Progress of genetic analysis of by-caught J-stock minke whales from Japan and Korea: seasonal pattern of migration

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ABSTRACT

We analyzed samples of minke whales from Japan (Sub-area 6 (SA6)) and Korea (SA5 and SA6) using mtDNA haplotypes and nine microsatellite loci in order to describe their genetic population structure. The samples were by-caught animals in set net fisheries along the Japanese coast in SA6 (mtDNA: N = 202, microsatellite: N = 202) from 2001 to 2004 and in coastal fishing gears along the Korean Peninsula (mtDNA: N = 287, microsatellite: N = 248) in SA6 and SA5 from 1999 to 2004. The microsatellite genotypes of the individuals were first standardized using reference samples in order to avoid scoring differences between the Japanese and Korean laboratories. In response to one of the suggestions offered at the 2006 SC meeting (analyse samples by month and season to investigate whether genetic heterogeneity could be due to seasonal or monthly difference in migration between two stocks), the statistical analysis of genetic data was conducted on samples divided into two temporal groups: 'early' = April to September, 'late' = October to March. This was done for samples taken in SA5 and SA6 in both Japan and Korea and for samples from both countries combined. For both mtDNA and microsatellites no significant heterogeneity was found for the different temporal combination of samples. We also examined genetic differences between Korea and Japan for the two temporal periods (to respond in part to other of the suggestions from the 2006 SC meeting), and no statistically significant heterogeneity was observed. Results of our genetic analyses are not consistent with the hypothesis of two stocks migrating in these sub-areas in different months or season. These results are more consistent with the single stock scenario in these sub-areas. Other suggestions offered last year such as the conduction of microsatellite analysis in a single laboratory and analysis by sex could not be completed at this stage. These tasks will be completed in the near future.

INTRODUCTION

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. Therefore Japanese and Korean scientists worked together in 2005 and 2006 to analyze the by-catch samples from Japan and Korea using mtDNA and microsatellites. Kanda *et al*. (2006) and Park *et al*. (2006) detected genetic differences between the 1999 Korean sample from sub-area 6 (99KBC-6) and the rest of the samples from Korea as well as from Japan. Two possibilities were raised by the authors: (i) this could indicate that there are genetically different stocks in this area, and (ii) this could have resulted from the 99KBC-6 sample not being representative of the whole J-stock given their high genetic diversity (IWC, 2007).

On the basis of the suggestions derived from discussions on these documents in the Working Group on the in-depth assessment of western North Pacific common minke whales, with a focus on J stock, a small group was established to discuss future work in order to better understand the source of the heterogeneity detected in the

Kanda *et al*. (2006) and Park *et al*. (2006). The group suggested six further analyses as follows:

(1) Analyse samples by month and season. The heterogeneity could be due to seasonal or monthly difference in migration between two stocks.
(2) Incorporate sex into analyses. This allows us to detect sex-specific migration.
(3) Compare between all of the bycatches from sub-area 5 and sub-area 6 as well as between all of the bycatches from Korea and from Japan. This increases the sample size and hence statistical power of the analyses.
(4) Investigate whether the heterogeneity was due to general aspect of the 1999 sample or due to a few unique individuals in the sample. This allows us to distinguish between a real biological difference and a chance effect.
(5) Score microsatellites in one of the two laboratories. No data standardization will then be required.
(6) Include samples, possibly identified as J-stock individuals, from the Pacific side of Japan. It is important to note that the initial task of our cooperative genetic analyses was to concentrate on the investigation of the stock structure in sub-area 5 and sub-area 6, but inclusion of the Pacific samples will eventually allow us to conduct a comprehensive analysis of the J-stock in future.

The small group expected items (1) to (5) to be conducted hopefully by this year's meeting and item (6) in the near future. Unfortunately we were not able to complete all of them in the intersessional period. On the basis of the results we presented in the 58SC meeting held in St. Kitts and Nevis (Kanda *et al*., 2006), possibility of two stocks that migrate in the area in different times of the season was raised (analysis 1 above). In this paper we tested this hypothesis using mtDNA and microsatellite data of the recently collected samples of the minke whales from SA5 and SA6 along Japanese and Korean coast. Also comparison between samples in Japan and Korea was made for two temporal groups (analysis 3 above). Analysis 4 above was also partially addressed.

MATERIALS AND METHODS

Samples

Minke whales used in this paper were bycaught individuals on set net fisheries 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 from July to December only, whereas other year samples included individuals from all months. Samples used were collected after the new regulation for incidental catches was changed in July 2001. 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).

Tables 1 and 2 show the number of samples used in mtDNA and microsatellite, respectively, by year and month. The approximate distribution of sampling locations in sub-area 5 and 6 is shown in Fig.1. It appears that there are two temporal peaks in the distribution of the sample: June-July and December-January.

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.

Sequencing of the mtDNA control region

The first half of control region of the mitochondrial genome was amplified by using the polymerase chain reaction (PCR). In order to amplify the approximately 500 bp minke whale mtDNA including control region, primers light-strand MT4 (*5′*-CCTCCCTAAgACTCAAggAAg-*3′*) and heavy-strand Dlp 5R (*5′*-CCATCgAgATgTCTTATTTAAggggAAC-*3′*) were used. PCR products were purified by MicroSpin S-400HR columns (Pharmacia Biotech). Cycle sequencing was performed with the same primers, using AmpliTaq FS Sequencing Kit (Perkin-Elmer, Inc). The cycle sequencing products were purified by AutoSeq G-50 spin Columns (Pharmacia Biotech). The labeled sequencing fragments were resolved by electrophoresis by the ABI 3100™ Automated DNA Sequencer (Applied Biosystems, Inc), following the protocols of the manufacture. For each sample both strands were sequenced.

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). Primer sequences and PCR profiles follow 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. Co-amplification of SRY locus located on Y chromosome and a single microsatellite locus allowed us to determine sex as males (PCR bands of both SRY and microsatellite loci) while females only showed the microsatellite locus band.

Data analysis
In order to test seasonal pattern of migration, we divided the samples into two groups on the basis of their collection date: Early = April to September, Late = October to March. Early and late roughly corresponded to spring-summer and fall-winter, respectively.

For the mtDNA analysis, we used the randomized chi-square test of Independence (Roff and Bentzen, 1989) to test genetic differences. A total of 10,000 randomizations of the original data set were performed. A 'p' value below 5% was used as criteria for rejecting the null hypothesis of panmixia.

For the microsatellite analysis, conventional hypothesis testing procedure was conducted using heterogeneity test in allele frequencies among the samples. Probability test (or Fisher's exact test) implemented in GENEPOP 3.2 (Raymond and Rousset, 1995) 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).

RESULTS AND DISCUSSION

mtDNA analysis
Table 3 shows the results of the heterogeneity tests for mtDNA analysis. Firstly, we examined if there was any evidence of genetic differences among the early and late samples collected from Korea SA6, Korea SA5/6, Korea SA5, and Japan SA6, respectively. No evidence of statistically significant heterogeneity was detected in each case. Secondly, we examined genetic differences among the early as well as among the late samples collected from Korea SA6, Korea SA5/6, and Korea SA5. No evidence of statistically significant heterogeneity was detected in each case, and the samples were combined as Korea early and Korea late, respectively. Thirdly, we examined genetic differences between the Korea early and Korea late, and no statistically significant heterogeneity was observed. Fourthly, we examined genetic difference in the early as well as in the late samples between Korea and Japan. No heterogeneity was observed, and the samples were combined as early and late. Finally, we examined genetic differences between the early and late samples, but failed to detect any evidence of genetic heterogeneity.

In these analyses, some cases including Korean SA6 Late group showed a relatively low chi-square p values. Kanda *et al*. (2006) and Park *et al*. (2006) detected significant genetic differences between the 1999 Korean sample from sub-area 6. Based on these results we investigated whether the heterogeneity was due to general aspect of the 1999 sample or due to a few unique individuals in the sample. The statistical significant difference in the previous analysis (chi-square P value =0.024) was detected in the comparison between Early (April-September) and Late group (October-March) samples in 1999. Therefore we assumed that the heterogeneity detected in our previous analyses was not due to a general aspect of the 1999 sample but due to a few individuals taken in that period.

Microsatellite analysis
Table 4 shows the results of heterogeneity tests for microsatellite analysis. No evidence of statistically significant heterogeneity was detected between the different temporal and geographical grouping. These results are similar to the results by the mtDNA analysis.

The results of both mtDNA and microsatellites provide no evidence of multiple stocks of common minke whales in this region (sub-areas 5 and 6).

Last year, we conducted the analysis to test 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 (Kanda *et al*., 2006). At the moment we favored the single stock interpretation. During the discussion of our study last year, it was suggested the possibility of two stocks migrating in the area in different times of the season. The results of our analysis are not consistent with such hypothesis rather they are consistent with the single stock scenario in this region.

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 and genetic sex determination for the several samples has not been completed yet. As a consequence we could not respond at this stage to some of the suggestions offered last year (see Introduction). In future, therefore, we expect that all the samples would be analyzed altogether at one time in either Japan, Korea, or both countries.

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LITERATURE CITED

Abe, H., Goto, M., Pastene, L.A., Dewa, K., and Naito, E. 2001. Practical use of multiplex fluorescent PCR for cetacean sex identification. *Mar. Mammal Sci.* 17:657-664.

Bérubé, M., Jørgensen, H., Mcewing, R., and Palsbøl, P.J. 2000. Polymorphic di-nucleotide microsatellite loci isolated from the humpback whale, *Megaptera novaeanglliae*. *Mol. Ecol.* 9:2181-2183.

Buchanan, F.C., Friesen, M.K., Littlejohn, R.P., and Clayton, J.A. 1996. Microsatellites from beluga whale *Delphinapterus leucas*. *Mol. Ecol.* **5:**571-575.

International Whaling Commission. 2007. Report of the Working Group on the In-Depth Assessment of Western North Pacific Common Minke Whales, with a Focus on J Stock. Annex G1. *J. Cetacean Res. Manage*. 9: xxx-xxx.

Kanda, N, Park, J.Y, Sohn, H., Kim, Z.G, Goto, M., Pastene, L.A. 2006. Preliminary microsatellite analysis of bycaught J-stock minke whales from Japan and Korea. Paper SC/58/NPM2 presented to the IWC Scientific Committee, May 2006, St. Kitts and Nevis (unpublished).

Palsbøll, P.J., Bérubé, M., Larsen, A.H., and Jørgensen, H. 1997. Primers for the amplification of tri- and tetramer microsatellite loci in baleen whales. *Mol. Ecol*. 6:893-895.

Park, J.Y., Goto., Kanda, N., Sohn, H., Kim, Z.G. and Pastene, L.A. 2006. Preliminary mitochondrial DNA control region sequencing analysis of by-caught J-stock minke whale from Korea and Japan. Paper SC/58/NPM3 presented to the IWC Scientific Committee, May 2006, St. Kitts and Nevis (unpublished).

Raymond, M. and Rousset, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* 83:248-249.

Rice, W.R. 1989. Analyzing tables of statistical tests. *Evolution* 43:223-225.

Roff, D.A. and Bentzen, P. 1989. The statistical analysis of mtDNA polymorphisms: chi-square and the problem of small samples. *Mol. Biol. Evol.* 6:539-45.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: a laboratory manual. 2ed.* Cold Spring Harbor Laboratory, New York, USA.

Sokal, R.R. and Rohlf, F.J. 1995. *Biometry: the Principles of Statistics in Biological Research.* Freeman and Company, New York.

Valsecchi, E. and Amos, W. 1996. Microsatellite markers for the study of cetacean populations. *Mol. Ecol.* 5:151-156.

Table 1: By-caught samples used in mtDNA analysis by country, sub-area, year and month.

Country	Sub-area	Year	Month												Total
			1	2	3	4	5	6	7	8	9	10	11	12	
Korea	KBC6	1999		3	3	3	6	2		4		8	4	10	43
		2000		1	8							1		2	12
		2001	12	1	1	1									15
		2002		4	2	2	4	5		2	6	4	8	9	46
		2003	5	3	5	6	5	10	7	6	11	4	5	5	72
		2004	5	2	4	5	4	1	1	1	3	4	2	8	40
	Subtotal		22	14	23	17	19	18	8	13	20	21	19	34	228
	KBC5/6	1999					1	1						2	4
		2000													
		2001						1	1						2
		2002		1				1		1			1		4
		2003				1	1	2	3	1					8
		2004				1	6	2					3		12
	Subtotal		0	1	0	2	8	7	4	2	0	0	4	2	30
	KBC5	1999													
		2000			1									2	3
		2001			1			1							2
		2002						4		1	1		1		7
		2003	2				1	2	1	1		1	1		9
		2004	1					1	3		2			1	8
	Subtotal		3	0	2	0	1	8	4	2	3	1	2	3	29
Total			25	15	25	19	28	33	16	17	23	22	25	39	287
Japan	JBC6	2001							1	6		2	9	8	26
		2002	9	4	5	7	5	3	1	2	4	2	2	5	49
		2003	10	9	3	5	4	5	2	1	4	7	6	5	61
		2004	16	7	7	11	8	5	1	1	4	1	2	3	66
Total			35	20	15	23	17	13	5	10	12	12	19	21	202

Table 2. By-caught samples used in microsatellite analysis by country, sub-area, year and month..

Country	Sub-area	Year	Month												Total
			1	2	3	4	5	6	7	8	9	10	11	12	
Korea	KBC6	1999		3	3	2	2	1		1		3	2	3	20
		2000			4							1		2	7
		2001	12	1	1	1									15
		2002		4	2	2	4	4		2	5	4	8	9	44
		2003	5	3	4	6	5	10	7	4	10	4	5	5	68
		2004	5	2	4	5	4	1	1	1	3	4	2	8	40
	Subtotal		22	13	18	16	15	16	8	8	18	16	17	27	194
	KBC5/6	1999					1							2	3
		2000													
		2001						1	1						2
		2002		1				1		1			1		4
		2003				1	1	2	3	1					8
		2004				1	6	2					3		12
	Subtotal		0	1	0	2	8	6	4	2	0	0	4	2	29
	KBC5	1999													
		2000												2	2
		2001			1			1							2
		2002						4		1			1		6
		2003	2					2	1			1	1		7
		2004	1					1	3		2			1	8
	Subtotal		3	0	1	0	0	8	4	1	2	1	2	3	25
Total			25	14	19	18	23	30	16	11	20	17	23	32	248
Japan	JBC6	2001							1	6		2	9	8	26
		2002	9	4	5	7	5	3	1	2	4	2	2	5	49
		2003	10	9	3	5	4	5	2	1	4	7	6	5	61
		2004	16	7	7	11	8	5	1	1	4	1	2	3	66
Total			35	20	15	23	17	13	5	10	12	12	19	21	202

Table 3. Results of heterogeneity tests conducted in the minke whales samples by mtDNA analysis. Sample sizes are shown in the parenthesis. Early: April- September, Late: October-March.

Samples	P
Korea SA6: Early (95) × Late (133)	0.0667
Korea SA5/6: Early (23) × Late (7)	0.9486
Korea SA5: Early (18) × Late (11)	0.4326
Korea Early : SA6(95) × SA5/6(23) × SA5(18)	0.7492
Korea Late : SA6(133) × SA5/6(7) × SA5(11)	0.6264
Korea: Early (136) × Late (151)	0.0837
Japan: 2 Early (80) × Late (122)	0.5470
Early : Korea(136) × Japan(80)	0.4089
Late : Korea(151) × Japan(122)	0.0914
All samples: Early (216) × Late (273)	0.4249

Table 4. Results of heterogeneity tests conducted in the minke whales samples by microsatellite analysis. Sample sizes are shown in the parenthesis. Early: April- September, Late: October-March.

Locality : tests	Microsatellite									
Samples	DlrFB14	EV1	EV14	EV21	EV94	GATA98	GT195	GT211	TAA31	all
Korea SA6: Early(81) × Late(113)	0.236	0.745	0.345	1.000	0.722	0.015	0.033	0.724	0.649	0.193
Korea SA5/6: Early(22) × Late(7)	1.000	0.639	0.235	0.417	1.000	0.425	0.426	0.950	1.000	0.958
Korea SA5: Early(15) × Late(10)	0.117	0.074	0.469	0.766	0.750	0.945	0.614	0.041	0.265	0.221
Korea Early: SA6(81) × SA5/6(22) × SA5(15)	0.158	0.526	0.349	0.206	0.850	0.183	0.214	0.084	0.872	0.220
Korea Late: SA6(113) × SA5/6(7) × SA5(10)	0.588	0.754	0.523	0.197	0.995	0.790	0.279	0.218	0.279	0.675
Korea: Early(118) × Late(130)	0.151	0.365	0.882	0.827	0.610	0.044	0.069	0.550	0.583	0.264
Japan: Early(136) × Late(151)	0.211	0.463	0.568	0.902	0.205	0.365	0.231	0.028	0.488	0.204
Early: Korea(118) × Japan(80)	0.366	0.324	0.498	1.000	0.707	0.259	0.224	0.070	0.379	0.373
Late: Korea(130) × Japan(122)	0.078	0.155	0.186	0.671	0.549	0.047	0.206	0.839	0.252	0.088
All samples: Early(198) × Late(252)	0.148	0.218	0.967	0.937	0.261	0.022	0.292	0.105	0.389	0.094

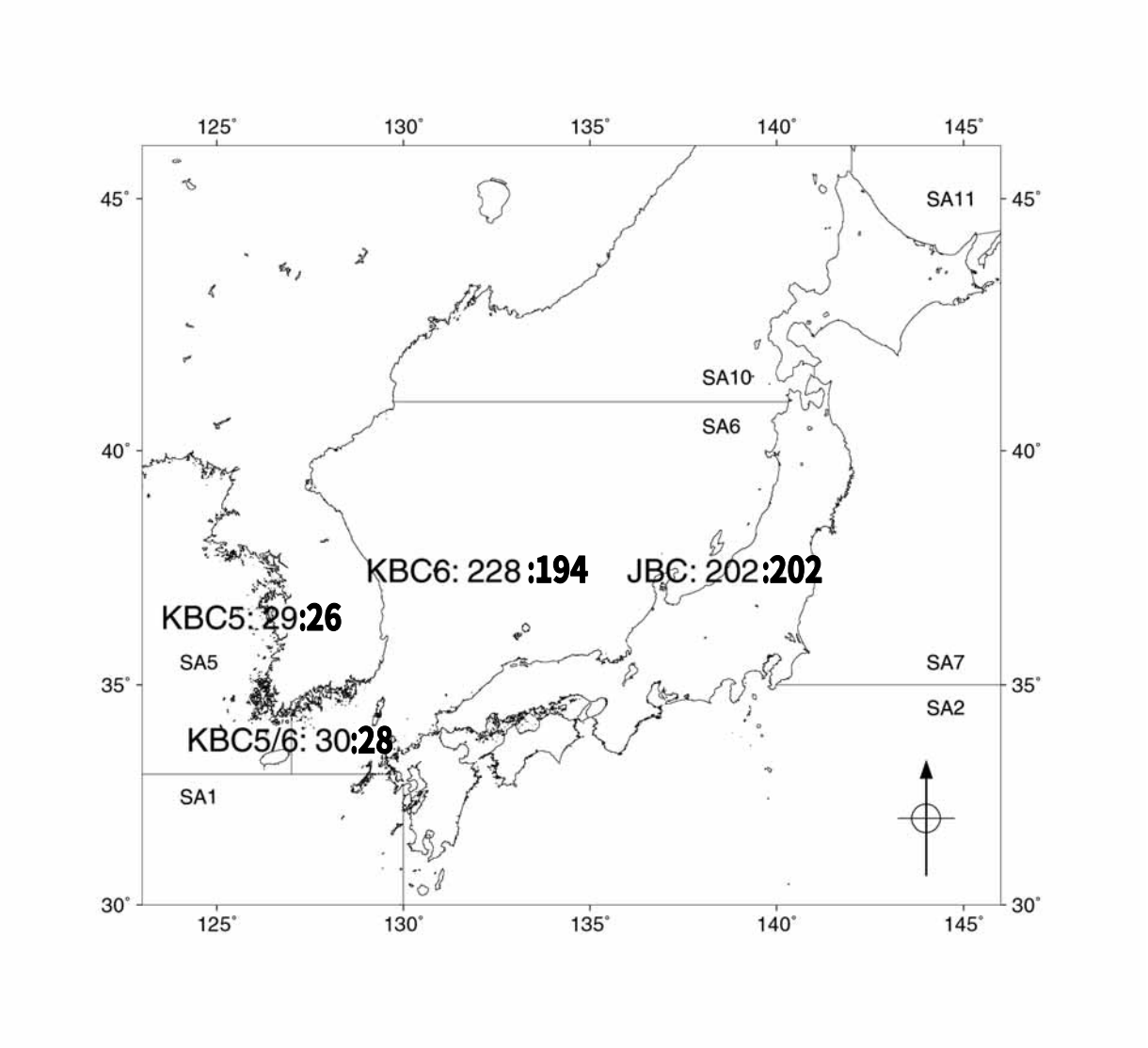

Fig 1. Approximate distribution of sampling locations in sub-area 5 and 6. Left and right number show the sample size using for mtDNA and microsatellite analyses, respectively.