Preliminary microsatellite analysis of bycaught J-stock minke whales from Japan and Korea

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Key words: minke whales, bycatch, Japan, Korea, genetic difference, microsatellites

ABSTRACT

We analyzed samples of minke whales from Japan (Sub-area 6 (SA6)) and Korea (SA5 and SA6) using nine microsatellite loci in order to describe their genetic population structure. The samples were bycaught in set net fisheries conducted along the Japanese coast in SA6 (N = 202) from 2001 to 2004 and in coastal fishing gears along the Korean peninsula (N = 278) in SA6 and SA5 from 1999 to 2004. The genotypes of the individuals were first standardized using reference samples in order to avoid scoring differences between the two laboratories. We then examined if there was any evidence of genetic differences among the samples collected on different years within Japanese and Korean samples, respectively, among the samples from different areas of Korea, and finally between the samples from Japan and Korea. No evidence of statistically significant temporal heterogeneity was detected in the Japanese samples (JBC). However, the Korean sample collected from SA6 in 1999 (99KBC-6) was different from both the JBC and the rest of the Korean samples (KBC), although we were not able to completely reject the possibility of a chance effect for this heterogeneity. Finally, no evidence of statistically significant heterogeneity was detected within the KBC as well as between the JBC and KBC. A Bayesian clustering method did not show evidence of multiple stocks in our samples. Although we observed the heterogeneity of the 99KBC-6, no strong evidence of the existence of an additional stock in SA5 and SA6 was indicated.

INTRODUCTION

It has been believed that only one population of minke whales exists in the sub-areas (SA) 5 and 6 between Japan and Korea (Omura and Sakiura, 1956; Ohsumi, 1977; Kato, 1992; Wada and Numachi, 1991; Goto and Pastene, 1997). This is J-stock that is genetically different from widely distributed O-stock in western North Pacific. The J- and O-stocks differ from each other in body size, conception dates, allozyme allele frequencies, and mitochondrial DNA (mtDNA) haplotype frequencies, suggesting their reproductive isolation. Although both stocks migrate to the Okhotsk Sea in spring and stay till the end of summer (Omura and Sakiura, 1956; Hatanaka and Miyashita, 1997), their temporal distribution in the area appears not to overlap completely (Omura and Sakiura, 1956; Goto and Pastene, 1997).

One of the tasks accomplished prior to the in-depth assessment of the J-stock minke whales recommended by the Committee during the 2004 meeting was to analyze genetic data to describe population structure of the J-stock. We therefore intersessionally worked together to analyze the samples from Japan and Korea using mtDNA and microsatellites. The objective of this study was to gain an understanding of the level of genetic variability and genetic population structure of the J-stock minke whales from Japanese and Korean waters through microsatellite data. We used recently and geographically widely collected samples of the minke whales from SA5 and SA6 along Japanese and Korean coast, which differed from the previous genetic studies that used only those collected from commercial whaling in a narrow area in 1982.

MATERIALS AND METHODS

Samples

Minke whales used in this paper were bycaught individuals on set net fisheries conducted along the Japanese coast in SA6 from 2001 to 2004 and in coastal fishing gears along the Korean peninsula in SA6 and SA5 from 1999 to 2004 (Table 1 and Fig.1). The 2001 Japanese sample contained the bycaught individuals only from July to December, whereas all the other samples included the bycaught individuals from all seasons. Korean samples were divided into three on the basis of where they came from: eastern side of the Korean Peninsula (SA6), southern side of the Korean Peninsula (SA5 and SA6), and western side of the Korean Peninsula (SA5).

DNA extraction

Genomic DNA was extracted from muscle or skin tissues, whichever was available, using the standard proteinase K, phenol-chloroform procedure (Sambrook *et al*., 1989) and then stored in the TE buffer.

Microsatellite analysis

Microsatellite polymorphisms were analyzed using nine sets of primers: EV1, EV14, EV21, EV94 (Valsecchi & Amos, 1996), GT195, GT211 (Bérubé *et al*., 2000), GATA98, TAA031 (Palsbøll *et al*., 1997), DlrFCB14 (Buchanan *et al*., 1996). All EV, GT, and DlrFCB primers are dinucleotide repeat, TAA31 is trinucleotide repeat, and GATA primers are tetranucleotide repeat. Primer sequences and PCR profiles follows those of the original authors with slight modifications. Because Korean samples were analyzed at the National Fisheries Research and Development Institute, Busan, and the Japanese ones at the Institute of Cetacean research, Tokyo, we first standardized the microsatellite allele data between the two countries in order to avoid laboratory differences in reading allele sizes generated from different fragment analyzers. This was done analyzing reference samples of known size.

Molecular genetic sexing (Abe *et al*., 2001) was used to determine sexes of each individual in the samples because it was required due to lack of professional knowledge of local people. Coamplification of SRY locus located on Y chromosome and a single microsatellite locus allowed us to determine sex as males show PCR bands of both SRY and microsatellite loci while females only showed the microsatellite locus.

Data analysis

Genetic diversity

Calculation of allele frequencies and tests for the deviations from expected Hardy-Weinberg genotypic proportions were conducted using GENEPOP 3.2 (Raymond and Rousset, 1995). Expected heterozygosity per locus, Weir and Cockerham estimator of Fst, and allelic richness were calculated using FSTAT 2.9.3 (Goudet, 1995). Decision of statistical significance on the H-W tests was made using the chi-square value obtained from summing the negative logarithm of p values over the nine microsatellite loci (Sokal and Rohlf, 1995). When simultaneous multiple tests were conducted, Rice (1989) correction for the multiple tests was performed.

Genetic divergence

Three procedures were performed to illuminate population structure of minke whales in SA5 and SA6.

First, conventional hypothesis testing procedure was conducted using heterogeneity test in allele frequencies among the samples. Our null hypothesis to be tested is if the samples came from a genetically same group of minke whales. If a statistically significant allele frequencies differences exist, then it could indicate these samples came from genetically different populations of minke whales. We examined if there was any evidence of genetic differences among the samples collected in different years within Japanese and Korean samples, respectively, among the samples from different areas of Korea, and finally between the samples from Japan and Korea. Probability test (or Fisher's exact test) implemented in GENEPOP was used to conduct the heterogeneity tests. Decision of statistical significance on the heterogeneity tests was made using the chi-square value obtained from summing the negative logarithm of *p* values over the nine microsatellite loci (Sokal and Rohlf, 1995). When pair-wise comparisons were conducted, the observed p-values from the heterogeneity tests were compared to the modified level of significance proposed by Rice (1989).

Second, genetic relationship among the samples was examined using unrooted neighbor-joining tree implemented in PHYLIP (Felsenstein, 1993). Cavalli-Sforza's cord measures (Cavalli-Sforza and Edwards, 1967) were used as the genetic distances between all pairs of samples. The genetic distance matrix was then used to create an unrooted neighbor-joining tree, and the obtained tree was visualized using TreeView PPC (Page, 1996). We chose this genetic distance measure because it needs no assumption of mutation process. Statistical inference was made based on 1000 replicates of bootstrap analysis

Third, a Bayesian approach was conducted using STRUCTURE (Pritchard *et al.*, 2000). STRUCTURE is a model-based clustering method for inferring population structure using multi-locus genotype data. Spatial differentiation of samples was assessed assuming that the sampled individuals belong to an unknown number of *K* populations. We conducted three independent runs for each value of *K* between 1 and 4 with no prior information (i.e., only genetic information was considered). All of the simulations were based on a burn-in period of 50,000 iterations and runs of 500,000 iterations. The ancestry model we used for the simulation was the admixture model, which assumes individuals may have mixed ancestry. The allele frequency model used was the correlated allele frequencies model, which assumes frequencies in the different populations are likely to be similar due to migration or shared ancestry.

RESULTS

Genetics diversity within samples

Genetic diversity indices for overall samples used in this study are shown in Table 2 and for each of the samples in Appendix 1. Averaged number of alleles per locus was 6.7 and heterozygosity per locus was 0.531 with Fst of 0.007, indicating the whales analyzed had high genetic diversity. Within each of the samples, the nine microsatellite loci analyzed were polymorphic except for one case (DlrFCB14 in the 00KBC-6). Average number of alleles per locus per sample ranged from 3.3 to 5.6 with an average of 4.7, but allelic richness index indicated that the differences in the number of alleles among the samples was mostly due to sample size effect. Average heterozygosity per locus per population was 0.531. Only one (DlrFCB14 in the 04JBC) out of the 108 cases showed evidence of deviation from the expected Hardy-Weinberg genotypic proportions after the correction for multiple tests.

Genetics divergence between samples

We first looked for any evidence of allele frequency differences among the samples collected from the same coast lines in different years. Among the six KBC-6s obtained from 1999 to 2004, statistically significant heterogeneity was obtained (chi-square = 55.976, d.f. = 18, $p = 0.00001$). Pair-wise comparisons indicated evidence of statistically significant heterogeneity between the 99KBC-6 and 03KBC-6 after the correction for multiple tests. Although insignificant, p values obtained from the comparisons involving the 99KBC-6 were all low. We, therefore, divided the KBC-6 into two groups, 99KBC-6 and 00-04KBC-6, for the next analysis. In contrast, no evidence of statistically significant heterogeneity in allele frequencies was detected among the four JBCs obtained from 2001 to 2004 (chi-square = 20.704, d.f. = 18, $p = 0.295$). We thus treated the four JBCs as a single sample.

Next, we looked for any evidence of statistically significant differences within SA5 and 6 of the Korean waters. In pair-wise comparisons, evidence of heterogeneity was detected only within the Korean SA6 that was already noted above. We therefore divided all Korean samples into two groups for the next analysis: 99KBC-6 and KBC (00-04KBC-6 + KBC-5/6 + KBC-5).

Finally, we looked for any evidence of statistically significant differences between Japanese and Korean samples. The JBC showed statistically significant heterogeneity only to the 99KBC-6 (chi-square = 42.010, d.f. = 18, $p = 0.0011$).

Phylogenetic relationship

Genetic relationship among the 12 samples was described in an unrooted neighbor-joining tree obtained from the frequencies of microsatellite alleles (Fig. 2). No geographically unique cluster was obtained, and almost all of bootstrap values were very low.

Bayesian clustering of samples

In order to estimate the number of populations extant in the SA5 and SA6, the bayesian clustering analyses without information on their sampling locations were conducted on the total data set. The analysis presented the highest likelihood probability at $K = 1$ (Table 3), indicating no signal of existence of multiple stocks in the study area.

DISCUSSION

Significance of this study was that we used recently and geographically widely collected samples of the minke whales from SA5 and SA6. The levels of allelic diversity and heterozygosity of the J-stock analyzed in this paper were much higher than predicted from most of the previous genetic studies of the same stock. In addition,

the genetic diversity levels of the J-stock were only slightly less than those of the O-stock minke whales (Kanda *et al*., unpublished data) as well as other whales species in the North Pacific, such as Bryde's whales (Pastene *et al.*, 2004) and sei whales (Kanda *et al*., 2006). This suggests that the effective population size of the J-stock is probably larger than we have thought based on the previous genetic studies.

Before conducting stock structure analyses, we raised three stock structure scenarios for our samples; 1) only a single stock in the SA5 and SA6, 2) two stocks, one along the Japanese coast and the other along the Korean coast, and 3) two stocks, one in SA6 and the other in SA5.

We found slight genetic differences within Korean SA6 that was mainly due to the heterogeneity of the 99KBC-6. Two possibilities were raised for the detected heterogeneity of the 99KBC-6. First, this could indicate that there are genetically different stocks in this area and that somehow we only captured the evidence in the 99KBC-6. Second, this could have resulted from the relatively small sample size of the 99KBC-6, suggesting it was not a good representative of the whole J-stock on the basis of their high genetic diversity.

In contrast, no evidence of genetic difference was observed among the four JBCs collected in different years as well as between the JBC and KBC (not include the 99KBC-SA6), indicating these samples came from a genetically same stock of minke whales. The result of STRUCTURE also supported the single stock scenario, and no geographically related cluster was observed in the phylogenetic tree. No strong indication of the existence of an additional stock in the SA5 and SA6 was therefore observed.

At this point we favor the single stock interpretation. Continued genetic monitoring of bycaught minke whales, however, is required to confirm which one of the above two possibilities is true for the heterogeneity we observed. In addition, it is important to note that this study is preliminary, and care is required in fully interpreting the data. For instance, the standardization of microsatellite allele data was more difficult than we had anticipated. In future, therefore, we expect that all the samples would be analyzed altogether at one time in either Japan, Korea, or both countries.

ACKNOWLEDGEMENTS

We thank all those in both Japan and Korea who were involved in sample collections and those who helped with laboratory work.

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Table 1. Bycaught minke whales collected from sub-areas (SA) 5 and 6 in Japan and Korea used in this study.

Locality	Sub-area	Sample	Year	N
Korea	SA6	99KBC-6	1999	20
		00KBC-6	2000	7
		01KBC-6	2001	15
		02KBC-6	2002	44
		03KBC-6	2003	68
		04KBC-6	2004	40
Korea	SA5 & 6	KBC-5/6	1999-2004	28
Korea	SA5	KBC-5	1999-2004	26
Japan	SA6	01JBC	2001	26
		02JBC	2002	49
		03JBC	2003	61
		04JBC	2004	66

Table 2. Genetic indices at the 10 microsatellite loci for overall samples.

	A	He	Fst
DlrFCB14	3	0.359	
EV1	17	0.668	
EV14	4	0.407	
EV21	2	0.372	
EV94	4	0.572	
GATA98	7	0.609	
GT195	10	0.799	
GT211	10	0.761	
TAA31	3	0.236	
Average	6.7	0.531	0.007(0.002, 0.013)

Table 3. Estimated posterior probability of number of populations (K) for the pooled samples computed using STRUCTURE.

K	Log P (x/k)	Probability
1	-8204.5	~1.0
2	-8534.4	~0.0
3	-8948.8	~0.0
4	-9898.6	~0.0

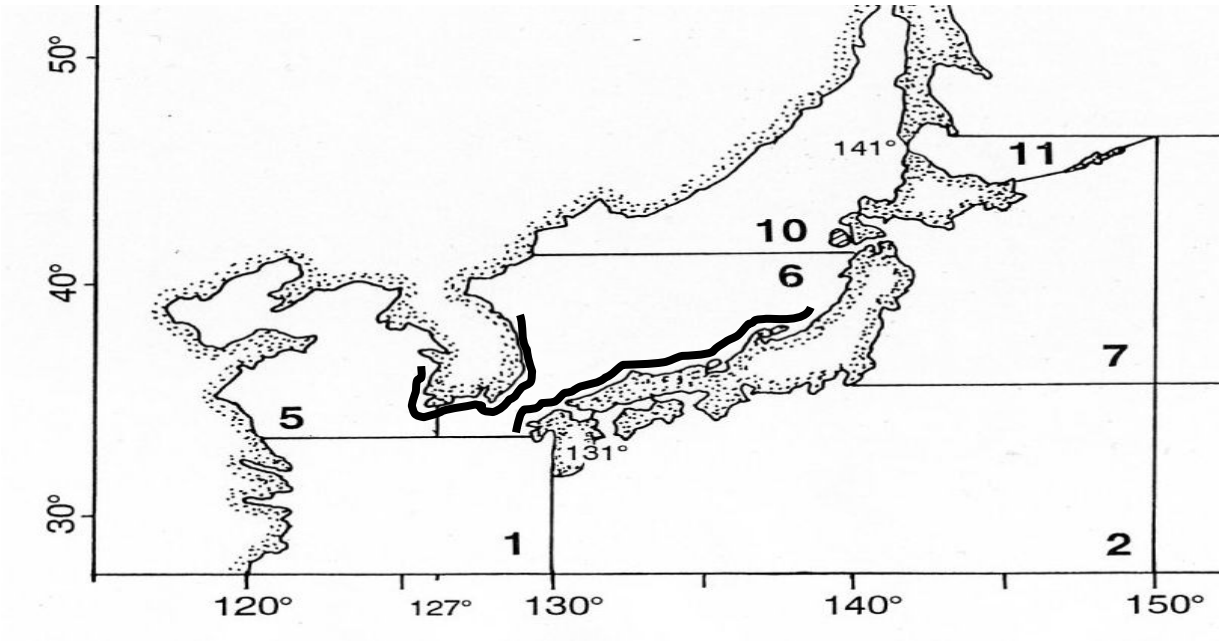

Fig 1. Approximate distribution of sampling locations in sub-area 5 and 6.

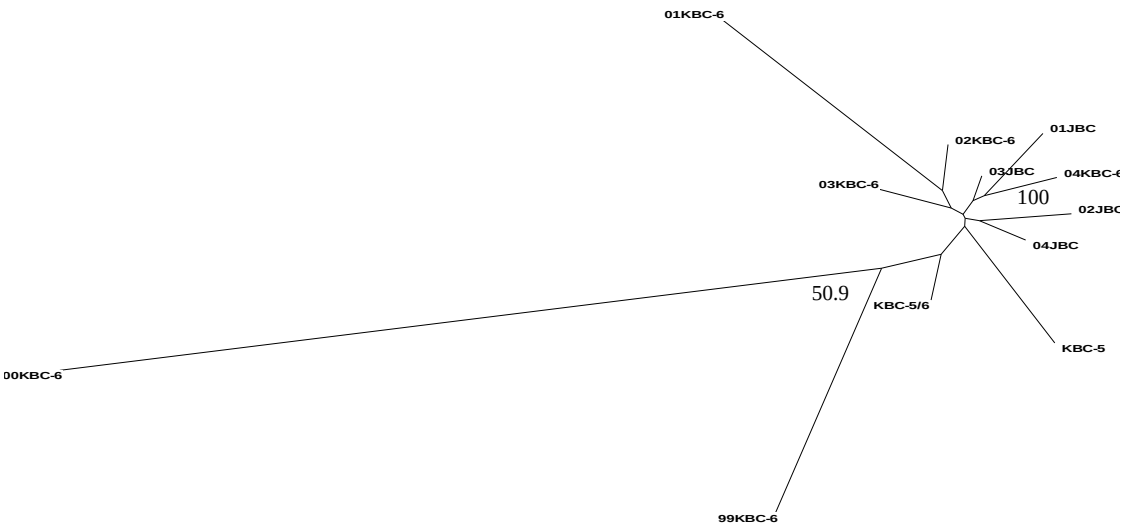

Fig 2. Un-rooted neighbor-joining tree of the 12 samples based on Cavalli-Sforza's cord measures. Bootstrapped values of over 50% were indicated.

Appendix 1. Genetic diversity indices in each of the 12 J-stock minke whales samples analyzed using nine microsatellite loci.

	99KBC-6				00KBC-6				01KBC-6				02KBC-6				03KBC-6				04KBC-6			
	A	AR	He	HW	A	AR	He	HW	A	AR	He	HW	A	AR	He	HW	A	AR	He	HW	A	AR	He	HW
DlrFCB14	2	1.5	0.108	NS	1	1.0	0.000	NS	2	1.9	0.253	NS	2	2.0	0.404	NS	2	2.0	0.448	NS	2	2.0	0.486	NS
EV1	9	4.9	0.776	NS	6	4.8	0.667	NS	7	4.3	0.624	NS	11	4.7	0.725	NS	10	4.2	0.660	NS	11	4.5	0.664	NS
EV14	3	2.7	0.494	NS	3	2.8	0.550	NS	3	2.5	0.393	NS	3	2.4	0.412	NS	3	2.3	0.320	NS	4	2.4	0.350	NS
EV21	2	1.8	0.224	NS	2	2.0	0.548	NS	2	2.0	0.451	NS	2	2.0	0.394	NS	2	1.8	0.258	NS	2	2.0	0.418	NS
EV94	3	2.6	0.544	NS	3	3.0	0.600	NS	4	2.9	0.558	NS	4	2.8	0.594	NS	4	2.8	0.588	NS	4	2.8	0.544	NS
GATA98	5	3.6	0.674	NS	4	3.8	0.679	NS	4	3.4	0.700	NS	5	3.2	0.572	NS	4	3.2	0.620	NS	5	3.4	0.614	NS
GT195	5	4.0	0.722	NS	5	5.0	0.800	NS	8	5.4	0.777	NS	7	5.1	0.828	NS	9	5.0	0.809	NS	7	5.0	0.824	NS
GT211	6	4.0	0.633	NS	4	4.0	0.650	NS	6	4.5	0.739	NS	8	4.3	0.731	NS	8	4.5	0.768	NS	8	5.0	0.813	NS
TAA31	2	1.7	0.193	NS	2	2.0	0.357	NS	2	1.8	0.198	NS	2	1.8	0.255	NS	2	1.8	0.220	NS	2	1.8	0.227	NS
Average	4.1	3.0	0.485		3.3	3.2	0.539		4.2	3.2	0.521		4.9	3.2	0.546		4.9	3.1	0.521		5.0	3.2	0.549	

	KBC-5/6				KBC-5				01JBC				02JBC				03JBC				04JBC			
	A	AR	He	HW	A	AR	He	HW	A	AR	He	HW	A	AR	He	HW	A	AR	He	HW	A	AR	He	HW
DlrFCB14	3	2.1	0.350	NS	2	2.0	0.417	NS	2	2.0	0.482	NS	3	2.1	0.451	NS	2	2.0	0.449	NS	3	2.1	0.429	Sig.
EV1	10	4.3	0.655	NS	9	3.7	0.522	NS	8	4.5	0.731	NS	11	4.4	0.685	NS	13	4.5	0.675	NS	14	4.0	0.620	NS
EV14	3	2.6	0.441	NS	3	2.4	0.340	NS	3	2.2	0.305	NS	3	2.8	0.559	NS	3	2.3	0.326	NS	3	2.4	0.420	NS
EV21	2	1.9	0.292	NS	2	2.0	0.472	NS	2	2.0	0.383	NS	2	1.9	0.340	NS	2	1.9	0.318	NS	2	2.0	0.385	NS
EV94	4	2.8	0.582	NS	4	3.0	0.569	NS	4	2.5	0.550	NS	4	2.7	0.580	NS	4	2.7	0.544	NS	4	2.9	0.584	NS
GATA98	4	3.5	0.659	NS	5	3.6	0.666	NS	4	2.8	0.517	NS	4	3.4	0.651	NS	5	2.9	0.491	NS	4	2.8	0.490	NS
GT195	8	4.7	0.793	NS	8	5.1	0.824	NS	7	4.4	0.780	NS	8	4.9	0.803	NS	8	4.7	0.795	NS	8	5.0	0.820	NS
GT211	8	4.9	0.768	NS	9	5.4	0.798	NS	8	5.1	0.818	NS	10	5.2	0.818	NS	8	5.1	0.821	NS	9	4.8	0.766	NS
TAA31	2	1.7	0.194	NS	2	1.5	0.115	NS	2	1.9	0.318	NS	2	1.8	0.247	NS	2	1.8	0.230	NS	3	1.9	0.274	NS
Average	4.9	3.2	0.526		4.9	3.2	0.525		4.4	3.1	0.543		5.2	3.2	0.570		5.2	3.1	0.517		5.6	3.1	0.532	