consistent trend was observed for this particular group. If some problems exist in the application of the randomization method, 'abnormal' results should appear in any of the several comparisons made and not for some specific group only.

b) There are marked yearly variation in the distribution of stocks in Area IVW and Area IIIE

It is possible that a substantial change in the distribution of stocks could have occurred in the region after 1989/90. This is consistent with the result that the three surveys combined in Area IIIEE were similar to adjacent Area IVWE. Consistently both of them were similar to the 'core stock' and different from the 'western stock'. Then the 'core stock' could have predominated in the region in recent years. The weak point of this explanation is the result of the analysis for the 1997/98 survey alone. In this particular year the sample from Area IVWE was different from adjacent Area IIIEE but also different from the 'western' and 'core' stocks. The inconsistency is given by the fact that adjacent Area IIIEE and IVWE were different.

Interpretation of the yearly variation in Area IV (especially in the western sector) could be assisted by the temporal analysis of ecological components in that Area. For example the temporal variation in the distribution of krill and the analysis of the oceanographic factors affecting the distribution of this prey species could be investigated.

c) Recent JARPA survey could not be covering all the geographical regions as covered in the 1989/90 survey

Goto *et al.* (1998) examined samples from Area IV from two JARPA surveys (1989/90 and 1991/92). Apart the longitudinal and temporal division they separated the samples as 'offshore' (more than 45n.miles from the ice-edge) and 'ice-edge' (within 45n.miles from the ice-edge). They found that the 'offshore' samples in Area IVWE were more informative on stock structure than the 'ice-edge' sample. The JARPA survey of 1989/90 in the early period covered widely both ice edge and offshore waters in the western part of Area IV. Furthermore the 'early' survey covered latitudes north and south of 60°S (see Fig 2). Recent sampling in Areas IV and IIIE have surveyed both ice edge and offshore waters but the sampling come mainly from the former and from latitudes south of 60°S only (especially in Area IIIE, see Figs. 5, 6 and 7). Then it is possible that the 'western stock' was distributed in Areas IIIEE and IVWE in recent years but because the limited sampling coverage (and limited sample size), it could not have been detected.

Another possibility is that a different stock could be commonly distributed in more northern latitudes and then the 1989/90 JARPA survey detected it because such survey covered waters north of 60°S.

Following a recommendation from the JARPA review meeting Abe *et al.* (1999) use a set of five microsatellites to examine a sub-set of the samples used in this study. Although this study found some degree of nuclear DNA (nDNA) heterogeneity, the geographic and temporal pattern of such heterogeneity was different from that observed in the mtDNA analysis. The nature of such differences should be investigated further in the future. The study by Abe *et al.* (1999) examined samples from two surveys in Area IIIE (1995/96 and 1997/98), one in Area IV (1989/90) and one in Areas V and VIW (1996/97). Additional samples, including those from Area IIIE from the 1999/00 JARPA survey, are being examined.

Because the mutation rate in the mtDNA is faster than in the nDNA we can expect a larger resolution using the former technique. However, there are some cases in our analysis of Antarctic minke in which significant differences are found using mtDNA (but not detected using nDNA), which are difficult to explain from the biological point of view of the species. There is the possibility that mtDNA is too sensitive to detect stock identity in some cases.

The genetic analysis of Antarctic minke whales is restricted by the problem of sample size. As suggested by Pastene *et al.* (1996b), a minimal of 150-200 samples are requested to detect significant differences. When the samples are divided into years, western and eastern sectors and into temporal groups, most sample sizes are lower than these figures. Then the power of the analysis decreases.

In general, interpretation of results of the genetic analyses is complicated due to several other factors: a) we are dealing only with sampling in the feeding grounds; b) we are dealing with an abundant species, where the degree of genetic differentiation among populations seems to be very small and c) while data are available for non-genetic studies on stock structure, these studies have not been conducted yet. Non-genetic studies would allow for comparison of the genetic results.

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Table 1: Results of the analysis by AMOVA on Antarctic minke whales sampled by JARPA in Areas IV and V between 1987/88 and 1994/95 (from Pastene *et al*, 1996a; Pastene *et al*., 1997). A= nested analysis, B= pairwise comparisons among longitudinal/temporal groups. The P-value is the probability of a more extreme variance component or PHIst than that observed, in comparison to a null distribution of these values on 2,000 random permutations of the data matrix. In Table B the sample size is shown in parenthesis. Below diagonal PHIst value, above diagonal P values. P values below 5% level are shown in bold.

A

	df	% total variance	PHI	P
Among sectors	3	-0.03	CT: -0.000	0.4698
Among area-time groups/long. sector	4	0.17	SC: 0.002	**0.0491**
Within area-time groups	2,116	99.87	ST: 0.001	**0.0340**

B

	IVWE (160)	IVWL (383)	IVEE (233)	IVEL (321)	VWE (208)	VWL (264)	VEE (76)	VEL (479)
IVWE		**0.0345**	**0.0295**	**0.0015**	**0.0060**	**0.0103**	**0.0493**	**0.0040**
IVWL	0.0052		0.4198	0.3628	0.2104	0.8676	0.5787	0.3058
IVEE	0.0072	-0.0000		0.2644	0.1674	0.2489	0.1724	0.3688
IVEL	0.0136	0.0001	0.0007		0.6327	0.6892	0.4218	0.5297
VWE	0.0105	0.0010	0.0017	-0.0009		0.4733	0.4673	0.7621
VWL	0.0076	-0.0015	0.0009	-0.0009	-0.0004		0.8361	0.3403
VEE	0.0087	-0.0015	0.0032	-0.0004	-0.0005	-0.0034		0.3093
VEL	0.0083	0.0003	0.0002	-0.0003	-0.0011	0.0003	0.0009	

Table 2: Samples of minke whales used for the mtDNA analysis in this study. They were grouped by Area, longitudinal sector, period within an austral summer season, sex and JARPA survey. See text for details of the groupings.

JARPA	IIIEE		IIIEL		IVWE		IVWL		IVEE		IVEL	
	F	M	F	M	F	M	F	M	F	M	F	M
1987/88											104	125
1989/90					30	88	50	42	49	33	7	8
1991/92					12	30	73	74	2	10	24	31
1993/94							56	88	62	77	7	15
1995/96	22	45	16	22	31	100	55	44			25	37
1997/98	29	57	1	12	21	51	48	70			52	74
1999/00	46	61			19	28	25	25			110	102
Total	97	163	17	34	113	297	307	343	113	120	329	392

Table 3: Results of the statistical analysis of yearly variation in the longitudinal/temporal groups, for two statistics Fst and PHIst. See Table 1 for the surveys and sample sizes used in each test. No test was conducted for group IIIEL because the small sample size available for this group. P values below 5% are shown in **bold**. P values below 10% are shown underlined.

	Fst	P	PHIst	P
III EE	-0.002	0.726	-0.001	0.526
IV WE	0.005	0.053	0.008	**0.017**
IV WL	0.002	0.121	0.001	0.295
IV EE	0.002	0.271	0.011	0.055
IV EL	-0.001	0.595	0.001	0.335

Table 4: Results of the statistical comparisons between groups in Areas III and IV and a 'core sample' from Area V (n=1,443). See text for definition of the 'core' sample. Results are shown for both Fst and PHIst statistics. P values below 5% are shown in **bold**. P values below 10% are shown underlined.

Groups	Fst	P	PHIst	P
IIIEE Total (260)	-0.001	0.658	-0.000	0.411
*IVWL Total (650)	0.001	0.066	0.001	**0.026**
IVEE Total (233-12)	-0.001	0.753	0.001	0.202
IVEL Total (721)	0.000	0.179	-0.000	0.485
*IVWE Total (410)	0.001	0.051	0.001	0.092
*IVWE 89/90 (118)	0.006	**0.012**	0.011	**0.002**
IVWE 91/92 (42)	-0.001	0.504	0.001	0.319
IVWE 95/96 (131)	-0.000	0.473	-0.001	0.683
*IVWE 97/98 (72)	0.006	0.051	0.009	**0.024**
IVWE 99/00 (47)	0.004	0.159	0.004	0.203

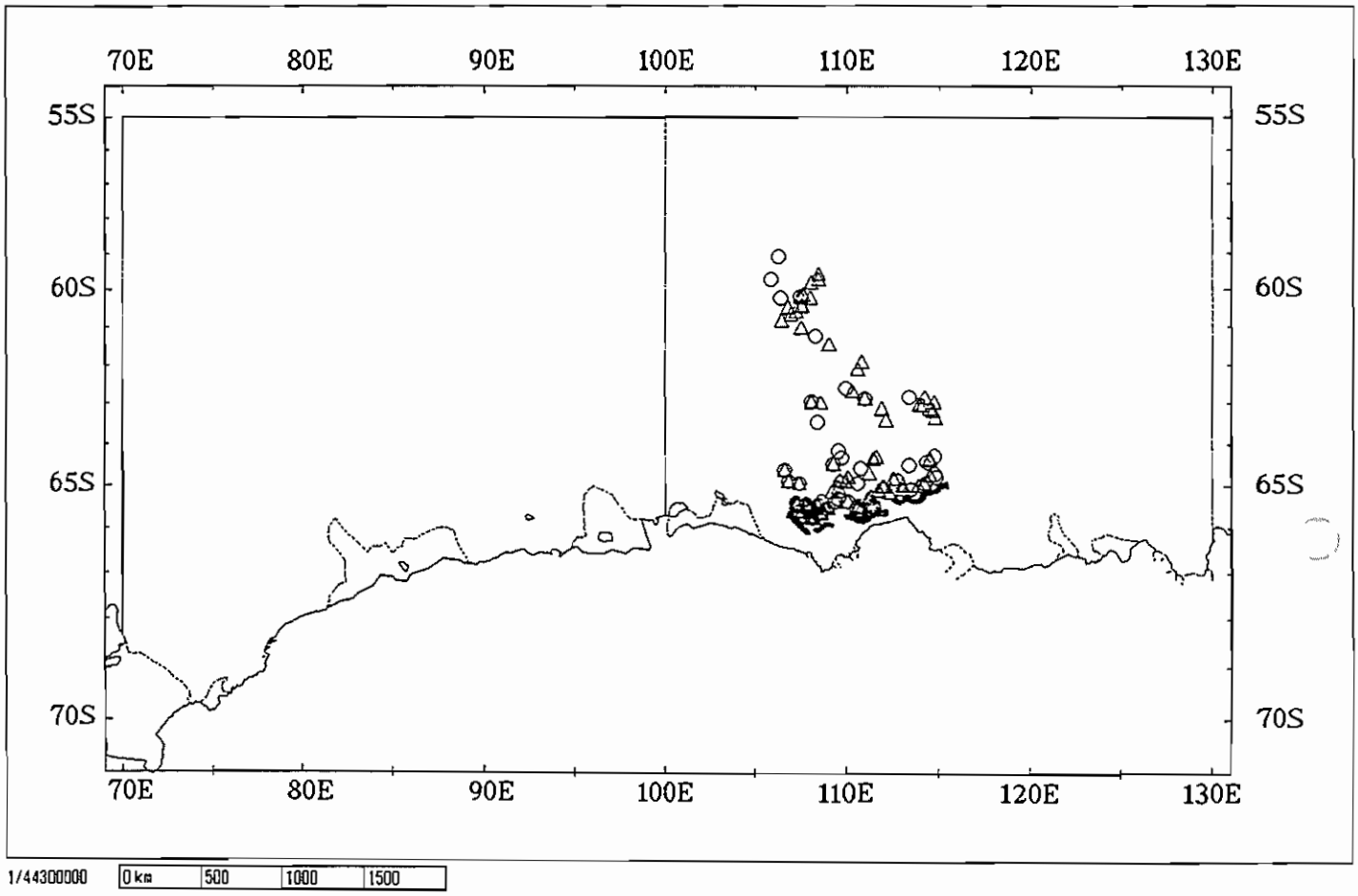

Fig. 1: Geographical distribution of longitudinal/temporal groups of Antarctic minke whales in Area IV in 1987/88, by sex. Area IV was divided into west and east at 100°E. Explanations of symbols is as follow: ● = early female; ▲= early male; ○= late female; △= late male. Position of the ice-edge in the early and late periods is indicated by line and gray-shaded figures, respectively.

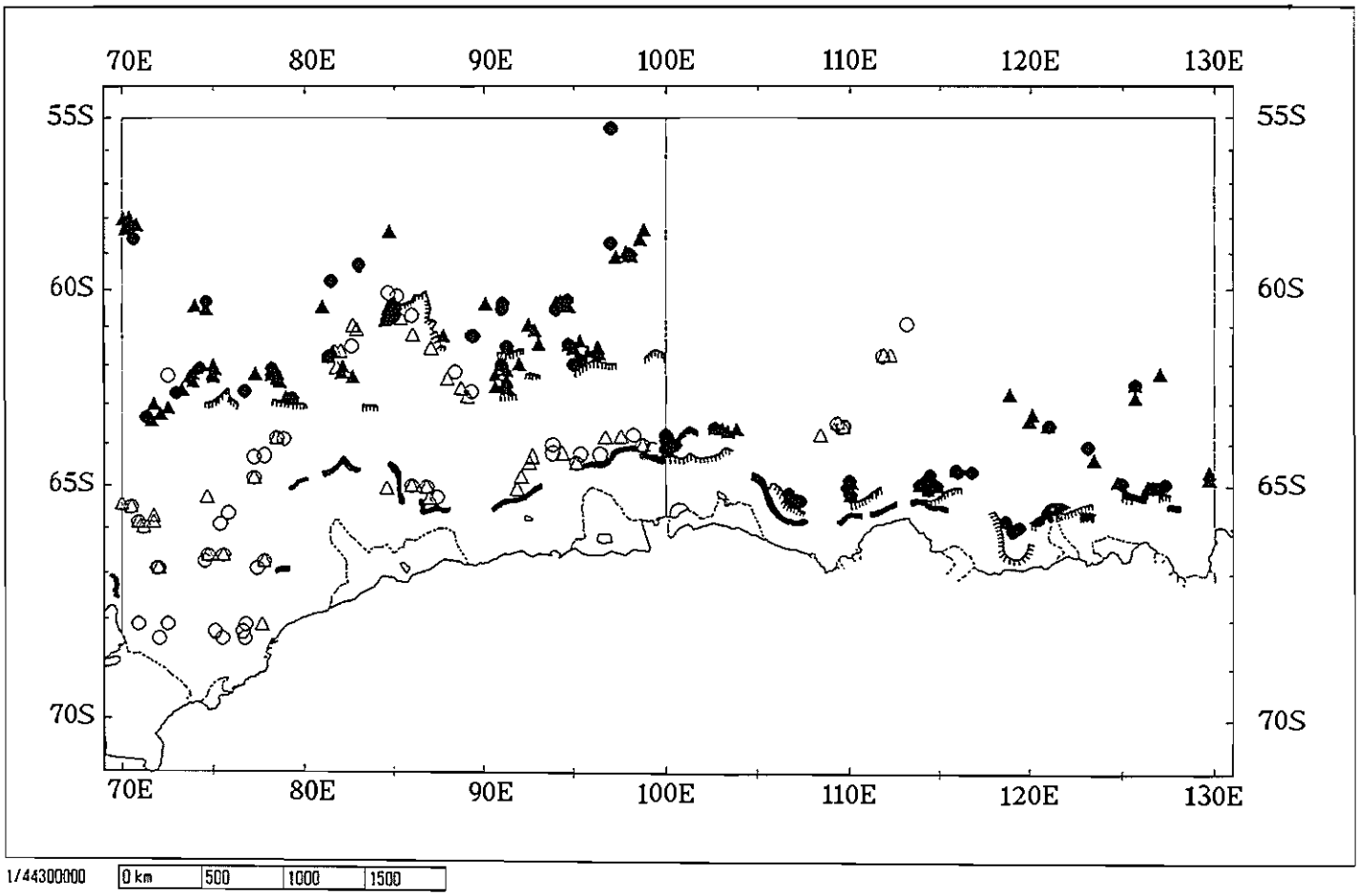

Fig. 2: Geographical distribution of longitudinal/temporal groups of Antarctic minke whales in Area IV in 1989/90, by sex. Area IV was divided into west and east at 100°E. Explanations of symbols is as follow: ● = early female; ▲= early male; ○= late female; △= late male. Position of the ice-edge in the early and late periods is indicated by line and gray-shaded figures, respectively.

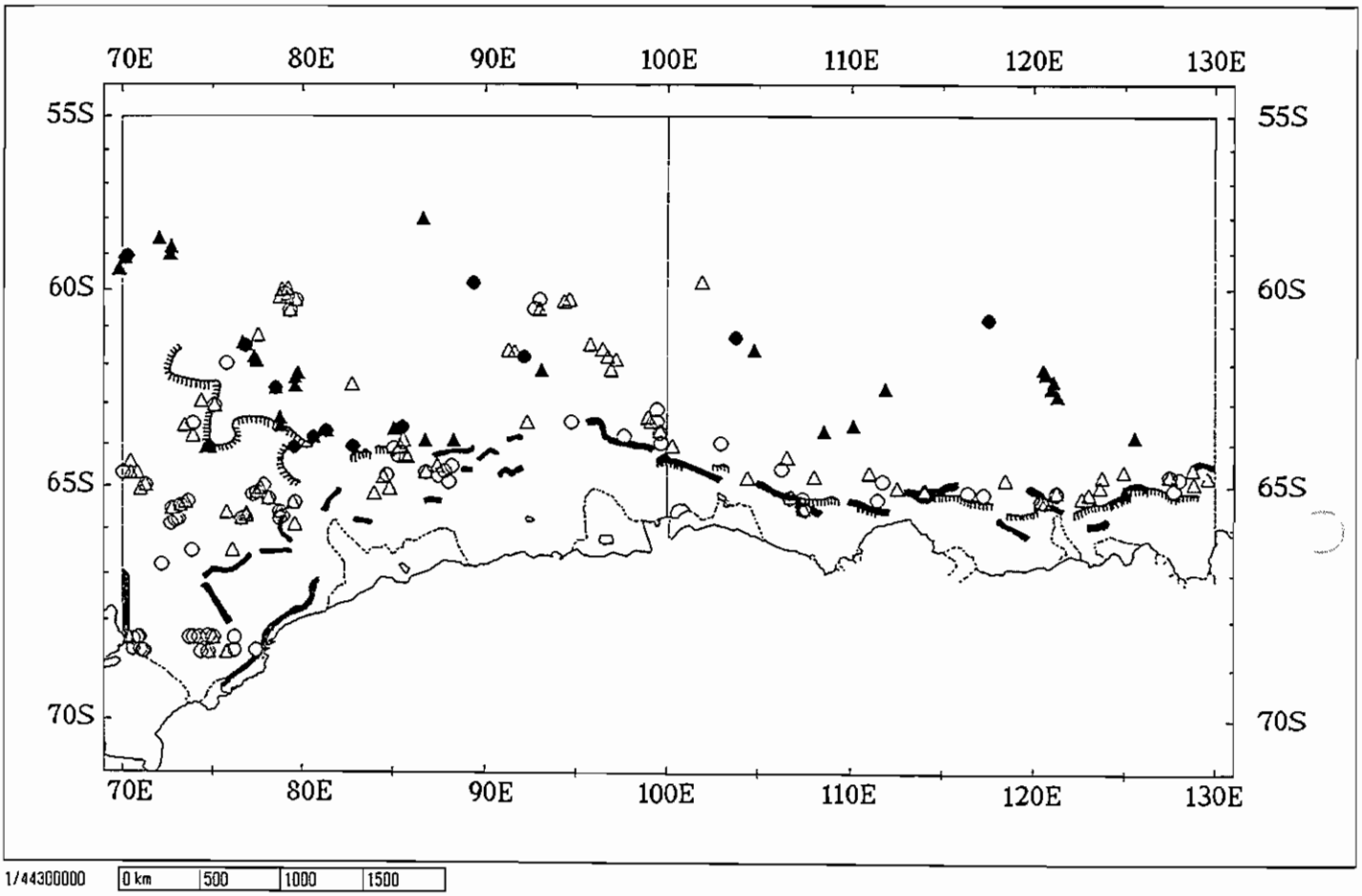

Fig. 3: Geographical distribution of longitudinal/temporal groups of Antarctic minke whales in Area IV in 1991/92, by sex. Area IV was divided into west and east at 100°E. Explanations of symbols is as follow: ● = early female; ▲= early male; ○= late female; △= late male. Position of the ice-edge in the early and late periods is indicated by line and gray-shaded figures, respectively.

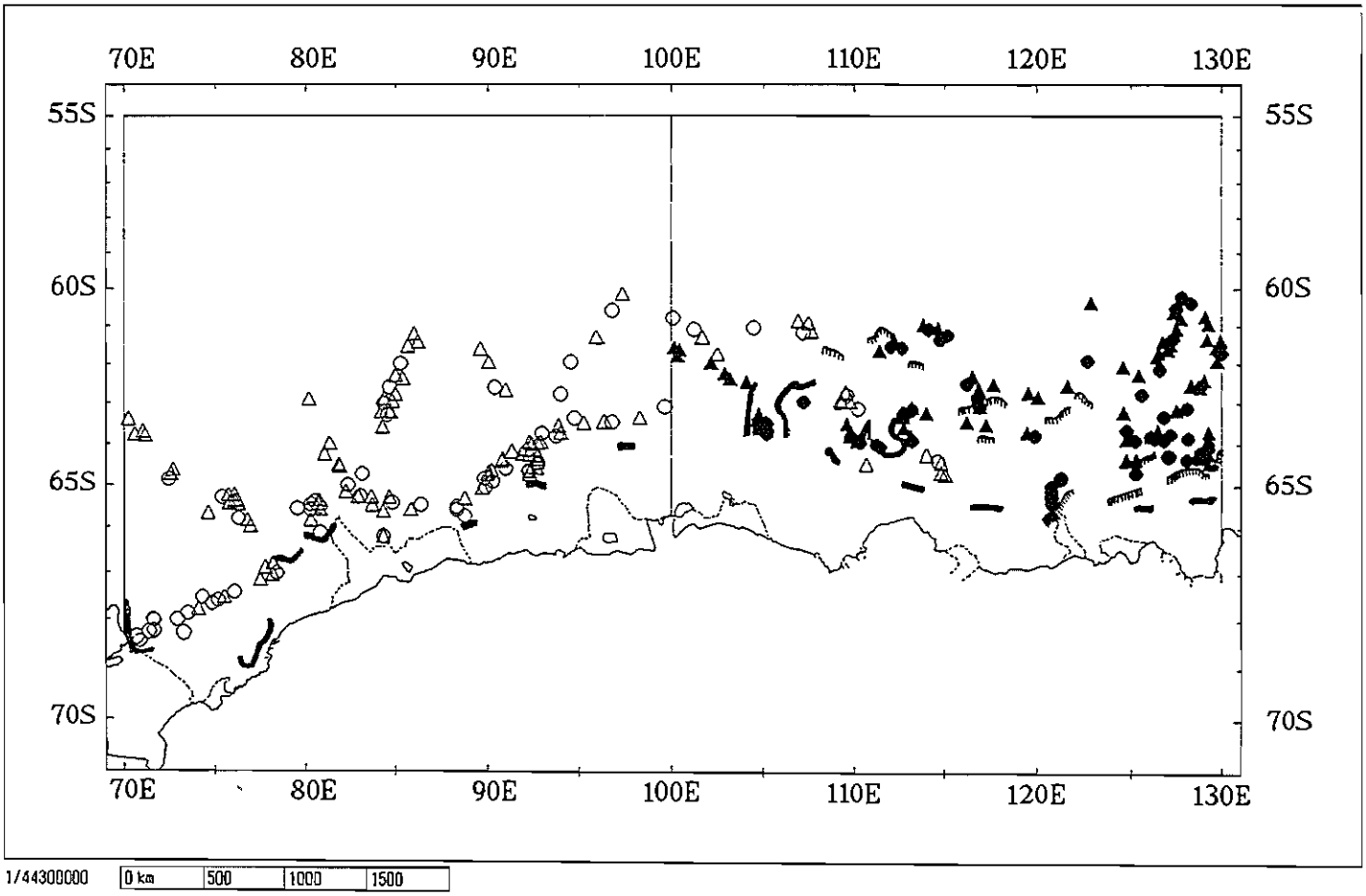

Fig. 4: Geographical distribution of longitudinal/temporal groups of Antarctic minke whales in Area IV in 1993/94, by sex. Area IV was divided into west and east at 100°E. Explanations of symbols is as follow: ● = early female; ▲= early male; O= late female; △= late male. Position of the ice-edge in the early and late periods is indicated by line and gray-shaded figures, respectively.

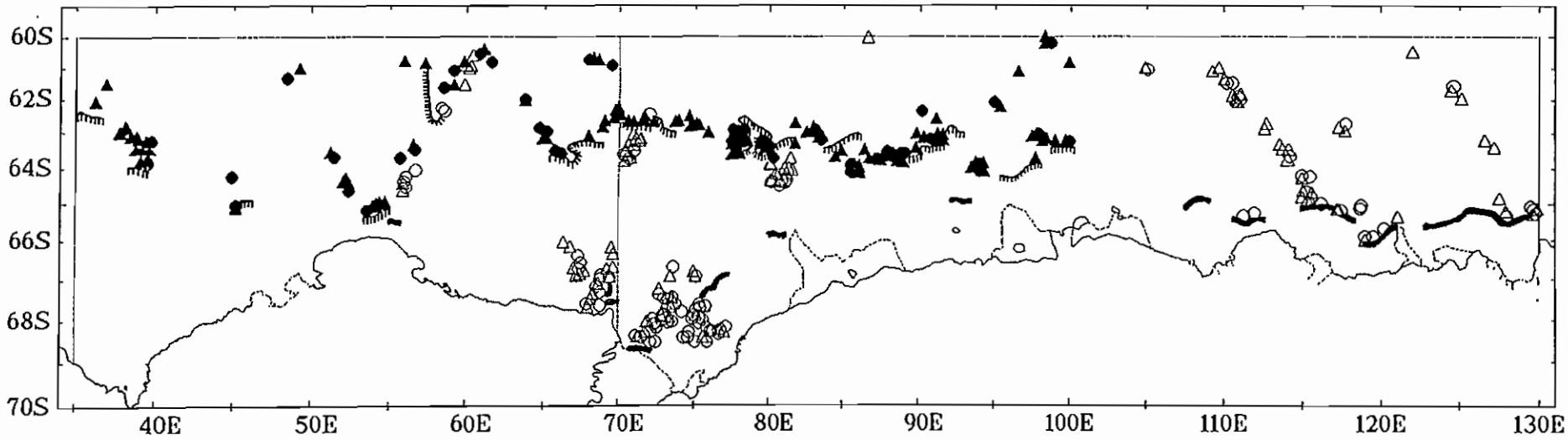

Fig. 5: Geographical distribution of longitudinal/temporal groups of Antarctic minke whales in Area IV and eastern part of Area III in 1995/96, by sex. Area IV was divided into west and east at 100°E. Explanations of symbols is as follow: ● = early female; ▲= early male; ○= late female; △= late male. Position of the ice-edge in the early and late periods is indicated by line and gray-shaded figures, respectively.

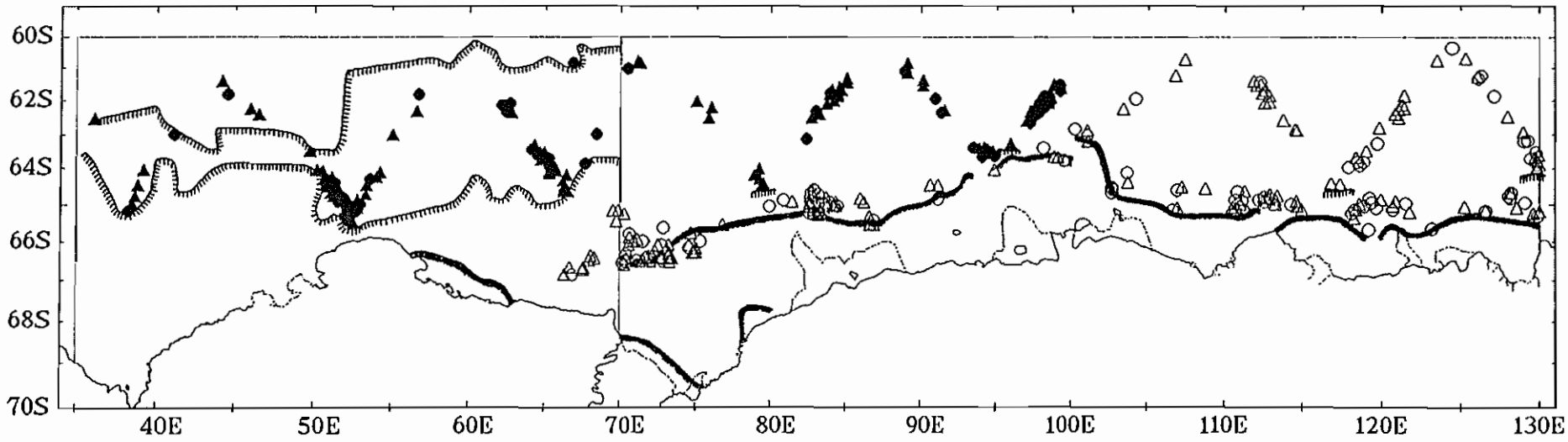

Fig. 6: Geographical distribution of longitudinal/temporal groups of Antarctic minke whales in Area IV and eastern part of Area III in 1997/98, by sex. Area 1V was divided into west and east at 100°E. Explanations of symbols is as follow: ● = early female; ▲= early male; ○= late female; △= late male. Position of the ice-edge in the early and late periods is indicated by line and gray-shaded figures, respectively.

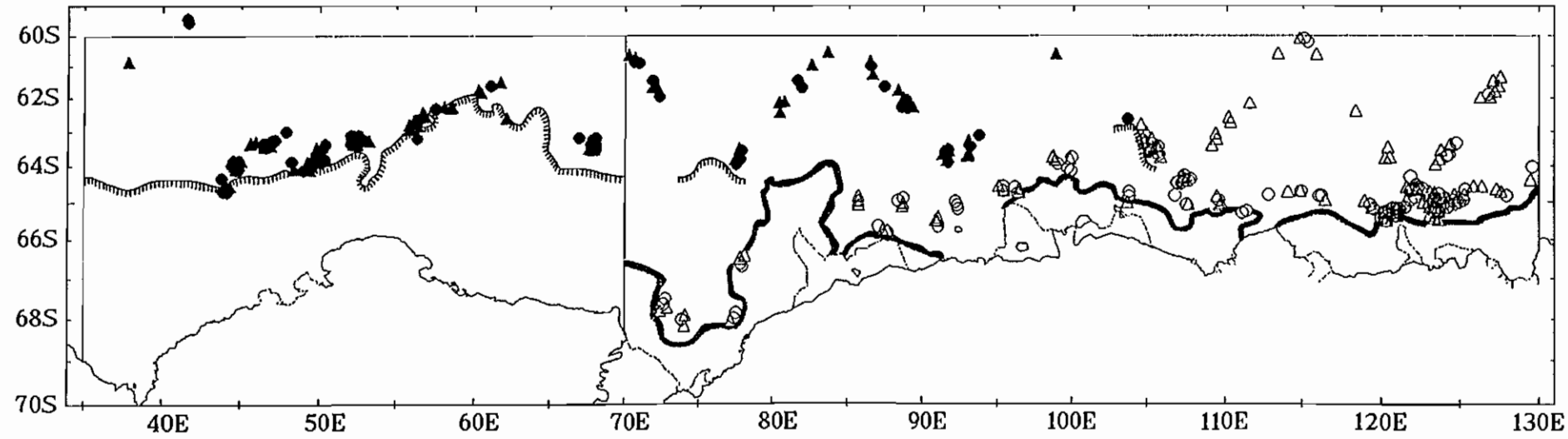

Fig. 7: Geographical distribution of longitudinal/temporal groups of Antarctic minke whales in Area IV and eastern part of Area III in 1999/00, by sex. Area IV was divided into west and east at 100°E. Explanations of symbols is as follow: ● = early female; ▲= early male; ○= late female; △= late male. Position of the ice-edge in the early and late periods is indicated by line and gray-shaded figures, respectively.