

## Genetic examination of western North Pacific minke whales including samples from JARPN II

Mutsuo Goto, Naohisa Kanda and Luis A. Pastene

*The Institute of Cetacean Research*

*4-18 Toyomi-cho, Chuo-ku, Tokyo 104-0055, Japan*

### ABSTRACT

Genetic analyses was conducted on 40 minke whales (n=24 from sub-area 7; n=16 from western part of sub-area 9) sampled during the JARPN II feasibility survey in 2000. Both mitochondrial DNA, mtDNA (sequencing of a 487bp segment of the control region) and nuclear DNA, nDNA (seven microsatellite loci) were used. For comparison we included genetic data for these markers available from the JARPN 1994-99. With regard the JARPN II samples, certain degree of mtDNA heterogeneity was found in the samples from sub-area 9. No significant nDNA heterogeneity was found. This situation is similar to the case of the 1995 JARPN survey where the sample from sub-area 9 west was heterogeneous with regard mtDNA but not with regard nDNA. Preliminary, we discuss two possible explanations for the results obtained: i) possible additional structure within the O stock and, ii) mtDNA heterogeneity reflect possible sampling bias. It should be noted that the sample size in the JARPN II survey in sub-area 9 is small (n=16) and that it is desirable the analysis of the samples from the JARPN II in 2001 before to reach a definitive conclusion.

### INTRODUCTION

Goto *et al.* (2000) conducted mtDNA sequencing control region analysis on minke whales sampled by JARPN in 1994-1999. They had found certain degree of mtDNA heterogeneity, which was attributed to samples of the western part of sub-area 9 in the 1995 JARPN survey. No other source of genetic heterogeneity was found. An analysis of microsatellite (Abe *et al.*, 2000) based on the same samples used in the mtDNA analysis failed to find any genetic heterogeneity in sub-area 7, 8 and 9.

These studies were presented and discussed during the JARPN Review Workshop (IWC, 2001). In the light of the results of mtDNA analysis, the Workshop agreed that the possibility of the existence of some group of minke whales to the east of Japan that differed from the 'O' stock could not be ruled out. The Workshop recommended several additional mtDNA analyses using the available samples (IWC, 2001). In response to these recommendations, two genetic studies were reported to the 52<sup>nd</sup> SC meeting. Goto and Pastene (2000a) examined the relationship between space and/or time and genetic distance for different partitions using 'isolation by distance' analyses and Mantel tests. Goto and Pastene (2000b) re-examined the mixing proportion between 'O' and 'J' stocks to investigate the sensitivity of the estimate to the omission of the samples from sub-area 9 taken in 1995. However, no conclusive results were derived from these studies.

One of the objectives of JARPN II is the elucidation of the stock structure in western North Pacific minke whales. This objective has two components: i) to investigate whether or not the W Stock exist in sub-area 9, and if so, to investigate the spatial and temporal extent of its occurrence; ii) estimate the mixing proportion between O and J Stocks in sub-area 7. JARPN II began with a feasibility study in 2000. A total of forty

samples of minke whales (n=24 in sub-area 7 and n=16 in sub-area 9) was collected.

We presented here the results of mtDNA sequencing and microsatellite analyses conducted on the samples obtained by the JARPN II in 2000. For comparison we used genetic data obtained during the JARPN 1994-1999.

## MATERIALS AND METHODS

### Samples and localities

During the 2000 JARPN II survey conducted from July to September, a total of 40 minke whales was taken from sub-area 7 (August: n=6, September: n=18) and sub-area 9 (August: n=16). The total number of samples examined in this study (JARPN and JARPN II) is shown in Table 1 by sub-area, month and sex. The geographical localities of minke whales collected in 2000 are shown in Fig. 1. The samples from sub-area 9 in 2000 were taken to the west of 162°E.

Samples from sub-area 11 collected during the 1996 and 1999 JARPN surveys, were excluded from this study, because J and O stock animals temporary mix within this sub-area (Pastene *et al.*, 1998; Goto *et al.*, this meeting). Our analysis was focused to the eastern side of Japan.

### MtDNA analysis

#### *Sequencing of the mtDNA control region*

The first half of control region of the mitochondrial genome (487bp) was sequenced by the same method used in our previous study (Goto and Pastene, 2000c). All the procedures for DNA extraction and amplification of mtDNA control region were the same as in the previous study.

#### *Data analysis*

The Kimura's two parameters method (Kimura, 1980) was used for estimating genetic distances between two sequences. The degree of mtDNA diversity within each sub-area was estimated using the index of nucleotide diversity (Nei, 1987 pp. 256). The net genetic distances between sub-areas were estimated from equation 10.21 of Nei (1987). Homogeneity tests were conducted using the randomized chi-square test (Rice, 1989) and Hudson *et al.* (1992)'s sequence (Kst\*) and haplotype (Hst) statistics. The degree of divergence was inferred as being larger than zero, if an equal or more extreme value of Kst\*/Hst was observed in less than 5% of 10,000 Monte Carlo simulations.

### Microsatellites

#### *Genotyping*

Microsatellite polymorphisms were analyzed using six primers: GT211, GT509 (Berube *et al.*, 2000), GATA28, GATA98, GATA417 (Palsboll *et al.*, 1997), EV1Pm and EV104Mn (Valsecchi and Amos, 1996). All of these primers, except EV1Pm, were developed from humpback whale, *Megaptera novaeangliae*. EV1Pm was developed from sperm whale, *Physeter macrocephalus*. These primers were already tested for amplification on minke whale by these authors. The GT and EV primers are dinucleotide repeat and the GATA primers are tetranucleotide repeat. Primer sequences and PCR profiles were as those suggested by these authors.

PCR amplifications were performed in 15  $\mu$ l reaction mixtures containing 10-100ng of DNA, 5pmol of

each primer, 0.625 units of EX Taq™ DNA polymerase (TaKaRa Shuzo), and 2mM of each dNTP, 25mM MgCl<sub>2</sub>, and 10x reaction buffer (TaKaRa Shuzo). An amplified product was electrophoresed with internal size standard (TAMRA 500) through 5% polyacrylamide denaturing gel (Long Ranger™) using an ABI 377 DNA Prism sequencer (PE Biosystems Japan).

#### *Data analysis*

Deviations from expected Hardy-Weinberg genotypic proportions at each locus were tested using the GENEPOP program (version 3.2a; Raymond and Rousset, 1995). GENEPOP was also used to test for the heterogeneity of microsatellite allele frequencies, based on the chi-square statistics. Table 7 shows the number of samples examined by the microsatellite analysis, by sub-area.

## RESULTS

### **Analysis of mtDNA**

#### *Variability of mtDNA control region sequences of JARPN II samples*

A 487 bp of mtDNA control region (the 5'-end) was analyzed in the 40 individuals sampled by JARPN II in 2000. Sixteen haplotypes were detected in 24 samples from sub-area 7 and eight in 16 samples from sub-area 9 (Table 2). Two and one new haplotypes were identified in sub-areas 7 and 9, respectively. Haplotype '9' was predominant (37.5%) in sub-area 9 in the 2000 sample. In sub-area 7, no particular predominant haplotype was observed.

#### *Homogeneity test*

Tables 3 and 4 show the results of the homogeneity tests for yearly variation including JARPN II samples within sub-areas 7 and 9, by the chi-square, Hst and Kst\* statistics, respectively. No significant differences were found among years. Analysis of yearly variation in sub-area 8 was shown in Goto and Pastene (2000c). In subsequent analyses, samples from different years were combined in these sub-areas.

Table 5 shows the results of the homogeneity tests among sub-areas 7, 8 and 9. No significant differences using three statistical tests were found between sub-areas 7 and 8. On the other hand, the comparison between sub-area 9 and other two sub-areas showed significant differences, except the comparison between sub-areas 8 and 9 using Kst\* statistics (P=0.0659).

#### *Inter- and Intra-population distances among sub-areas*

The net interpopulation distance among sub-areas in the eastern side of Japan ranged from negative value to 0.00002. Table 6 shows the nucleotide diversity in sub-areas 7, 8 and 9. Nucleotide diversities were 0.00846 (SE: 0.00037), 0.00832 (SE: 0.00050) and 0.00723 (SE: 0.00027) for sub-areas 7, 8 and 9, respectively. Estimated nucleon diversity was 0.9565 for sub-area 7, 0.9602 for sub-area 8 and 0.9377 for sub-area 9.

### **Microsatellite analysis**

#### *Homogeneity tests*

No significant deviations from H-W expectation at 5% level was detected in each sub-area and year, although sub-area 9 in 2000 was close to significant (p=0.057) (Table 8). In the subsequent analyses we therefore combined all the samples collected from different years in each sub-area. Table 9 shows the results of homogeneity test for allele frequencies within and between sub-areas. For all loci combined no

statistically significant differences were found within sub-areas 7 and 9. Furthermore, no significant differences were found among sub-areas 7, 8 and 9.

## DISCUSSION

In this study we examined the stock structure of western North Pacific minke whale including 40 new samples collected from JARPN II in 2000. For this research we used two different genetic markers, mtDNA and microsatellite. We found certain degree of mtDNA heterogeneity in the samples of sub-area 9 obtained by the JARPN II survey. No such heterogeneity was found by microsatellite analysis. The mtDNA pattern observed is similar to that observed in the western part of sub-area 9 in the 1995 JARPN survey. In both cases the frequency of haplotype '9' was higher in comparison with that observed in other sub-areas. This situation is difficult to interpret from the biological point of view. In the case of 1995 the heterogeneity was observed in the western part of sub-area 9. The eastern part was similar to sub-area 7 and 8. The mtDNA heterogeneity observed in the western part of sub-area 9 could be due to additional stock structure within the O stock or could be due to sampling bias.

If the first explanation is correct then we have a scenario in where a different stock distributes in part of sub-area 9 at least in some years. The sub-area 9 has been surveyed in 1994, 1995, 1997 and 2000 and the mtDNA heterogeneity has been detected in only two of these surveys (1995 and 2000) and in only a part of sub-area 9. In both cases genetic heterogeneity has been detected by the mtDNA but not by the microsatellite analysis. However there are several differences in these genetic markers, which could explain the different results found;

- i) Analysis of mtDNA shows female-mediated gene flow. Minke whale females may have stronger site fidelity to breeding grounds than males have. No heterogeneity of microsatellite allele frequencies among minke whale populations suggests male-mediated gene flow.
- ii) Because females pass on only a single mtDNA genotype to progeny, effective population size for mtDNA is smaller than for nDNA, indicating that genetic divergence in mtDNA will accumulate faster than that in nDNA.

The maternal inheritance of mitochondrial haplotypes makes mtDNA analysis a powerful tool in detecting stock structure. However, it must not be forgotten that the picture obtained depends strongly both on the relative movements of males and females and on the sampling regime employed. Majerus *et al.* (1996) discussed about the influence of sampling regime and breeding system on inferences from mtDNA studies. In these hypothetical examples, four different sampling regimes were employed to study species showing various breeding behaviors.

It is noted here that the most of the samples (88.2%) from sub-area 9 were males. Majerus *et al.* (1996) mentioned that if just males are sampled, the stock structure is detected when both sexes show maternal site fidelity and non-breeding sub-adults are mobile. In this case, female do not disperse. If this hypothesis is correct, some heterogeneity will be revealed by both mtDNA and microsatellite analyses. However, results from microsatellite analyses indicate that there is no significant deviations from H-W expectation in sub-area 9 as well as sub-areas 7 and 8, and also no significant differences among sub-areas 7, 8 and 9 by the homogeneity test using allele frequencies data.

The alternative explanation is that there could have been a bias related to the sampling scheme that affected only the mtDNA analysis in our case. When conducting a chi-square test, we assume that each of the existing haplotypes in a stock have been randomly sampled depending on its frequency. This assumption is easily violated because individuals in a stock constrained in time and space, and thus chances of appearing in the sample differ by individuals (or haplotypes), producing over-dispersion. In this situation, conducting chi-square test without considering the over-dispersion may result in type I error. Further considerations on this matter should be given in future.

The samples examined in this study were available from the first feasibility survey of JARPN II. The second feasibility survey is being conducted in year 2001 and it was planned additional sampling from sub-area 9. Before to reach a definitive conclusion on whether or not additional structure occur in sub-area 9, the analysis of the additional samples from the 2001 survey should be carried out first.

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Table 1. Number of samples collected during the JARPN surveys from 1994 to 1999 and JARPN II in 2000, by sub-area, year, month, period and sex. All these were examined in this study for sequencing and microsatellite analyses, except sub-area 11.

Sub-area	Year	Month										Total
		Early				Late						
		May		June		July		August		September		
		Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	
7	1996					1			15	2	13	31
	1997				2							2
	1998	7	49									56
	1999			7	43							50
	2000							1	5	4	14	24
8	1996						11		5			16
	1997					1	30					31
	1998	1	7	3	33							44
9	1994					2	6	1	8		4	21
	1995					5	56	4	21			100
	1997	7	20	5	35							67
	2000								16			16
11	1996							11	19			30
	1999					22	28					50
Total		15	76	15	127	31	131	17	89	6	31	538

Table 2. MtDNA haplotype frequencies in samples taken by JARPN (1994-1999) and JARPN II (2000).

JARPN 1994-1999				1995JARPN SA9(Boundary=162E)			2000JARPN II		
Hap.	SA7	SA8	SA9	Hap.	West	East	Hap.	SA7	SA9
1	6	1	2	1	0	0	1	2	0
2	0	0	0	2	0	0	2	0	0
3	0	0	0	3	0	0	3	0	0
4	0	1	0	4	0	0	4	0	0
5	0	0	0	5	0	0	5	0	0
6	8	1	9	6	4	0	6	2	1
7	15	10	15	7	5	4	7	2	3
8	6	1	3	8	1	2	8	0	0
9	13	7	30	9	18	0	9	2	6
10	1	0	0	10	0	0	10	0	0
11	5	5	10	11	7	1	11	1	0
12	4	3	7	12	2	2	12	0	0
13	13	8	4	13	1	1	13	0	0
14	6	6	11	14	4	1	14	0	0
15	4	4	6	15	2	0	15	2	0
16	8	4	11	16	4	1	16	0	0
17	6	5	16	17	5	3	17	2	2
18	2	2	3	18	2	0	18	0	0
19	2	1	1	19	0	0	19	0	0
20	3	0	0	20	0	0	20	0	0
21	3	0	3	21	1	0	21	0	0
22	1	0	0	22	0	0	22	0	0
23	1	0	1	23	0	1	23	0	0
24	1	0	0	24	0	0	24	0	0
25	1	2	4	25	2	0	25	0	0
26	1	1	0	26	0	0	26	0	0
27	1	0	1	27	0	0	27	0	0
28	1	0	0	28	0	0	28	1	0
29	2	4	5	29	0	0	29	1	0
30	7	1	9	30	1	2	30	2	1
31	2	0	2	31	2	0	31	0	0
32	1	0	0	32	0	0	32	0	0
33	2	0	0	33	0	0	33	0	0
34	2	4	3	34	1	1	34	0	1
35	2	1	0	35	0	0	35	0	0
36	3	3	1	36	1	0	36	1	0
37	2	0	2	37	1	0	37	0	0
38	2	1	1	38	1	0	38	0	0
39	1	2	5	39	3	0	39	0	0
40	0	1	0	40	0	0	40	0	0
41	0	1	0	41	0	0	41	0	0
42	0	1	0	42	0	0	42	0	0
43	0	1	0	43	0	0	43	1	0
44	0	1	0	44	0	0	44	0	0
45	0	2	0	45	0	0	45	0	0
46	0	1	0	46	0	0	46	1	0
47	0	1	0	47	0	0	47	0	0
48	0	1	1	48	1	0	48	0	0
49	0	1	1	49	0	0	49	0	0
50	0	1	1	50	0	0	50	0	1
51	0	1	1	51	0	0	51	0	0
52	0	0	5	52	2	1	52	0	0
53	0	0	4	53	3	0	53	1	0
54	0	0	1	54	1	0	54	0	0
55	0	0	1	55	1	0	55	0	0
56	0	0	1	56	1	0	56	0	0
57	0	0	1	57	1	0	57	0	0
58	0	0	1	58	0	1	58	0	0
59	0	0	2	59	0	1	59	0	0
60	0	0	2	60	0	0	60	0	0
61	0	0	1	61	0	0	61	0	0
62	1	0	0	62	0	0	62	0	0
<b>Total</b>	<b>139</b>	<b>91</b>	<b>188</b>	<b>Total</b>	<b>78</b>	<b>22</b>	<b>81</b>	<b>2</b>	<b>0</b>
							<b>87</b>	<b>1</b>	<b>0</b>
							<b>88</b>	<b>0</b>	<b>1</b>
				<b>Total</b>			<b>24</b>	<b>16</b>	



Table 3. Analysis of yearly variation in sub-area 7 using three different statistics. P values are shown in parenthesis.

	Year	Sample size	$\chi^2$	Hst	Kst*
Sub-area 7	1996	31			
	1998	56	-	-0.0037	-0.0043
	1999	50	(0.2203)	(0.8810)	(0.8464)
	2000	24			

Table 4. Analysis of yearly variation in sub-area 9 using three different statistics. P values are shown in parenthesis.

	Year	Sample size	$\chi^2$	Hst	Kst*
Sub-area 9	1994	21			
	1995	100	-	0.0008	-0.0017
	1997	67	(0.6618)	(0.3509)	(0.6310)
	2000	16			

Table 5. Comparison among sub-areas 7, 8 and 9 using  $\chi^2$  (upper), Hst (middle) and Kst\* (lower). P values are shown in parenthesis.

		Sub-area 7 n=163		Sub-area 8 n=91	
Sub-area 8	n=91	-	(0.2902)		
		-0.0005	(0.6536)		
		-0.0010	(0.7356)		
Sub-area 9	n=204	-	(0.0096)	-	(0.0360)
		0.0023	(0.0200)	0.0022	(0.0386)
		0.0024	(0.0495)	0.0025	(0.0659)

Table 6. Nucleotide ( $\pi$ ) and haplotypic ( $H$ ) diversity by sub-area.

Sub-area	Sample size	$\pi$	SE( $\pi$ )	$H$
7	163	0.00846	0.00037	0.95645
8	91	0.00832	0.00050	0.96020
9	204	0.00723	0.00027	0.93765

Table 7. Samples of western North Pacific minke whales examined by the microsatellite analysis.

Sample	No.
SA7 96+98+99*	139
SA7 00	24
SA7 Total	163
SA8 96+97+98*	91
SA9 94+95+97*	188
SA9 00	16
SA9 total	204

\* :After Abe *et al.* (2000)

Table 8 Results of Hardy-Weinberg equilibrium test applied to western North Pacific minke whale in different sub-areas and years.

Sample	Microsatellite locus							All
	EV104	GT211	GATA28	EV1	GATA417	GATA98	GT509	
SA7 96+98+99*	0.558	0.264	0.553	0.274	0.010	0.391	0.580	0.138
SA7 00	0.194	0.347	0.842	0.256	0.292	0.929	0.683	0.619
SA7 Total	0.257	0.608	0.479	0.281	0.030	0.609	0.369	0.218
SA8 96+97+98*	1.000	0.107	0.273	0.753	0.986	0.129	0.190	0.372
SA9 94+95+97*	0.522	0.809	0.986	0.507	0.865	0.183	0.900	0.934
SA9 00	0.159	0.100	0.014	0.933	0.995	0.362	0.120	0.057
SA9 total	0.346	0.941	0.957	0.692	0.892	0.077	0.787	0.837

\* :After Abe *et al.* (2000)

Table 9. Heterogeneity test for allele frequencies within and between sub-areas in the western North Pacific minke whales.

	Microsatellite locus							All
	EV104	GT211	GATA28	EV1	GATA417	GATA98	GT509	
Within SA7	0.932	0.102	0.908	0.433	0.023	0.281	0.160	0.121
Within SA9	0.358	0.828	0.181	0.233	0.713	0.039	0.147	0.138
SA7xSA8	0.045	0.939	0.018	0.733	0.681	0.622	0.161	0.118
SA7xSA9	0.095	0.932	0.035	0.317	0.929	0.417	0.619	0.273
SA8xSA9	0.464	0.687	0.228	0.314	0.824	0.799	0.047	0.513

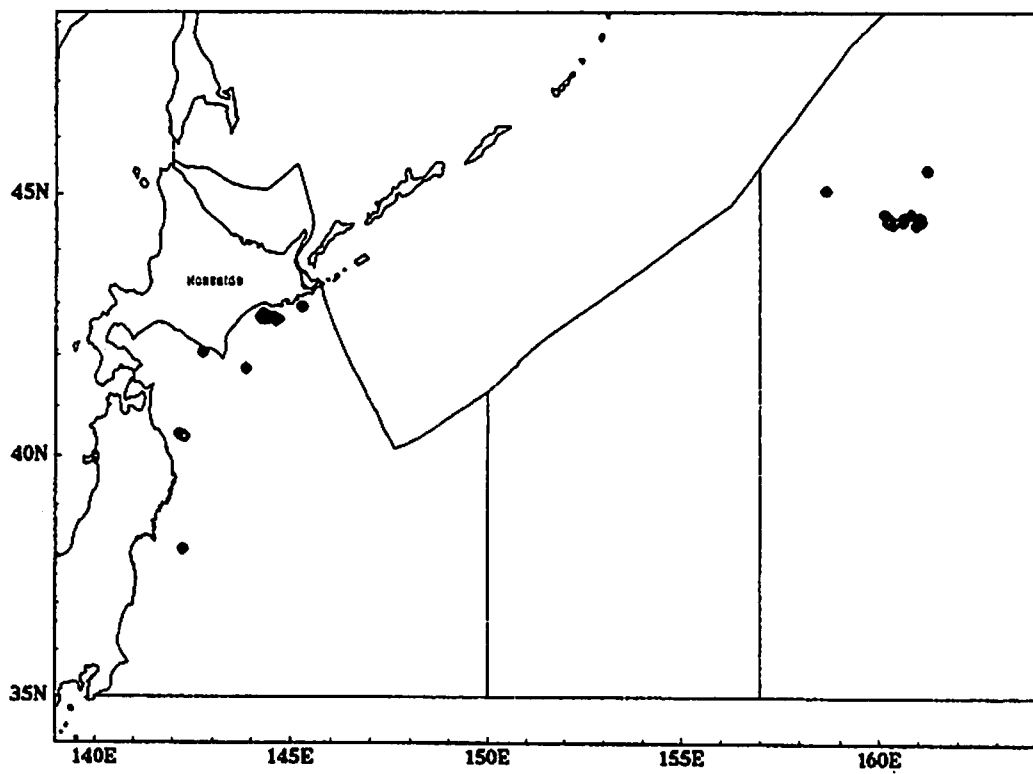


Fig. 1: Geographical position of minke whales sampled in the 2000 JARPN II survey.