An update of the mitochondrial DNA RFLP analysis in the Antarctic minke whales from Areas V and VI

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ABSTRACT

A restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) in the Antarctic minke whale from Areas V and VIW was conducted using samples from the 1988/89-2000/01 JARPA surveys. Samples were divided arbitrarily as follow: Area V Western (130°-165°E), Area V Eastern (165°E-170°W) and Area VI Western (145°W-170°W) and two temporal periods (Early and Late). A total of 2,228 samples was examined in six longitudinal/temporal groups as follow: VWE, n=215; VWL, n=703; VEE, n=200; VEL, n=844, VIWE, n=194 and VIWL, n=72. As out-group, we used a sample from Area IVWE (n=160). 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. First we compared female and male samples in each of the longitudinal/temporal/year groups. If no significant differences were found we pooled male and female samples and then we compared a particular longitudinal/temporal group among years. If no significant yearly differences were found we pooled longitudinal/temporal groups from several years. Finally a hierarchical analysis was conducted using the total samples and considering both geographical and temporal criteria. Overall no significant mtDNA heterogeneity was found in Areas V and VIW. Some degree of yearly variation was detected in group VWE using the Fst. However no significant mtDNA heterogeneity was found in the hierarchical analysis after use different combinations for group VWE. Each of the longitudinal/temporal groups in Areas V and VIW differed significantly from the out-group sample from Area IVW in both Fst and PHIst statistics.

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

INTRODUCTION

One of the main research objectives of the JARPA (the Japanese Whale Research Program under Special Permit in the Antarctic) is the estimation of biological parameters required for the stock management of minke whale. The accuracy of such estimation depends, among other factors, on information on stock identity in the research area. Several studies on stock identity have been conducted using samples and data collected during JARPA surveys (see review of these studies in Pastene and Goto, 1997).

These studies have been based mainly on mitochondrial DNA (mtDNA) RFLP analysis. Results 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). In addition to the genetic analyses, a study on morphology and morphometry found also some degree of heterogeneity in Area IVW (Fujise, 1995).

Since 1995/96 the JARPA surveys were extended geographically to the eastern part of Area III and western part of Area VI. Such expansion was needed for a better interpretation of the pattern of genetic heterogeneity found in Areas IV and V. Of particular interest has been the investigation of possible temporal and geographical boundary of the hypothesized 'core stock', which occupy the core of Areas IV and V (Pastene *et al.* 1996a). An update of the mtDNA analysis conducted in Areas IIIE and IV was

presented to the Scientific Committee last year (Pastene *et al.* 2001). Here we present an update of mtDNA analyses in Areas V and VIW by using the total JARPA samples available from these Areas. The goal of this analysis is to investigate whether or not an eastern boundary for the 'core stock' can be defined.

MATERIALS AND METHODS

Samples

Samples of the Antarctic minke whale from Areas V and VIW were available from seven JARPA surveys (see Table 1). For each year and sex, samples were grouped into Area V Western Early (group VWE, n=215), Area V Western Late (group VWL, n=703), Area V Eastern Early (group VEE, n=200), Area V Eastern Late (group VEL, n=844), Area VI Western Early (group VIWE, n=194) and Area VI Western Late (group VIWL, n=72). Thus a total of 2,228 samples was examined in this study. Area V was divided into western and eastern sectors at 165°E; '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. Group VEL was further divided into north (VELn, n=335) and south (VELs, n=509) at 69°S (Table 1). The southern sample in this group corresponds to minke whales sampled in the Ross Sea. In the1998/99 survey, it was not possible to sample minke whales from the Ross Sea. The VEL samples in that survey were from the north strata only (although some few samples were taken just south of 69°S). For comparison we used an out-group sample from Area IV (Area IV Western Early 1989/90+1991/92, n=160).

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. In the text we will refers to these simply as 'group'.

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 2,000 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. As mentioned earlier, group VEL was further divided into two latitudinal strata. First we tested for differences between sexes in each of the groups. If no significant differences were found both sexes were pooled in these groups. Second we tested for yearly differences in the groups. If no significant yearly differences were found, then samples from different years were pooled in the subsequent analyses.

Finally a hierarchical analysis was conducted using the total samples grouped into three longitudinal sectors and two temporal groups in each sector.

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.

MtDNA of the sexes

None of the longitudinal/temporal/year groups showed significant differences between male and female samples. Male and female samples were combined in the subsequent analyses.

Yearly variation in the longitudinal/temporal groups

Results of the statistical analysis of yearly variation in the longitudinal/temporal groups are shown in Table 2, for both Fst and PHIst statistics. Results for the south and north strata in group VEL are also shown. The only evidence for yearly variation is given for group VWE where the P-value for the Fst was below the 5% critical level. Pairwise yearly comparison using Fst for group VWE (data not shown) showed that group VWE 1990/91 (n=30) differed significantly from the other two surveys (1992/93 and 1994/95, n=83 and 102, respectively). Thus in the subsequent hierarchical Fst analysis we considered two options for group VWE: 1990/91 and 1992/93+1994/95.

No significant differences were found between latitudinal strata in the group VEL (Fst=0.002, P=0.061; PHIst=-0.000, P=0.433). In the subsequent hierarchical analysis both latitudinal strata were combined in a single VEL group.

Hierarchical analysis

Table 3 shows the results of the hierarchical analysis by AMOVA. Tables 3A and 3B show the results of Fst for the two options of group VWE (1990/91 only and 1992/93+1994/95), respectively. Table 3C shows the results for PHIst using the total samples. Results for both statistics are similar in the three cases, with most of the molecular variance attributed to within groups. In the PHIst analysis the P-values of the three tests conducted were high. Although none of the P-values in the Fst analyses were significant, it is noted that for the tests 'among longitudinal sector' and 'within longitudinal/temporal sector', the P-values in the case of Table 3B were smaller than in case of Table 3A. Overall these results indicate that minke whales from Area V are similar in mtDNA composition to those from Area VIW.

DISCUSION

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). Based on these results it was hypothesized that more than one stock is involved in Areas IV and V, with a 'western stock' distributed early in the austral summer season in Area IVW (at least in some years) and a 'core stock' occupying the core of these Areas. It was also proposed a temporal component in the distribution of these stocks in Area IVW. Considering the main objective of the JARPA, which is the estimation of the biological parameters in the minke whale, there is an interest in defining a biological stock on which these estimation can be made. For such reason we were interested to investigate the geographical and temporal boundaries of the hypothesized 'core stock' by investigating the genetic composition of minke whales distributed in adjacent areas, e.g. Area IIIE and Area VIW. In that context in this study we examined all the samples available from Areas V and VIW, with the objective to define an eastern boundary for this stock.

Unlike the case of Areas IIIE and IV we found little mtDNA heterogeneity in Areas V and VIW. Fst found the only heterogeneity in the analysis of yearly variation for group VWE only). Whales from survey 1990/91 seemed to differ in haplotype frequencies from the surveys 1992/93 and 1994/95. In the hierarchical analysis smaller (but not significant) P-values were found when the latter groups were considered (Table 3B). Pairwise comparisons using Fst revealed some degree of heterogeneity between

Group VWE (92/93+94/95) and the other groups in Areas V and VIW. Additional analysis conducted to investigate further this result indicated that the inclusion/exclusion of samples from the 1998/99 survey affected the results. For instance the P-values in Table 3B changed to 0.267, 0.523 and 0.227, respectively, when samples from that survey were excluded. It should be noted that the sampling coverage (geographical and temporal) in the 1998/99 survey was smaller than the other surveys in Areas V and VIW. Samples in that survey were obtained mainly around the ice-edge (Fig. 6). It has been suggested that samples taken from restricted areas or periods could be not representative of the genetic diversity (Goto *et al.*,1998; Pastene and Goto, 2000).

Individual longitudinal/temporal groups in Areas V and VIW were then compared with a sample from Area IVWE (1989/90+1991/92). Previous studies showed that this sample is differentiated in their mtDNA from most other groups in Areas IV and V. Almost all comparisons with this out-group sample revealed significant mtDNA differences, for both Fst and PHIst. In summary no significant mtDNA heterogeneity was found in Areas V and VIW but groups in these Areas seemed to be differentiated from Area IVWE.

One of the aspects requesting further consideration, is that related with the way as the samples were grouped. In our analysis the issue of yearly variation was addressed first. The question addressed here was whether or not the whales occupying a determined longitudinal sector (in a determined period) in different years (surveys) have a similar mtDNA composition. The results of this first examination were used for grouping the samples for the subsequent hierarchical analysis. An alternative way to conduct the analysis is by addressing the geographical and temporal heterogeneity within a year (survey) first and then the issue of yearly variation could be addressed. The first approach (used in our study) has the merit that the analysis of temporal and geographical variation in Areas V and VIW is based in a larger number of samples, and then the power of the statistical analysis is increased. The second approach has the merit that the analysis of temporal and geographical variation will consider whales sampled in a same year (survey), under the same conditions. This alternative way of grouping the samples should be further explored.

The other issue requesting consideration is the sample size. As suggested by Pastene *et al.* (1996b), a minimal of 150-200 samples is requested to detect significant differences. The groups considered in the hierarchical analysis involved sample sizes within or larger than this range. However, analysis to investigate differences between sexes as well differences among years, in some cases involved sample sizes smaller than this range. Sample sizes were larger for Area V than Area VIW. In the latter Area only 194 samples were available for group VIWE. Based on these differences in sample sizes, we feel more confident on the results obtained for Area V.

It should be noted that sample size is a complex issue and it depend on a combination of factors, among them, the level of differentiation between putative stocks and whether the samples were taken in offshore areas or around the ice-edge (Goto *et al.* 1998).

Finally we should remind of some important issues affecting the research on stock identity of minke whale in the Antarctic: a) we are dealing only with samples in the feeding grounds. Analysis of samples from lower latitudes would facilitate the interpretation of the pattern of variation found in the Antarctic; b) the analysis of additional genetic marker (e.g. Abe *et al.*, 1999) as well of other non genetic marker would contribute for a better interpretation and comprehensive view on stock structure in the area; c) patterns of genetic variation in the Antarctic could be correlated with the dynamic of prey species and oceanographic feature in the research area (e.g. Murase *et al.* 2002). It is noted that the study by Abe *et al.* (1999) revealed some degree of nuclear DNA heterogeneity in the Antarctic and such results deserve further consideration.

ACKNOWLEDGMENTS

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Table 1: 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	VWE		VWL		VEE		VEL				VIWE		VIWL	
	F	M	F	M	F	M	Fs	Ms	Fn	Mn	F	M	F	M
1988/89					5	5	31	9	26	30				
1990/91	19	11	56	93	4	16	68	9	5	28				
1992/93	41	42	40	70	12	19	52	30						
1994/95	20	82	5	18	7	6	79	24	14	51				
1996/97			66	53	26	41	88	17	5	19	31	60	5	8
1998/99			81	89					34	105			20	39
2000/01			40	92	10	49	80	22	4	14	35	68		
Total	80	135	288	415	64	136	398	111	88	247	66	128	25	47

Table 2: Results of the statistical analysis of yearly variation in the longitudinal/temporal groups, for two statistics, Fst and PHIst. See Table 1 for the number of surveys and sample sizes used in each test. P values below 5% are shown in **bold**.

	Fst	P	PHIst	P
VWE	0.014	**0.021**	0.005	0.120
VWL	-0.001	0.659	-0.003	0.939
VEE	-0.009	0.970	0.000	0.443
VEL-south	0.003	0.112	0.003	0.127
VEL-north	0.001	0.313	-0.002	0.589
VIWE	-0.001	0.483	-0.002	0.596

Table 3: Results of the nested analysis of molecular variance of minke whale mtDNA haplotypes in Areas V and VIW. 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. PHIct and the among longitudinal sectors variance component involves the permutation of whole longitudinal/temporal groups among sectors; the PHIsc and the among temporal groups within sectors involves the random permutation of individuals among temporal groups within sectors; the PHIst and the within longitudinal/temporal groups components involves the random permutation of individuals among the six longitudinal/temporal groups. Group VWE differed among years using the Fst statistics (see Table 2 and text). Thus the Fst hierarchical analysis was conducted considering two set of sample for this group: VWE 90/91 (n=30) (Tables 3A) and VWE 92/93+94/95 (n=185) (Table 3B). Table 3C shows the results for PHIst using the total sample.

3A: Fst

	df	% total variance	Fst	P
Among long. Sector	2	-0.07	CT: -0.001	0.608
Among temp. in sector	3	0.11	SC: 0.001	0.234
Within long./temp. gr	2,037	99.97	ST: 0.000	0.237

3B: Fst

	df	% total variance	Fst	P
Among long. Sector	2	0.02	CT: 0.000	0.072
Among temp. in sector	3	0.07	SC: 0.001	0.230
Within long./temp. gr	2,192	99.91	ST: 0.001	0.085

3C: PHIst

	df	% total variance	Fst	P
Among long. Sector	2	-0.09	CT: -0.001	0.929
Among temp. in sector	3	0.04	SC: 0.000	0.335
Within long./temp. gr	2,222	100.05	ST: -0.001	0.556

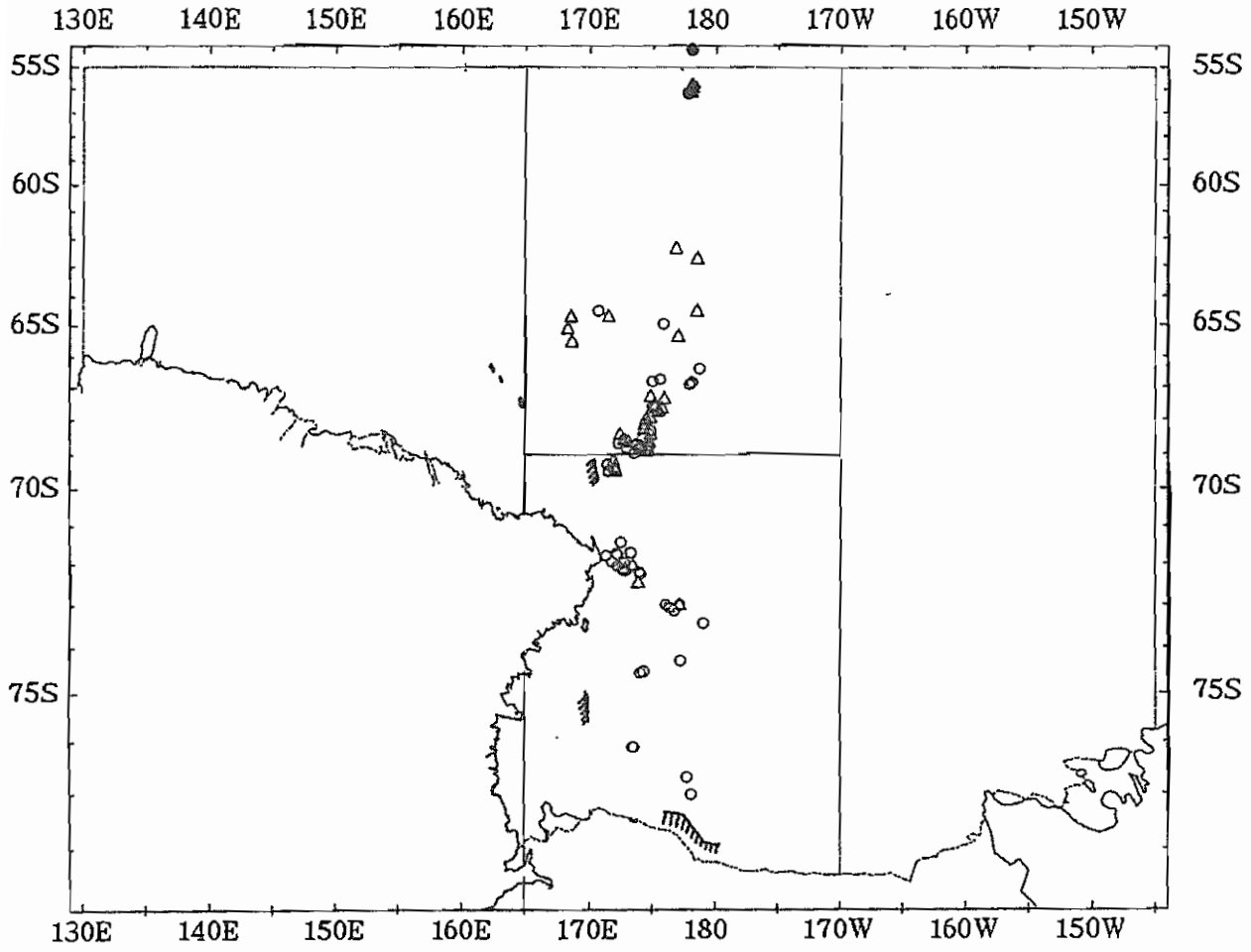

Fig. 1: Geographical distribution of longitudinal/temporal groups of minke whales in Area V in JARPA 1988/89, by sex. Area V was divided into west and east at 165°E. Group VEL was divided into two latitudinal strata at 69°S. Explanation of symbols is as follow:● = early female; ▲ = early male;○ = late female; Δ = late male.

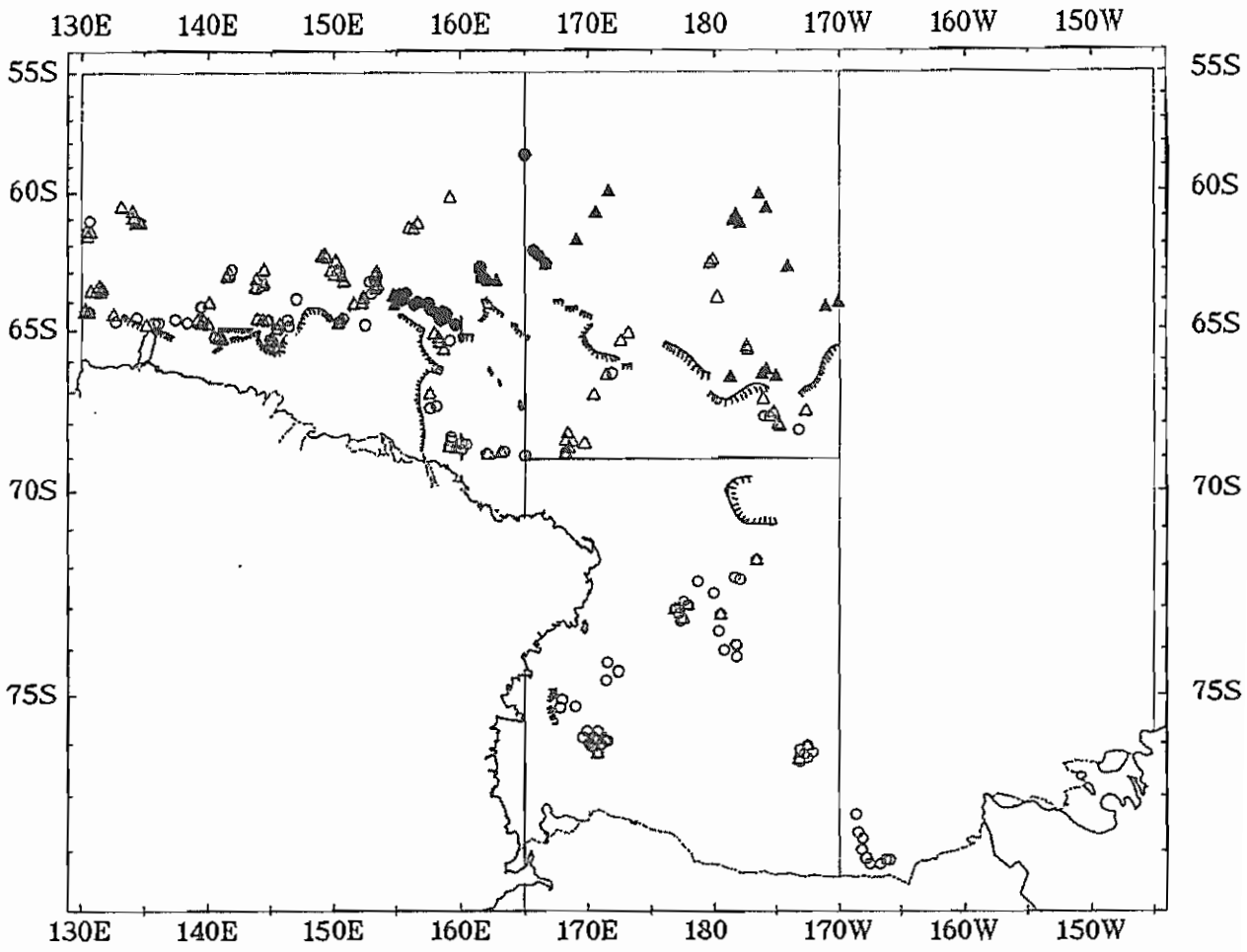

Fig. 2: Geographical distribution of longitudinal/temporal groups of minke whales in Area V in JARPA 1990/91, by sex. Area V was divided into west and east at 165°E. Group VEL was divided into two latitudinal strata at 69°S. Explanation of symbols is as follow: ● = early female; ▲ = early male; ○ = late female; △ = late male.

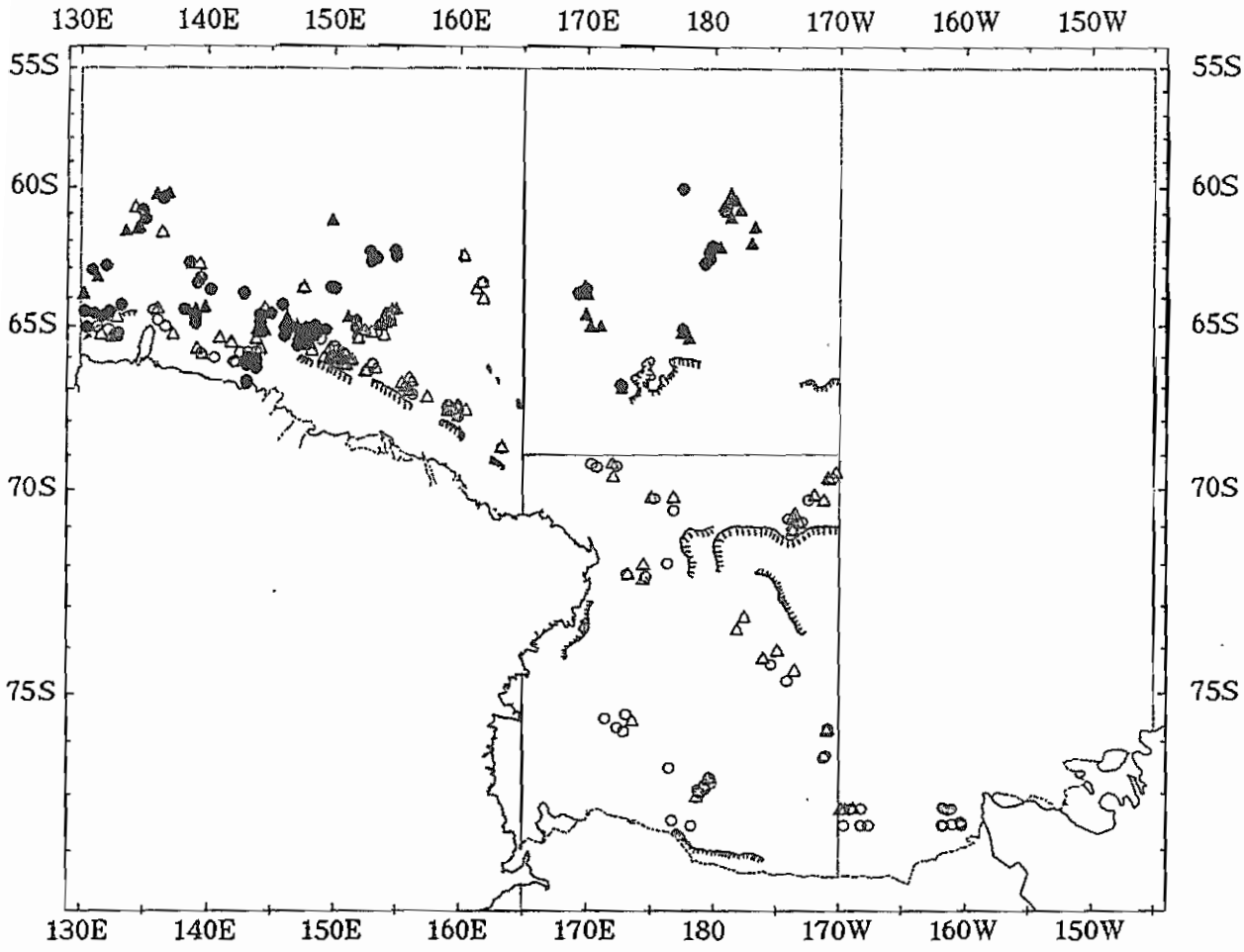

Fig. 3: Geographical distribution of longitudinal/temporal groups of minke whales in Area V in JARPA 1992/93, by sex. Area V was divided into west and east at 165°E. Group VEL was divided into two latitudinal strata at 69°S. Explanation of symbols is as follow:● = early female; ▲ = early male;○ = late female; Δ = late male.

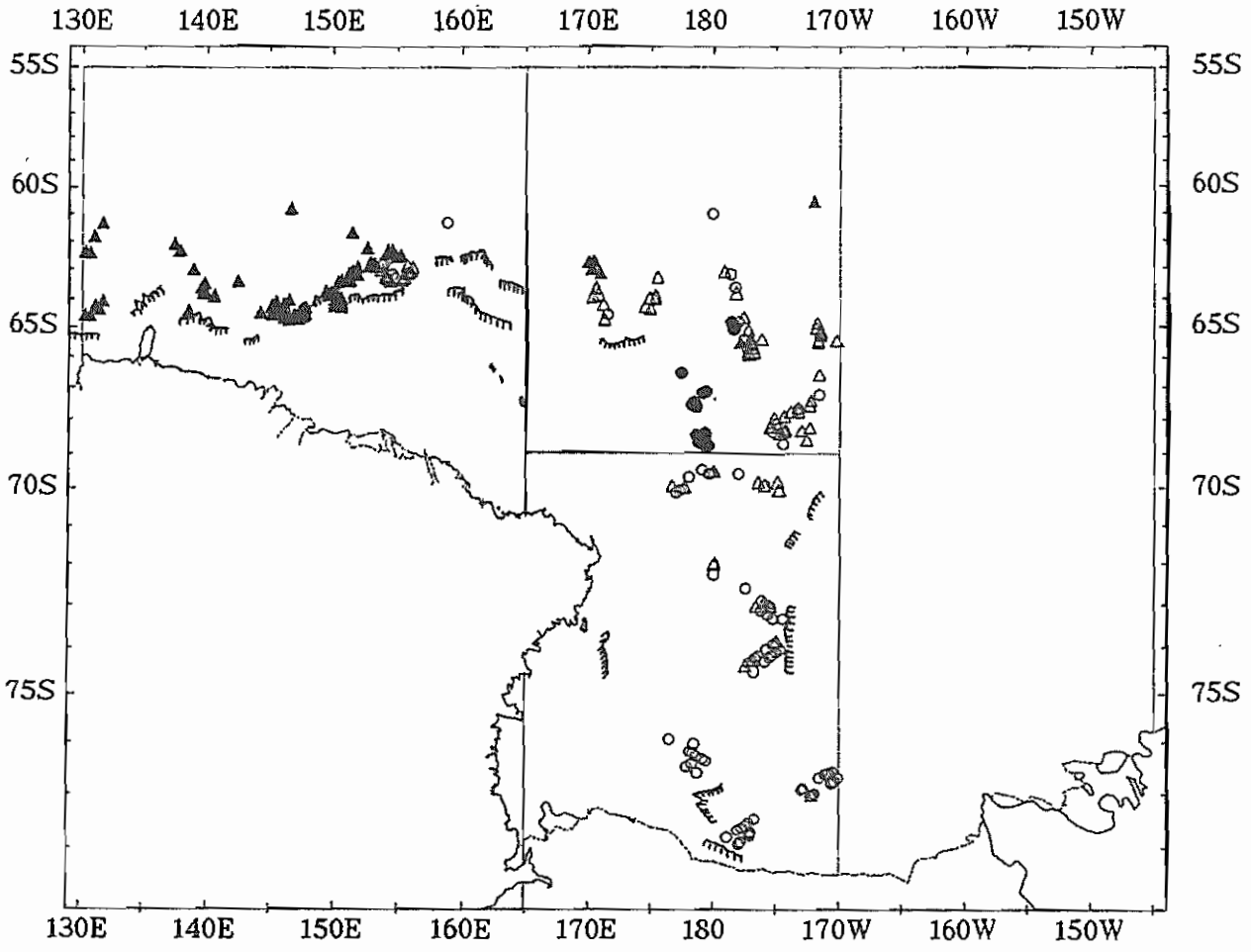

Fig. 4: Geographical distribution of longitudinal/temporal groups of minke whales in Area V in JARPA 1994/95, by sex. Area V was divided into west and east at 165°E. Group VEL was divided into two latitudinal strata at 69°S. Explanation of symbols is as follow:● = early female; ▲ = early male;○ = late female; Δ = late male.

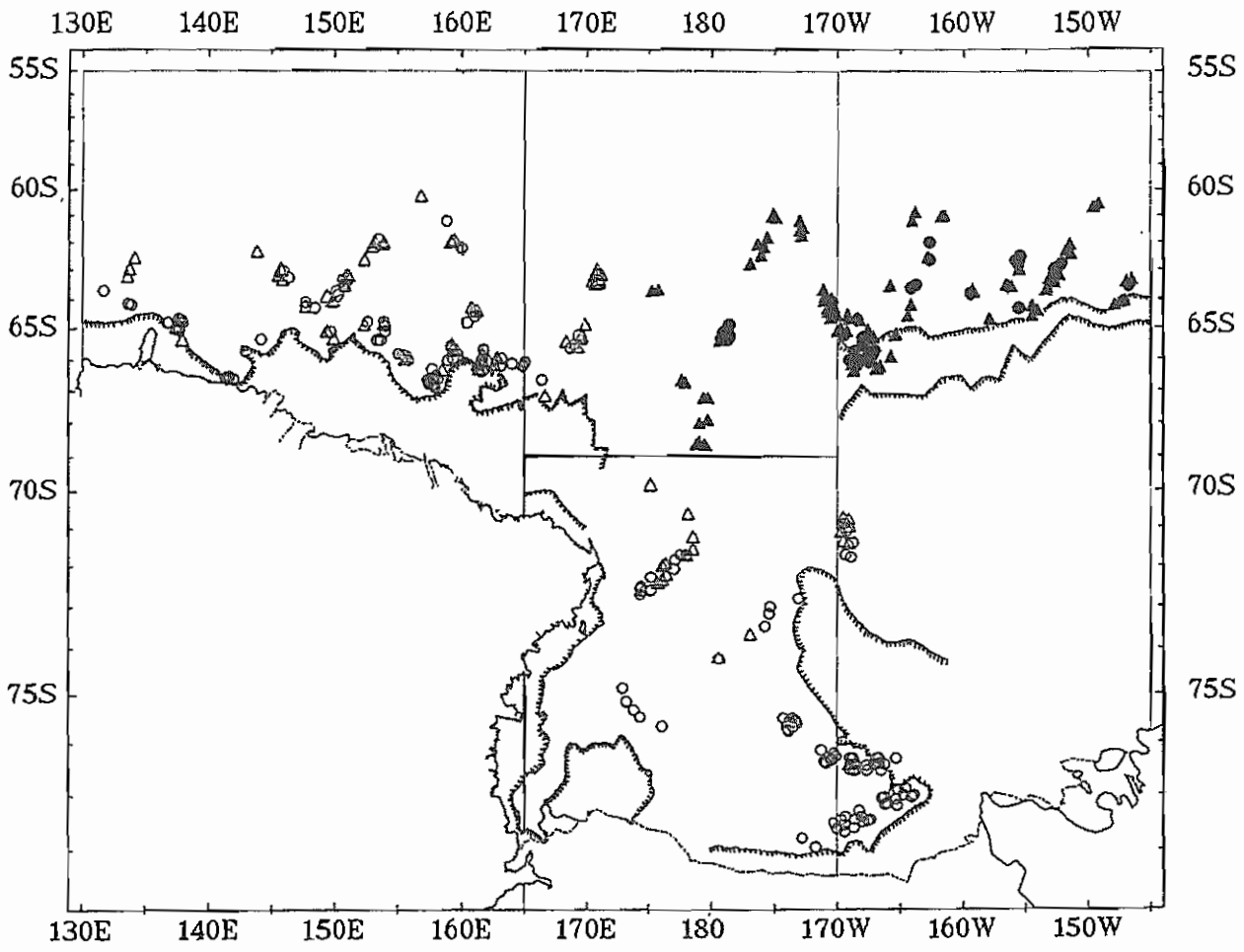

Fig. 5: Geographical distribution of longitudinal/temporal groups of minke whales in Areas V and VIW in JARPA 1996/97, by sex. Area V was divided into west and east at 165°E. Group VEL was divided into two latitudinal strata at 69°S. Explanation of symbols is as follow: ● = early female; ▲ = early male; o = late female; Δ = late male.

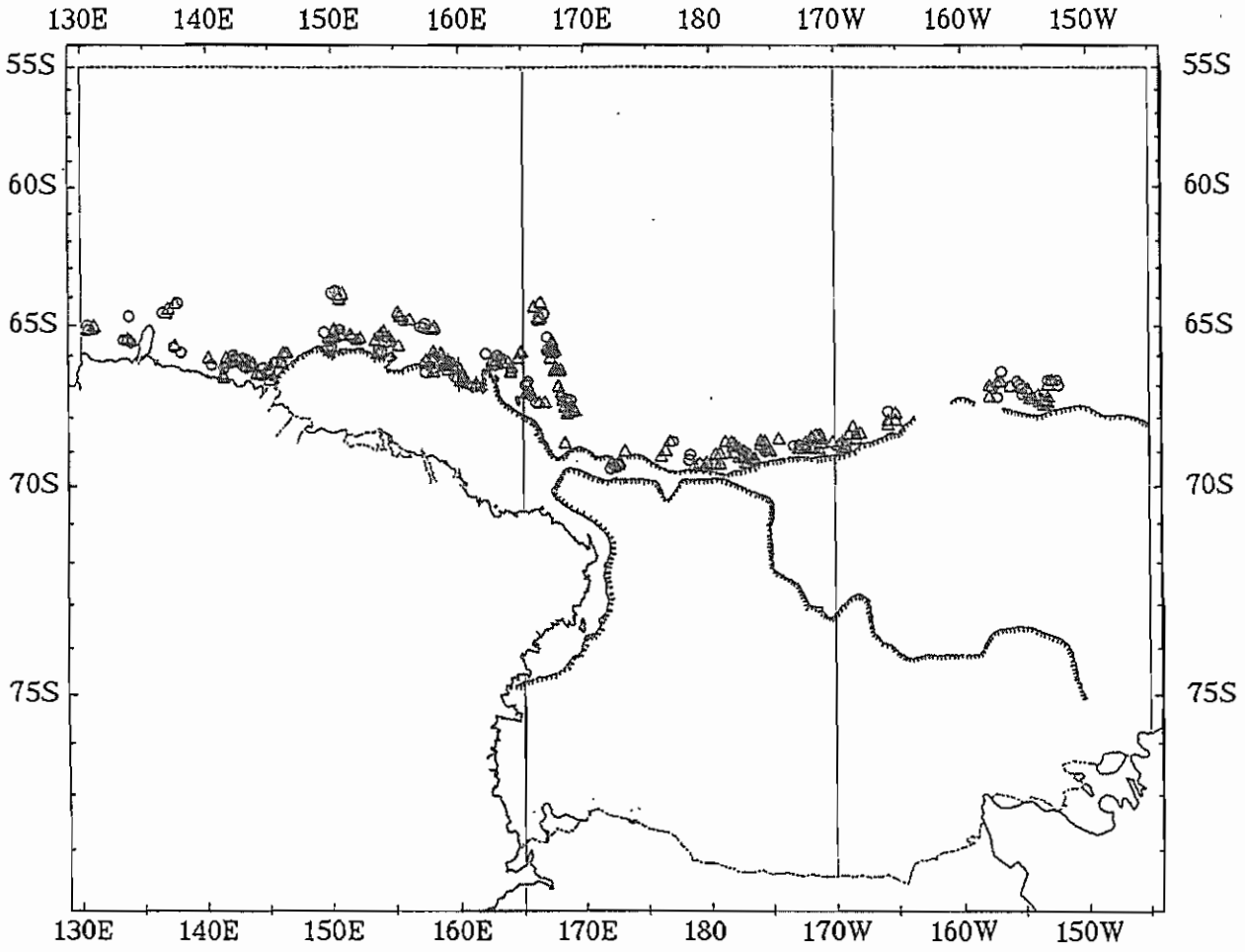

Fig. 6: Geographical distribution of longitudinal/temporal groups of minke whales in Areas V and VIW in JARPA 1998/99, by sex. Area V was divided into west and east at 165°E. Group VEL was divided into two latitudinal strata at 69°S. Explanation of symbols is as follow: ● = early female; ▲ = early male; ○ = late female; Δ = late male.

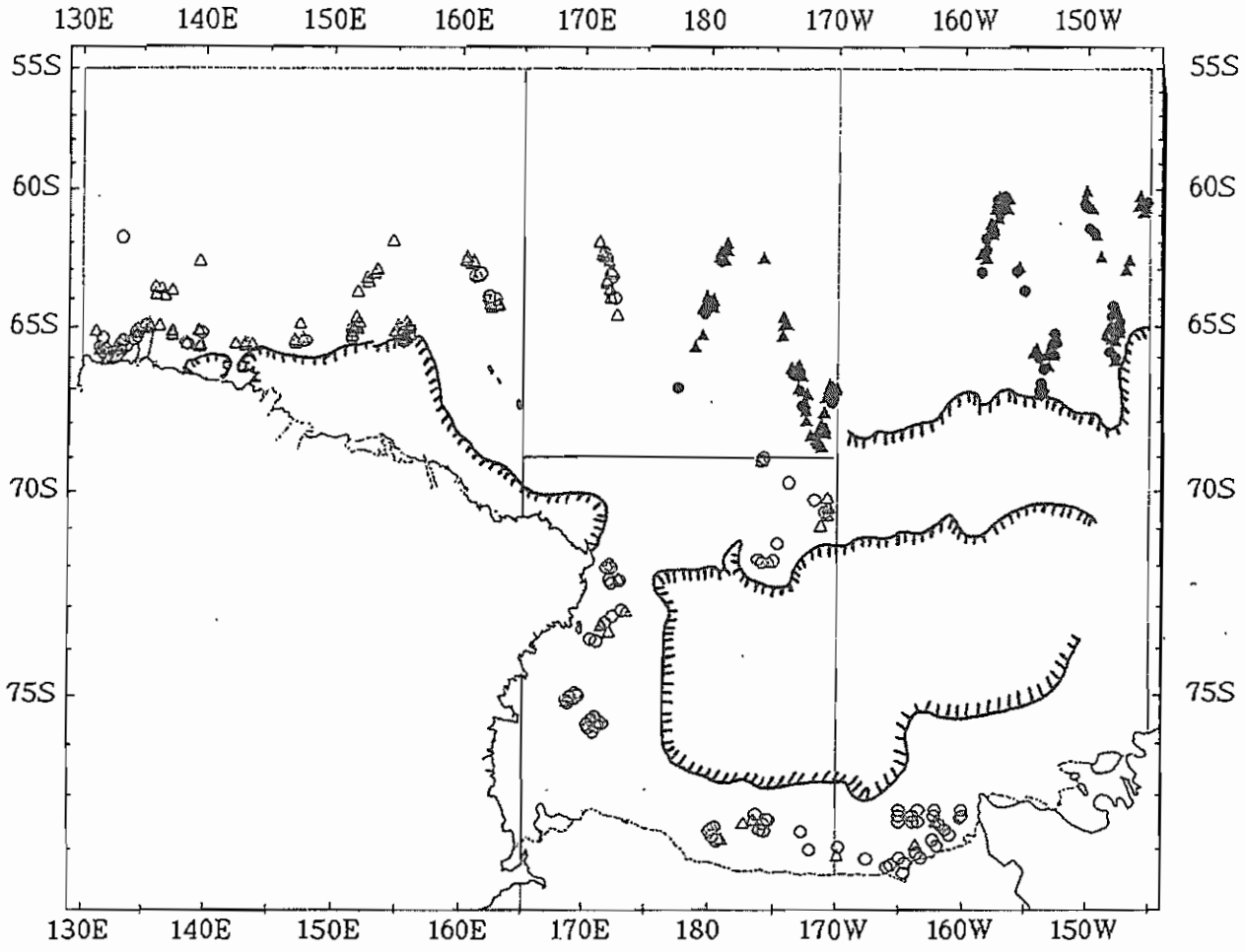

Fig. 7: Geographical distribution of longitudinal/temporal groups of minke whales in Areas V and VIW in JARPA 2000/01, by sex. Area V was divided into west and east at 165°E. Group VEL was divided into two latitudinal strata at 69°S. Explanation of symbols is as follow: ● = early female; ▲ = early male; ○ = late female; △ = late male.