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Update of the mitochondrial DNA analysis on sub-stock structure of the J stock common minke whales from the Japanese waters

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ABSTRACT

Genetic variation at the mtDNA control region in the western North Pacific common minke whales around Japanese waters was examined. Samples used were from JARPN/JARPNII-offshore component obtained in sub-areas7W (140-147°E) and 11 from 1994 to 2007, JARPNII-coastal component obtained in Sanriku region in spring of 2003 to 2007 and Kushiro region in fall of 2002 to 2007, and from bycatches of the set net fishery along the Japanese coast from 2001 to 2007. The main objective of the study was to investigate the possibility of sub-structure of the J stock common minke whale. Based on the results of individual assignment using microsatellites, heterogeneity tests were conducted for two options: a) all individuals (n = 1,807) and b) only the suspected J stock individuals (n = 768). Heterogeneity tests were based on the randomized chi-square test and Fst as recommended by the JARPN II Review Workshop. Results of the analysis that used all by-catch individuals suggested seasonal genetic differences in SA7 between early (April to September) and late (October to March) samples most likely due to the different proportion of the J and O stocks in the early and late samples. Sub-area 2 in the Pacific side was mainly occupied by the J stock. No significant differences were found among J stock animals from SA2 (Pacific side), SA6 (Sea of Japan) and SA7 (Pacific side), which suggest that a single J stock distributes around Japanese waters. It is important to note that analysis of mtDNA marker independent from the assignment procedure conducted in Kanda *et al.* (2009a) showed the same pattern of the stock structure to microsatellite analysis (Kanda *et al.*, 2009b).

KEYWORDS: COMMON MINKE WHALE, MITOCHONDRIAL DNA, SCIENTIFIC PERMITS, INCIDENTAL CATCHES

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. This is the J-stock, which is genetically different from widely distributed O-stock in western North Pacific. The J- and O-stocks differ from each other in body size (Omura and Sakiura, 1956; Ohsumi, 1983), conception dates (Kato, 1992), allozyme allele frequencies (Wada, 1984; 1988; 1991; Wada and Numachi, 1991) and mitochondrial DNA (mtDNA) haplotype frequencies (Goto and Pastene, 1997; 1998) 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).

Following earlier Committee recommendations, Japanese and Korean scientists worked together in 2005 and 2006 to analyze 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 discussions of these documents at 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 on stock structure, in particular to better understand the source of the genetic heterogeneity detected in the Kanda *et al.* (2006) and Park *et al.* (2006) analyses.

In 2008 the following tasks were listed by the Working Group:

(1) Standardize Japanese and Korean microsatellite data (end of 2008);
(2) Conduct heterogeneity tests on samples stratified by month and season, as well as sex;
(3) Include recent data from 2005-2007 to increase sample sizes and power for mtDNA and microsatellite analyses;
(4) Investigate whether previously found heterogeneity is due to the 1999 data in general or just a few individuals in this year;
(5) Analyse the 1982 Korean commercial samples (27) together with recent samples (recommended at SC59); and
(6) Include samples from the Pacific side of Japan in work related to the JARPN-II review.

Unfortunately it was not possible to conduct a mtDNA analysis for the combined samples of Korea and Japan because at this stage there was no confidence in the accuracy of both data set combined. Therefore in this study the mtDNA analysis was conducted using only the Japanese samples. It was thus difficult for us to cover all of the tasks listed by the Working Group in 2008.

MATERIALS AND METHODS

Samples

Table 1 shows the number of samples used in the present mtDNA analysis by sex, month, year, sub-area in by-catch (BC) and JARPN & JARPNII. Table 2 also shows sample sizes used in this study by sex, month, year, sub-area in by-catch (BC) and JARPN & JARPNII assigned to J animals by Kanda *et al*. (2009a). Details of the surveys design and methodology in the offshore and coastal components of JARPNII were described in Tamura *et al*. (2009) and in Kishiro *et al*. (2009), respectively. During the *IST* specification conducted in 2003, eighteen sub-areas were set for management purpose of the western North Pacific common minke whale (Figure 1). As of July 1st 2001, the new regulation governed by the Japanese Government has allowed the set net fishermen to harvest whales found in their set net and to sell these on to the market after DNA registration of these for individual identification. The bycatches used were obtained from the SA2, SA6, SA7, SA10, and SA11 all the year round.

Sequencing of the mtDNA control region

We followed the IWC guidelines for DNA data quality (IWC, 2009) as much as possible. Using established protocols (Sambrook *et al*., 1989), total-cell DNA was extracted from skin tissue samples. The extracted DNA was used for both mtDNA and microsatellite analyses. The first half of control region of the mitochondrial genome was amplified using the polymerase chain reaction (PCR). In order to amplify an approximately 500 bp of the mtDNA control region, primers light-strand MT4 (Árnason *et al*., 1993) and heavy-strand Dlp5R (5′-CCATCgAgATgTCTTAT-TTAAggggAAC-3′), were used. PCR products were purified by MicroSpin S-400HR columns (Pharmacia Biotech). Cycle sequencing was performed with the same primers, using BigDye terminator cycle sequence Kit (Applied Biosystems, Inc). The cycle sequencing products were purified by AutoSeq G-50 spin Columns (Pharmacia Biotech). The labeled sequencing fragments were resolved by electrophoresis through a 5% denaturing polyacrylamide matrix on an ABI 377™ and ABI3100 Automated DNA Sequencer (Applied Biosystems, Inc), following the protocols of the manufacturer. For each sample both strands were sequenced.

Data analysis

The randomized chi-square Test of Independence (Roff and Bentzen, 1989) was used to investigate the temporal/spatial differentiation of mtDNA variation. F_{ST} was calculated using the software of Analysis of Molecular Variance (AMOVA) (Excoffier *et al*. 1992). The strata with less than 4 individuals were excluded from the analyses. In each test a total of 10,000 permutations of the original data were performed. A P-value smaller than 0.05 was used as a criterion to reject the null hypothesis of panmixia.

The following steps were followed: a) analysis of yearly genetic differences of the bycatch samples in sub-areas 2, 6 and 7 for total, early (April – September) and late (October - March) samples; b) analysis of seasonal genetic differences in the bycatch samples (all years combined); c) analysis of genetic differences between the bycatch (BC) and JARPN/JARPNII (NP) samples in SA7W and SA11; and d) analysis of genetic differences among the samples collected from the different sub-areas (BC and NP samples combined).

Assignment for J or O stock types

In the case of sub-area 7W the analyses were conducted for two options: a) 'all individuals' and b) 'only J stock individuals'. Individual assignment to O and J stocks was based on analysis of microsatellite and the computer program STRUCTURE (Kanda *et al*., 2009a).

RESULTS

Genetic divergence between samples

Yearly and seasonal genetic differences within the subareas

No statistically significant genetic differences were detected for BC samples collected in different years in SA2, SA6 and SA7 for total, early and late samples, under both options 'all individuals' and 'only J stock individuals' (Table 3). For all years combined a significant difference was found between early and late samples for option 'all individuals'. However no significant differences were found when only J stock individuals were considered. Same results were found for both statistics (Table 3).

Genetic difference between bycatch and JARPN/JARPNII samples

In both SA7 and SA11 significant differences were found between BC and NP samples when option 'all individuals' was considered. Such significant differences were not found when only J stock individuals were considered (Table 4). Same results were found for both statistics

Genetic difference among subareas

Several instances of significant differences among sub-areas were found under the option 'all individuals'. No significant differences were found when only J stock individuals were considered. Same results were found for both statistics.

DISCUSSION

Results of this study suggested some seasonal genetic differences of common minke whales in SA7. This is most likely due to the different proportion of the J and O stocks in the early and late samples. This study also found that SA2 in the Pacific side was mainly occupied by the J stock. Comparison among J stock animals from SA 2, 6 and 7 resulted in no significant differences, which suggest a single J stock around Japanese waters. It is important to note that analysis of mtDNA marker independent from the assignment procedure conducted in Kanda *et al*. (2009a) showed the same pattern of the stock structure to microsatellite analysis (Kanda *et al*., 2009b)

The Working Group on the in-depth assessment of western North Pacific common minke whales, with a focus on J stock, agreed the following four stock structure hypotheses (IWC, 2007).

1) One stock in the SA5 and SA6.
2) Two stocks in the SA5 and SA6. The one stock migrates along the Japanese coast, and the other migrates along the Korean coast.
3) Two stocks. One migrates up to the SA5 and the other migrates further north in the SA6 along the both Japanese and Korean coast.
4) Two stocks. Both migrate through the SA6 in different time of the year.

Among these hypotheses, only the fourth one suggests that minke whales from two different stocks migrate, but the different time of the year, along the Japanese coast in the Sea of Japan. This study did not support this hypothesis because we found no seasonal genetic difference in the SA6 samples. We believe that the sample sizes for this test (Early = 188, Late = 223) was reasonably large to say that. In regard to the stock structure only within the SA5 and SA6, even this study that used only the Japanese data set can conclude that the fourth hypothesis is the most unlikely.

Furthermore, all of these four hypotheses assume that the SA2 is occupied by the O stock, which is different from our observation, because these were raised before the sample from the SA2 were used for the analysis. With respect to this, our study indicates all four hypotheses are equally unlikely.

Although the working group disagreed to list as standing hypotheses, some members of the group raised two additional stock structure scenarios that extended covering geographic area to the Pacific east of Japan, i.e., SA2 and SA7 (IWC, 2009). One of them assumes two different stocks in addition to the O stock: one in the Sea of Japan side (SA6) and the other in the North Pacific side (SA2) of Japan. Again, this study did not support this hypothesis because no evidence of the genetic difference was found between the samples from the SA2 and SA6.

It should be noted, however, that this study used only samples from Japan. Future analyses should incorporate samples from Korea once the combined data set is accurate enough for conducting such analyses. Furthermore, the power analysis which examined in the microsatellite analysis (Kanda *et al.*, 2009b) would be conducted until the next SC meeting.

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Table 1. Sample sizes used in this study by sex, month, year, sub-area in by-catch (BC) and JARPN & JARPNII (NP).

Source	Sub-area	Year	Jan.		Feb.		Mar.		Apr.		May		Jun.		Jul.		Aug.		Sep.		Oct.		Nov.		Dec.		Total
			F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	
BC	2	2001*													1	1							4	1	1	2	10
		2002	2	2	4		1	1		1			1		1		1				1			2	2	3	22
		2003	4	1				2		2	1		1		2	1									4	3	21
		2004	3		4	1	2		1		1		1		3						1		1		3	2	23
		2005	7	1	1	2		3		2	3				2		2	1			1	1		1	4	2	33
		2006	4	2	2	1	1	1	2		4	4				1		1					1		4	1	29
		2007	4	2	8	3	1	2			1	1	2	1	1							1	4	4	7	4	46
		sum	24	8	19	7	5	9	3	5	10	5	5	1	10	3	3	2			3	2	10	8	25	17	184
	6	2001*														1	3	3			1	1	5	4	6	2	26
		2002	7	2	2	2	1	4	2	5	5		2	1	1		2			4	1	1	1	1	3	2	49
		2003	6	4	4	5	2	1	3	2	1	3	4	1	1	1		1	2	2	4	3	5	1	3	2	61
		2004	10	6		7	3	4	6	5	4	4	4	1		1		1	2	2		1	1	1		3	66
		2005	4	2	2	4	1	1	6	4	3	3	4	2	1	2	1	1					1	3	9	4	59
		2006	7	4		5	10	6	6	5	9	8		2	1	1	4	3					1	1	4	1	78
		2007	5	4	3	1	2	3	9	7	3	5	2	3	3	3	2	3		1	1	1	3	3	1	4	72
		sum	39	22	11	24	19	19	32	28	25	23	16	10	7	9	12	12	4	9	7	7	17	14	26	18	411
	7	2001*														1			1	1		1	3	1	2	1	11
		2002	1	1		2	1	1	1	2	4	3	2	3	1	1					1	1	2	2	3	2	34
		2003		2			1		5	3	4	2	3	3	3							1	1	2	2	3	35
		2004							2	1	5	4	6	1	1	1		1		2				1	1		26
		2005	2	1			1		2	2	1	3	6	3	2	1	1		1				1	2	2		31
		2006	1			2	2		5	4	2	3	6		3	2	1	3	1			1		2		1	39
		2007	1	1	1		2		1	1	5	4	5	2	2		2	1			2		1	1	2	1	35
		sum	5	5	1	4	7	1	16	13	21	19	28	12	12	6	4	5	3	3	3	4	8	11	12	8	211
	10	2001*																				1	1	2			4
		2002																					1			1	2
		2005																					1	1	1		3
		sum																				1	3	3	1	1	9
	11	2001*																		1		1					2
		2002																				1	1				2
		2003											1								1	1	2	1			7
		2005																					1	1			2
		2006																				1					1
		2007																			1		1				2
		sum											1							1	2	4	5	2			16
BC sum			68	35	31	35	31	29	51	46	56	47	50	23	29	19	19	19	7	13	15	18	43	38	64	44	832
JARPN & JARPNII	1	1996															11	19									30
		1999													22	28											50
	7W	1996													1			15	2	13							31
		1997												1													1
		1998									3	22															25
		1999											7	43													50
		2000															1	5	4	14							24
		2001									3	25		15													43
		2002															1	5	24	67	6	7					110
		2003							29	20	2	16															67
		2004																	10	41	4	20					75
		2005							19	13	18	10	2	12	2	16			10	25	5	20					152
		2006							11	8	24	33		9	2	9			7	15	3	10					131
		2007							7	3	29	18	13	66					9	12	8	21					186
JARPN(II) sum									64	44	79	124	22	146	5	25	2	25	66	187	26	78					975
Grand total			68	35	31	35	31	29	115	90	135	171	72	169	34	44	21	44	73	200	41	96	43	38	64	44	1807

*:samples collected from July

Table 2. Sample sizes used in this study by sex, month, year, sub-area in by-catch (BC) and JARPN & JARPNII (NP) assigned to J animals by microsatellite analysis.

Source	Sub-area	Year	Jan.		Feb.		Mar.		Apr.		May		Jun.		Jul.		Aug.		Sep.		Oct.		Nov.		Dec.		sum
			F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	
BC	2	2001*													1	1							3	1	1	1	8
		2002	2	1	2		1	1		1			1		1		1				1			1	2	3	18
		2003	4	1				2					1		2	1									4	2	17
		2004	3		4	1	2		1		1				2						1		1		3	2	21
		2005	5	1		2		2		1	2				2		1	1			1	1		1	4	2	26
		2006	4	2			1		2		3	2				1		1					1		4	1	22
		2007	3		7	2	1	1			1	1	1	1	1								4	3	5	2	33
		sum	21	5	13	5	5	6	3	2	7	3	3	1	9	3	2	2			3	1	9	6	23	13	145
	6	2001*														1	3	2			1	1	4	3	6	2	23
		2002	6	2	1	2	1	4	2	4	5		2	1	1		2			4	1		1	1	3	1	44
		2003	6	4	4	5	2	1	3	1	1	2	4	1	1	1		1	2	2	3	3	5	1	2	2	57
		2004	9	6		6	3	3	6	5	4	4	4	1		1		1	2	2		1	1			2	61
		2005	3	2	2	3	1	1	6	3	3	2	4	2	1	2	1	1					1	3	9	4	54
		2006	6	4		5	8	6	6	5	9	8		2	1	1	3	3					1	1	4	1	74
		2007	5	4	3	1	2	3	8	5	3	5	2	3	3	3	2	3		1	1	1	3	3	1	4	69
		sum	35	22	10	22	17	18	31	23	25	21	16	10	7	9	11	11	4	9	6	6	16	12	25	16	382
	7	2001*																					3	1	1		5
		2002		1		2		1				1	2								1	1	2	2	2	2	17
		2003		1					1	1	1		1	1	1							1	1	2	1	2	14
		2004										1	3	1		1		1		2				1	1		11
		2005	2	1						1		1	3		1	1	1		1				1	2	1		16
		2006	1			1	1		3		1	1	1		1	1	1					1		2		1	16
		2007	1		1						1		1	1	2		1	1			1		1	1	1	1	14
		sum	4	3	1	3	1	1	4	2	3	4	11	3	5	3	3	2	1	2	2	3	8	11	7	6	93
	10	2001*																				1		1			2
		2002																					1			1	2
		2005																					1	1	1		3
		sum																				1	2	2	1	1	7
	11	2001*																		1		1					2
		2002																				1	1				2
		2003											1								1	1	1	1			6
		2005																					1	1			2
		2006																				1					1
		2007																			1		1				2
		sum											1							1	2	4	4	2			15
BC sum			60	30	24	30	23	25	38	27	35	28	31	14	21	15	16	15	5	12	13	15	39	33	56	36	642
JARPN & JARPNII	1	1996															1	7									8
		1999													10	5											15
	7W	1996																		1							1
		1999											1	1													2
		2000															2			1							3
		2001									1																1
		2002															1		2	6	2						11
		2003							2	4	1																7
		2004																	1	3		3					7
		2005							2	6	2	4		1	2				2	8		2					29
		2006								1	5	7			2					4							19
		2007							2		4	2	1	3					2		1	8					23
ARPN(II) sum									6	11	13	13	2	5	4		3		7	23	3	13					103
Grand total			60	30	24	30	23	25	44	38	48	41	33	19	25	15	19	15	12	35	16	28	39	33	56	36	768

*:samples collected from July

Table 3. Statistical comparison of yearly variation in sub-areas 2, 6 and 11for total, early (April-September) and late (October-March) bycatch samples. The table also shows the results of a statistical test to compare early versus late samples in the same sub-areas for all years combined (E x L). Results are shown for both options 'all individuals' and 'only J stock individuals'.

	All individuals											
	Total			Early			Late			E x L		
	Chi-square P value	Fst	P value	Chi-square P value	Fst	P value	Chi-square P value	Fst	P value	Chi-square P value	Fst	P value
BC-SA2	0.986	-0.006	0.848	0.987	-0.037	0.982	0.728	0.011	0.120	0.054	-0.001	0.497
BC-SA6	0.852	0.001	0.362	0.980	-0.010	0.958	0.740	0.007	0.120	0.653	0.001	0.195
BC-SA7	0.152	-0.004	0.867	0.101	0.002	0.364	0.881	-0.011	0.772	0.012	0.014	0.001

	Only J											
	Total			Early			Late			E x L		
	Chi-square P value	Fst	P value	Chi-square P value	Fst	P value	Chi-square P value	Fst	P value	Chi-square P value	Fst	P value
BC-SA2	0.952	-0.007	0.779	0.955	-0.060	0.957	0.625	0.008	0.270	0.067	-0.000	0.403
BC-SA6	0.770	0.001	0.327	0.929	-0.008	0.890	0.526	0.007	0.160	0.596	0.002	0.227
BC-SA7	0.377	-0.014	0.801	0.429	-0.012	0.574	0.562	0.006	0.440	0.769	-0.008	0.750

Table 4. Statistical comparison among the bycatch (BC) and JARPN/JARPNII (NP) samples in the SA7W and SA11. Results are shown for both options 'all individuals' and 'only J stock individuals'.

Sub-area	All animals			Only J		
	Chi-square P value	Fst	P value	Chi-square P value	Fst	P value
SA7	***	0.012	***	0.615	-0.006	0.920
SA11	0.460	0.057	***	0.781	-0.021	0.813

***: <0.0001

Table 5. Statistical comparison among sub-areas for samples collected from bycatch (BC) and JARPN/JARPNII (NP). Results are shown for both options 'all individuals' and 'only J stock individuals'.

(A) Chi-square statistics

All individuals: **

	BC2	BC6	BC7	BC10	BC11	NP7	NP11
BC2	-	***	***	0.668	0.925	***	***
BC6		-	***	0.578	0.981	***	***
BC7			-	0.373	0.977	***	0.715
BC10				-	0.283	0.081	0.317
BC11					-	0.259	0.669
NP7						-	0.534
NP11							-

***: <0.0001

**: <0.001

Only J: P=0.533

(B) Upper diagonal: P value of hypothecal test of Fst, lower diagonal: Fst value

All individuals: Fst value; 0.033, P value of hypothecal test; ***

	BC2	BC6	BC7	BC10	BC11	NP7	NP11
BC2	-	***	0.003	0.350	0.127	***	***
BC6	0.008	-	***	0.277	0.540	***	***
BC7	0.008	0.017	-	0.216	0.009	***	0.539
BC10	0.001	0.013	0.011	-	0.181	0.022	0.092
BC11	0.017	-0.005	0.031	0.031	-	***	***
NP7	0.037	0.059	0.012	0.036	0.085	-	0.558
NP11	0.018	0.038	0.000	0.017	0.057	-0.001	-

***: <0.0001

Only J: Fst value; 0.002, P value 0.173

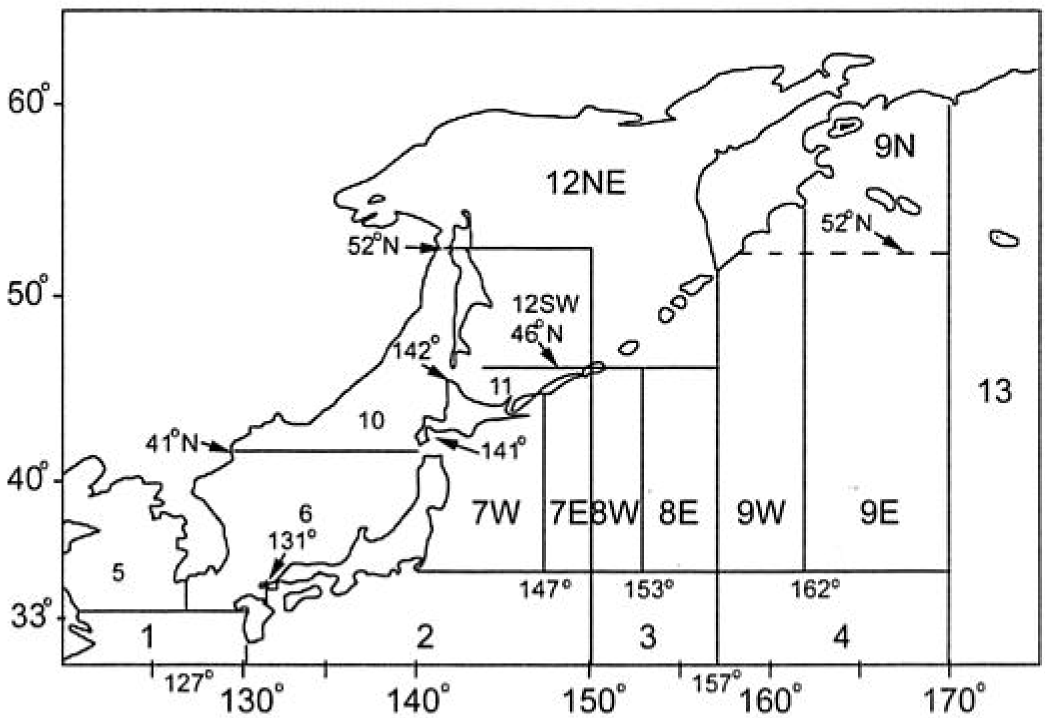

Fig. 1. The 18 sub-areas used for the *Implementation Simulation Trials* for North Pacific minke whales.