# Further microsatellite analyses in the western North Pacific minke whales, *Balaenoptera acutorostrata*

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## ABSTRACT

Here we present the results of a microsatellite analysis to examine stock structure in the western North Pacific minke whales that used a larger number of samples and loci than in a previous study. Genotyping of three groups (sub-area 6;n=26-28, sub-area 7;n=184-206 and sub-area 9;n=177-188) at six microsatellite loci (GATA417, GATA28, GATA98, GT23, EV1*Pm*, EV37*Mn*) revealed significant differences in the levels of allelic diversity and expected heterozygosity between groups from Korea (J stock) and Pacific Ocean (O stock). No significant differences in allele frequencies were found between sub-areas 7 and 9. By contrast to the previous study, we did not detect significant deviation from the expected Hardy-Weinberg proportions in sub-area 9, though some degree of deviations was still detected in sub-area 7.

## INTRODUCTION

In the western North Pacific, the existence of two distinct populations — J stock ( the Sea of Japan and East China Sea) and O stock ( the Pacific coast of Japan and in the Sea of Okhotsk) of minke whales were well supported by genetic approaches (Goto and Pastene, in press; Wada, 1983; Wada, 1984) as well as morphological studies (Ohsumi, 1983; Kato *et al.*, 1992).

International Whaling Commission (IWC) regarded these distinguishable populations as two independent 'stocks' even though these populations might mix in southern part of the Okhotsk Sea in certain proportions (Pastene *et al.*, 1997).

In 1993, a Working Group on North Pacific Minke Whale Management Trials proposed to introduce the possibility of a third stock, W stock (West Pacific). In addition to this proposal, they also assumed that the stocks should be divided into more than one sub-stock (IWC, 1994). From a practical point of view, over-partition of the region to be managed (the western North Pacific ) made the correct understanding of these stocks more difficult.

In order to clarify this situation, two different kinds of genetic markers (mtDNA, allozyme) had been used to verify the genetic differentiation between minke whales from coastal Japan (sub-area 7) and from offshore area (sub-area 9, equal to the hypothetical W stocks). But no evidence to support the existence of a W stock was obtained by mtDNA analyses (Goto and Pastene, this meeting). These data are

necessary but not sufficient.

As a clue to elucidate the stock structure in western North Pacific minke whales, more sensitive molecular marker was required to further investigate the results obtained using mtDNA and allozymes (IWC, 1997).

Microsatellites are highly polymorphic markers consisting of various numbers of tandem repeats (Tautz,1989). For the sake of a wide range of applications, microsatellites were vigorously isolated even from some sort of cetaceans (Velsecchi *et al.*, 1996; Palsbøll *et al.*, 1997; Buchanan *et al.*, 1996; Richard *et al.*, 1996). Recently, detection of variation at microsatellite loci has proved to be an extremely sensitive measure of genetic variation in wild species (Houlden *et al.*, 1996), including the whale (Velsecchi *et al.*, 1997).

In a previous study, we conducted a preliminary analysis of western North Pacific minke whales using four microsatellite loci (Abe *et al.*, 1997). Results showed striking similarities in allele frequencies between sub-area 7 and sub-area 9. Nevertheless, we detected significant and close-to-significant deviations from the expected Hardy-Weinberg genotypic proportions under panmixis in the two sub-areas 7 and 9 respectively.

However, we could not reach conclusion at that point that these sub-areas were not drawn from a single panmictic population. Because it was clear that more microsatellite loci and additional samples were needed to be analysed to increase our understanding of spatial and temporal structure in this region.

Here we present the results of an additional analysis with six microsatellite loci and increased sample size both in sub-areas 7 and 9 for western North Pacific minke whales.

## MATERIALS AND METHODS

### Samples and DNA extraction

A total of 356 minke whales were obtained from three distinct areas of North Pacific (Fig.1): coastal (sub-area 7;n=184~206) and offshore (sub-area 9;n=177~188) areas of eastern side of Japan and coastal Korea (sub-area 6;n=26~28).The number of samples used in this study were slightly different in each locus due to exclusion of ambiguous data from analysis. These samples were collected from small-type coastal whaling operations (sub-area 6 and 7, in 1982~87) or from JARPN surveys (Japanese Whale Research Program under Special Permit in the North Pacific in sub-areas 7 and 9, in 1994~97). Genomic DNA was extracted from several kinds of tissues (muscle, liver, heart) using standard proteinase K, phenol-chloroform procedure (Sambrook *et al.*, 1989).

### Genotyping

Six microsatellite primers, including two newly added (EV1*Pm* and EV37*Mn*) in this study, are listed in Table 1. Three of these microsatellite loci contained dinucleotide

motifs [GT023 (Palsbøll, unpublished), EV1 *Pm* and EV37*Mn* (Valsecchi *et al.*, 1996)] and the others composed of tetranucleotide motifs [GATA028, GATA098 and GATA417 (Palsbøll *et al.*, 1997)]. These loci were analyzed by fluorescent detection. One primer of each pair was synthesized with either 6-FAM, TET, or HEX dye at their 5' end. Genotype was determined using an ABI377 sequencer and scoring was accomplished by GeneScan™ software (Ver 2.0.1). Details of the reaction mixture and conditions for PCR amplification were described in our previous paper (Abe *et al.*,1997).

Statistical analysis

The allele frequencies and expected heterozygosity were calculated in each of the three groups for each of six microsatellite locus. Consequently, deviation from Hardy-Weinberg proportions and population differentiation were estimated using the Genepop Version 3.1 (Raymond and Rousset, 1995). We used Guo & Thompson's (1992) Markov chain method (2000 for dememorization, 100 for batches) to evaluate exact tests for deviation from Hardy-Weinberg equilibrium at each locus.

## RESULT

Genetic diversity

The six microsatellite loci were all polymorphic, revealing various numbers of alleles and heterozygosity (Table 2). We observed between 3~25 alleles within each population at different loci. The average number of alleles detected by six microsatellites was 12.2 per locus and the average heterozygosity was 0.70, with a range of 0.29~0.88. Additional two loci used in this study indicated a similar trend to that observed in previous analysis—the number of different alleles, and the expected heterozygosity amongst the Korean coastal samples was lower than those estimates observed in the combined sub-areas 7 and 9.

Hardy-Weinberg proportions

The $\chi^2$ test for Hardy - Weinberg equilibrium detected significant deviation ($P<0.001$) at the EV1 *Pm* locus in sub-area 7 and close-to-significant deviation ($P<0.05$) was found at GATA417 locus in the same sub-area (Table 2).

Genetic differentiation among sub-areas

Despite the larger sample size and the additional loci used, we did not detect significant changes in genetic differentiations compared between sub-areas 7 and 9 (Table 3).

## DISCUSSION

The results of the microsatellite analysis of western North Pacific minke whales presented here were consistent with the genetic data, such as mtDNA and allozymes (Goto and Pastene,in press; Wada,1983; Wada,1984). These results indicate a clear genetic differentiation in minke whales between the Korean and eastern side of Japan. We observed a significant low level of variations in the Korean samples at six

microsatellite loci. This definitive distinction between two populations may be explained as the consequence of founder effect in the Sea of Japan. From a paleoenvironmental point of view, it should be noted that the Sea of Japan had experience of being completely isolated from North Pacific Ocean by Japanese land mass at the middle of Pleistocene (Riss II stadial; Fujii, 1990). It is plausible that migration of a limited number of minke whales into this area occurred as the rise of sea level have severely affected their genetic variation mainly due to founder effect. To confirm this hypothesis, more microsatellite data from the Korean Peninsula are required.

The results of the homogeneity tests revealed no significant differences in allele frequencies between coastal (sub-area 7) and offshore (sub-area 9) areas of eastern side of Japan at all of the six independent loci. Then the microsatellite analysis provide no evidence for additional stock structures within the O stock in the eastern side of Japan.

One of the main purpose of this paper was to examine the significant deviations from Hardy-Weinberg equilibrium observed in our previous study (Abe *et al.*, 1997). By a series of microsatellite analyses, we did not detected significant deviation from the expected Hardy-Weinberg proportions under panmixis in sub-area 9.

On the other hand, the significant deviations from Hardy-Weinberg equilibrium in sub-area 7 remained after increasing sample size and number of loci. There are at least two possible explanations for this situation:

(1) The significant deviation for Hardy-Weinberg equilibrium could reflect the excessive number of alleles in locus EV1*Pm*. The National Research Council (1996) suggested that the existence of large number of minor alleles could play a crucial role in the results of statistical tests, especially in $\chi^2$-based tests.
(2) On the other hand, the significant deviations could reflect a true biological phenomenon. In sub-area 7, we combined data from different years extending from 1982 to 1997. If some of the J stock animals invaded into this sub-area in certain year(s), the Hardy-Weinberg equilibrium could reflect partial mixing of J and O stock animals in this sub-area. This is analogous to the hypothesis derived from mtDNA and allozyme analyses (Pastene *et al.*, 1997; Wada, 1991).

Temporal and spatial re-grouping of microsatellite data might be necessary for further understanding of stock structure in western North Pacific minke whales.

## ACKNOWLEDGMENTS

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Table 1. Microsatellite allele frequency data for all six loci at the three western North Pacific sub-areas 6,7,9.
n ; sample size, - ; allele not observed.

GT023

| Area | 6 | 7 | 9 |
|---|---|---|---|
| Allele | n=26 | n=184 | n=188 |
| 88 | - | 0.004 | 0.003 |
| 94 | 0.115 | 0.004 | 0.009 |
| 96 | 0.096 | 0.009 | 0.006 |
| 98 | - | 0.119 | 0.082 |
| 100 | 0.019 | 0.017 | 0.021 |
| 102 | - | 0.034 | 0.033 |
| 104 | 0.077 | 0.140 | 0.139 |
| 106 | 0.173 | 0.085 | 0.109 |
| 108 | 0.038 | 0.072 | 0.064 |
| 110 | 0.058 | 0.111 | 0.142 |
| 112 | 0.423 | 0.166 | 0.185 |
| 114 | - | 0.119 | 0.13 |
| 116 | - | 0.106 | 0.070 |
| 118 | - | 0.013 | 0.003 |
| 120 | - | - | 0.003 |

GATA098

| Area | 6 | 7 | 9 |
|---|---|---|---|
| Allele | n=28 | n=206 | n=188 |
| 100 | - | 0.002 | 0.008 |
| 104 | 0.054 | 0.097 | 0.090 |
| 108 | 0.732 | 0.568 | 0.500 |
| 112 | 0.214 | 0.199 | 0.277 |
| 116 | - | 0.124 | 0.120 |
| 120 | - | 0.010 | 0.005 |

EV1 *Pm*

| Area | 6 | 7 | 9 |
|---|---|---|---|
| Allele | n=28 | n=203 | n=187 |
| 123 | - | 0.002 | 0.003 |
| 129 | - | 0.002 | 0.005 |
| 133 | - | 0.145 | 0.134 |
| 135 | - | 0.022 | 0.024 |
| 137 | - | 0.007 | 0.011 |
| 143 | - | 0.007 | 0.003 |
| 145 | 0.036 | 0.042 | 0.064 |
| 147 | 0.107 | 0.106 | 0.096 |
| 149 | 0.839 | 0.372 | 0.358 |
| 151 | - | 0.074 | 0.075 |
| 153 | - | 0.027 | 0.027 |
| 155 | - | 0.037 | 0.029 |
| 157 | - | 0.007 | - |
| 159 | - | 0.010 | - |
| 161 | - | 0.015 | 0.016 |
| 163 | - | 0.002 | 0.003 |
| 165 | - | 0.002 | 0.003 |
| 167 | 0.018 | 0.049 | 0.035 |
| 169 | - | 0.022 | 0.032 |
| 171 | - | 0.017 | 0.040 |
| 173 | - | 0.015 | 0.019 |
| 175 | - | 0.005 | 0.011 |
| 177 | - | 0.005 | 0.003 |
| 179 | - | 0.002 | 0.005 |
| 183 | - | 0.002 | 0.005 |

EV37 *Mn*

| Area | 6 | 7 | 9 |
|---|---|---|---|
| Allele | n=27 | n=202 | n=181 |
| 179 | 0.315 | 0.302 | 0.334 |
| 181 | - | 0.002 | 0.014 |
| 193 | - | 0.069 | 0.064 |
| 197 | - | 0.010 | 0.022 |
| 199 | 0.333 | 0.399 | 0.406 |
| 201 | 0.037 | 0.062 | 0.050 |
| 203 | - | 0.020 | 0.011 |
| 205 | 0.315 | 0.111 | 0.083 |
| 207 | - | 0.015 | 0.003 |
| 209 | - | 0.010 | 0.014 |

GATA028

| Area | 6 | 7 | 9 |
|---|---|---|---|
| Allele | n=28 | n=195 | n=177 |
| 194 | - | 0.008 | 0.006 |
| 198 | - | 0.056 | 0.031 |
| 202 | 0.268 | 0.184 | 0.153 |
| 206 | - | 0.153 | 0.147 |
| 210 | 0.268 | 0.166 | 0.178 |
| 214 | 0.446 | 0.302 | 0.288 |
| 218 | - | 0.074 | 0.130 |
| 222 | - | 0.043 | 0.048 |
| 226 | - | 0.013 | 0.020 |

GATA417

| Area | 6 | 7 | 9 |
|---|---|---|---|
| Allele | n=28 | n=190 | n=177 |
| 200 | - | 0.015 | 0.023 |
| 204 | - | 0.051 | 0.045 |
| 208 | 0.034 | 0.091 | 0.102 |
| 212 | 0.466 | 0.035 | 0.395 |
| 216 | 0.483 | 0.323 | 0.285 |
| 220 | 0.017 | 0.149 | 0.144 |
| 224 | - | 0.008 | 0.006 |
| 228 | - | 0.008 | - |

Table 2. Expected heterozygosities and $\chi^2$ test statistic for differences in allele frequencies of six microsatellite loci.

| | Heterozygosity | | | $\chi^2$ test | | |
|---|---|---|---|---|---|---|
| | **6** | **7** | **9** | **6** | **7** | **9** |
| GT 023 | 0.772 | 0.883 | 0.881 | ns | ns | ns |
| GATA 028 | 0.659 | 0.783 | 0.795 | ns | ns | ns |
| GATA 098 | 0.423 | 0.614 | 0.653 | ns | ns | ns |
| GATA417 | 0.578 | 0.729 | 0.712 | ns | P<0.05 | ns |
| EV1 *Pm* | 0.288 | 0.818 | 0.830 | ns | P<0.001 | ns |
| EV37 *Mn* | 0.702 | 0.730 | 0.711 | ns | ns | ns |

Table 3. Probability values of genotypic differentiation calculated by Genepop software.

| | GT023 | GATA028 | GATA098 |
|---|---|---|---|
| Sub-area 6 & sub-area 7 | <0.00001 | <0.00001 | 0.00339 (SE 0.00064) |
| Sub-area 6 & sub-area 9 | <0.00001 | <0.00001 | 0.00105 (SE 0.00022) |
| Sub-area 7 & sub-area 9 | 0.53818 (SE 0.01068) | 0.35319 (SE 0.00998) | 0.17009 (SE 0.00621) |



| | GATA417 | EV1 *Pm* | EV37 *Mn* |
|---|---|---|---|
| Sub-area 6 & sub-area 7 | 0.00051 (SE 0.00024) | <0.00001 | 0.00238 (SE 0.00062) |
| Sub-area 6 & sub-area 9 | 0.00025 (SE 0.00012) | <0.00001 | 0.00027 (SE 0.00011) |
| Sub-area 7 & sub-area 9 | 0.55704 (SE 0.00951) | 0.81644 (SE 0.00951) | 0.20536 (SE 0.00946) |

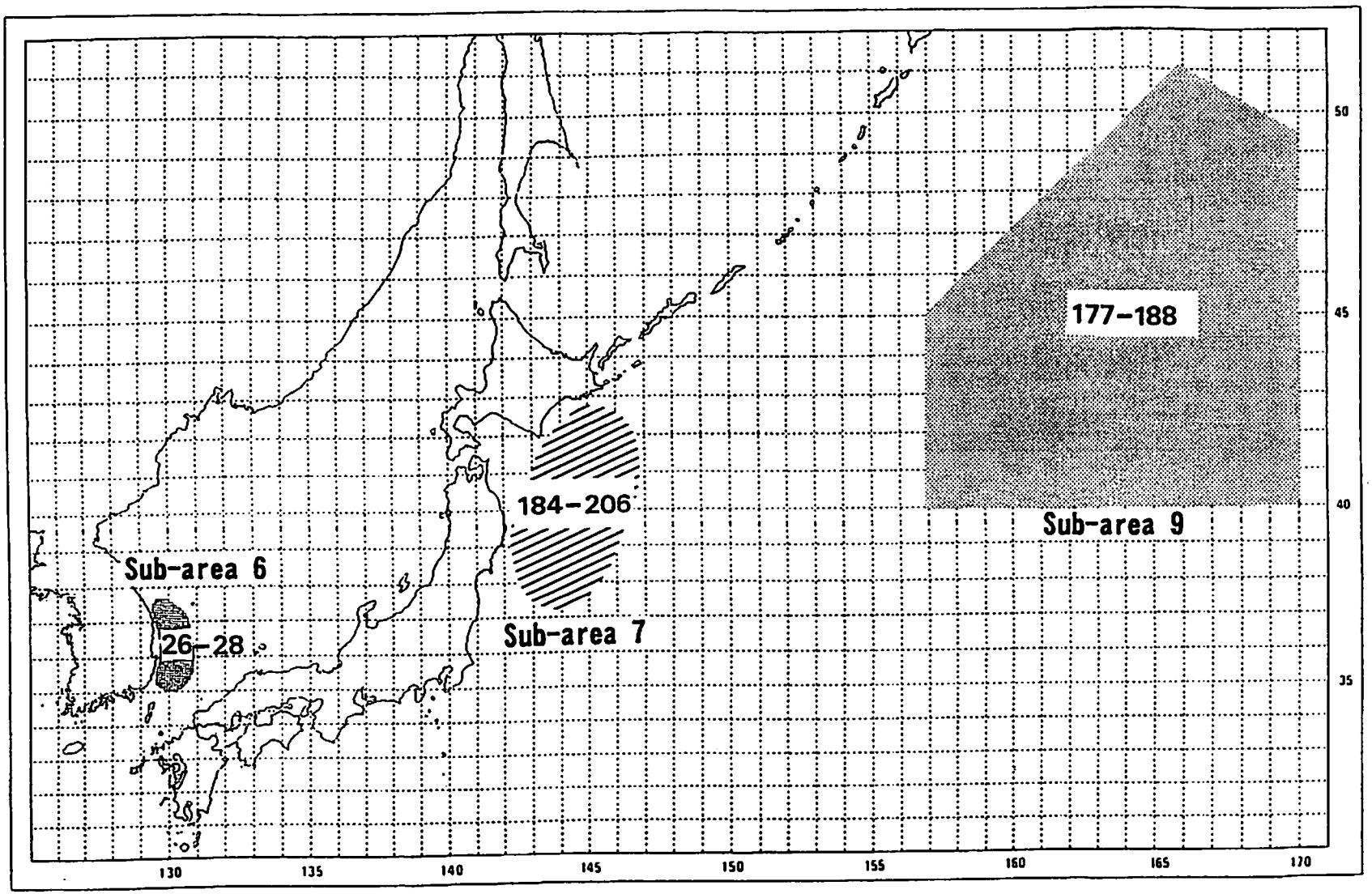


Fig. 1. Geographical origin of the samples. Numbers indicate sample sizes for each locality. Korea correspond to sub-area 6, the Japanese coastal waters to sub-area 7 and the offshore waters to sub-area 9 as defined by the Working Group on North Pacific Minke Whale Management Trials (IWC, 1994).