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

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ABSTRACT
This paper examined the stock structure of common minke whales existing around the Japanese waters. Past studies indicated that two different stocks of minke whales existed around the Japanese coast: O stock in the western North Pacific and the J stock in the Sea of Japan. Kanda *et al.* (2009, i.e., SC/61/JR5) indicated by utilizing genetic variation observed at 16 microsatellite loci and a Bayesian clustering approach that only the J and O stocks of common minke whales occupied around the Japanese waters and the proportion of these two stocks varies among the subareas. In this paper, we used the offshore JARPN and JARPNII samples from 1994 to 2007 at the SA7W (140-147°E) and SA11, the coastal JARPNII (7W) samples conducted at Sanriku region in spring of 2003 to 2007, and at Kushiro region in fall of 2002 to 2007, and the bycatch samples from set net fishery along the Japanese coast from 2001 to 2007 to look for evidence of sub-structuring of the J stock. On the basis of the results from Kanda *et al.* (2009), whales in these samples were assigned to either the J stock, the O stock, or unknown origins. We conducted heterogeneity tests with all individuals included (total N = 1,805) and with only the suspected J stock individuals included (total N = 768). This paper found the seasonal genetic difference in the SA7 between the minke whales from April to September and from October to March most likely due to the different proportion of the J and O stocks between the seasons. No evidence of the genetic differences was detected in the samples from all of the subareas when we used only the J stock individuals for the tests. This paper found that the SA2 was mainly occupied by the J stock that was not genetically different from the J stock in the Sea of Japan. Our simulation study indicated that from genetics standpoint the statistical power for testing the J sub-stocks (especially in the SA2 and SA6) with our data set was quite high. This study demonstrated that only the single J stock with no sub-structuring existed around the Japanese waters.

KEYWORDS: COMMON MINKE WHALE, MICROSATELLITE, 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 (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 the 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).

Japanese and Korean scientists have worked together intersessionally to analyze the samples from Japan and Korea using genetic data (mtDNA and microsatellites) in order to investigate stock structure of common minke whales in the Japanese and Korean waters. Kanda *et al*. (2006), Park *et al*. (2006), and Goto *et al.* (2007) detected no genetic difference among the samples from different sampling areas as well as different sampling years except significant difference between the 1999 Korean sample from SA6 (99KBC-6) and the rest of the samples from Korea and 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).

In 2008, the following future work was planned in the Working Group on the in-depth assessment of western North Pacific common minke whales, with a focus on J stock:

(1) Standardize Japanese and Korean microsatellite data,
(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 that year,
(5) Analyze the 1982 Korean commercial samples (27) together with recent samples, and
(6) Include samples from the Pacific side of Japan in work related to the JARPNII review.

Plans were made to conduct analyses to cover these future works for the SC61 meeting using the Japanese and Korean samples. With respect to the microsatellite standardization between the two laboratories, Korean scientists re-analyzed all of the individuals they possessed with several Japanese samples, as references, for the 16 microsatellite loci we regularly used for making our genetic database, and sent raw output data from the DNA sequencer (ABI3100) to us in Japan to read genotypes. Although the microsatellite allele standardization has been done successfully for most of the loci from this way, the Korean data set was still incomplete for proceeding with further analyses. In this paper, therefore, we presented updated, yet preliminary, microsatellite analysis of common minke whale stock structure using only the Japanese samples. It was thus difficult for us to cover all of the tasks suggested during the 2008 SC meeting. These tasks will be completed in future when the Korean data set is finalized.

Kanda *et al*. (2009, i.e., SC/61/JR5) attempted to distinguish sampled minke whales into genetically distinct stocks using a combination of microsatellite analysis and a Bayesian clustering approach implemented in the computer program STRUCTURE (Pritchard *et al.*, 2000). Samples of 2542 minke whales were collected during the offshore component of JARPN and JARPNII from 1994 to 2007, during the coastal component of JARPNII from 2002 to 2007, and from bycatches in the set net fishery along the Japanese coast from 2001 to 2007, and were analyzed using 16 microsatellite loci. Result of the clustering analysis indicated that our samples came from two genetically differentiated groups of minke whales. More than 90% of the individuals were assigned into the either stocks based on their high membership probabilities obtained from the program. All other individuals with the lower membership probabilities to the either groups were assigned as individuals of unknown origin. Both of the assigned and unassigned individuals were then grouped based on their sampling origins. Spatial distribution of these individuals clearly indicated that these two stocks were the J and O stocks because individuals from the Sea of Japan and offshore North Pacific tended to be assigned in the different group (see also Table 2 in this paper). In this paper, we conducted heterogeneity tests among these samples grouped based on the assignment results from Kanda *et al*. (2009) to look for evidence of sub-structuring of the J stock around the Japanese waters.

MATERIALS AND METHODS

Sample collections

As samples of common minke whales existing around the Japanese waters (Table 1), we used those collected during offshore component of JARPN and JARPNII from 1994 to 2007 at SA7W (140-147°E) and SA11, those collected during coastal component of JARPNII (SA7W) conducted at Sanriku region in spring of 2003, 2004, 2005, 2006, and 2007, and at Kushiro region in fall of 2002, 2004, 2005, 2006, and 2007, and those bycaught on set net fishery conducted along the Japanese coast from 2001 to 2007 (bycatch) (Table 1). Details of the recent JARPNII surveys can be found in Tamura *et al*. (2009) and Kishiro *et al.* (2009). 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. Fig. 1 shows the 18 sub-areas set for management purpose of the western North Pacific common minke whale.

Microsatellites analysis

We used genetic data obtained from the 16 microsatellite loci: EV1, EV14, EV21, EV37, EV94 (Valsecchi and Amos, 1996), GT23, GT195, GT211, GT310, GT509, GT575 (Bérubé *et al*., 2000), GATA28, GATA98, GATA417, TAA31 (Palsbøll *et al*., 1997), and DlrFCB14 (Buchanan *et al*., 1996). Laboratory procedure used for this analysis and the level of genetic diversity of these loci in our samples can be found in Kanda *et al*. (2009).

Data analysis

In order to detect genetic differences in the samples of minke whales, we performed conventional hypothesis testing procedure using heterogeneity test in frequencies of the microsatellite alleles among samples. Null hypothesis to be tested is if the samples came from a genetically same group of common minke whales. If genetic differences exist, then it could indicate these samples came from genetically different stocks of minke whales. Markov chain method implemented in the GENEPOP (Rousset, 2008) was used to conduct the heterogeneity tests. Statistical significance was determined using the chi-square value obtained from summing

the negative logarithm of *p*-values over the 16 microsatellite loci (Sokal and Rohlf, 1995). F_{ST} values were also calculated using ARLEQUIN 2.0 (Schneider *et al.*, 2000) or FSTAT 2.9.3 (Goudet, 1995). The samples with less than 4 individuals when we divided into smaller groups for the tests were excluded from the test procedure. File conversions were conducted using either computer program CREATE (Coombs *et al.*, 2008) or CONVERT (Glaubitz, 2004).

Assessment of statistical power for the tests of homogeneity

In order to assess statistical power for tests of homogeneity (e.g., Waples and Gaggiotti, 2006), we generated genotypic data using computer software EASYPOP (Balloux, 2001) and conducted heterogeneity tests with these generated data. We assumed two populations, each of which consists of diploid individuals with a constant size and equal sex ratio with random mating. We assumed ratio of effective population size to census population size to be 1/3 to 1/4 (Roman and Palumbi, 2003). The effective population size of the populations was thus set as it becomes 1/3 or 1/4 of the census population size. We used census population size of 9000. This population size was derived from Hakamada *et al*. (2009), which estimated abundance of the J stock in the area as over 17,000. At each generation, simulation produces genotype data set for 16 independent nuclear gene loci (microsatellites) for each individual. The number of the loci simulated and maximum number of the allelic states (18) was set based on the observed data in this study. Bidirectional migration was assumed with an equal migration rate (m). Migration rates ranged from 0.01 to 0.2, some of which (0.1- 0.2) were quite high for genetic method to detect. We specified a range of genetic divergence using F_{ST} values estimated assuming island model between the two populations by changing migration rate. Mutation rate of $5x10^{-4}$ was chosen to represent microsatellite loci. For each simulation parameter set, we made 100 replicates. We ran 5,000 generations for each replicate before collecting data. In the final generation of each replicate, a sample of 140 individuals was taken from each population for genetic analysis. The sample size of 140 was come from the number of the suspected J stock individuals in the SA2 (Table 2). We conducted homogeneity tests for the generated data set using pairwise tests of differentiation option in the FSTAT. In this option, for each pair of samples, multiloci genotypes are randomized between the two samples. The overall loci G-statistic is given and statistical significance was decided with a table wide level of significance at 5%. We conducted this simulation in order to assess the statistical power to detect the genetic difference between common minke whales from the SA2 and SA6 for the case that genetically different sub-stocks might exist.

RESULTS

On the basis of the results from the individual assignments to the stocks according to the criteria in Kanda *et al*. (2009), we conducted the tests with two different kinds of sample groups: 1) one that included all the analyzed individuals and 2) one that used only the suspected J stock individuals. Although the proportions of the individuals of unknown origins were similar to each of the subareas, the proportions of the J and O stock individuals were quite different among the subareas (Table 2).

Genetic divergence between samples

Yearly and seasonal genetic differences within the subareas. We first looked for evidence of genetic differences among the bycatch samples collected from the different years within the SA2, SA6, and SA7W respectively. No statistically significant genetic differences were detected within each of the subareas for the all individuals and the only J stock individuals (Table 3).

We then divided the individuals in the samples into those collected from April to September (Early) and from October to March (Late). We first looked for evidence of genetic difference among the samples collected from the different years of the same seasons. No statistically significant genetic differences were detected within each season for both the all individuals and only J stock individuals, so we combined the samples as Early and Late samples, respectively, and looked for evidence of genetic difference between them (Table 3). For the all individuals, although no statistically significant difference was detected in the SA2 and SA6, highly significant heterogeneity was observed in the SA7W. F_{ST} between the Early and Late samples was also significantly larger than 0 in the SA7W. For the only J stock individuals, no statistically significant difference was detected within each of the subareas.

Genetic difference between bycatch and JARPN/JARPNII samples in the SA7W and SA11. We looked for evidence of genetic difference between the bycatch and JARPN/JARPNII samples in the SA7W as well as SA11 where the samples of both sources were available (Table 4). For the all individuals in the SA7, we compared total, Early, and Late bycatch samples, respectively, to the JARPN/JARPNII sample on the basis of the results shown above. The bycatch sample from the SA11 was too small to do so. In both the SA7W and SA11, when all individuals were used for the tests, highly significant difference was observed from the all tests. In contrast, when only J stock individuals were used, no statistically significant difference was observed within the both

SA7W and SA11.

Genetic difference among the subareas. We finally looked for evidence of genetic difference among the samples collected from the different subareas (Table 5). For the all individuals, highly statistically significant difference was observed among them. F_{ST} was also significantly larger than 0. We thus conducted pair-wise comparisons as the next step. Only seven out of the 21 pairs showed no statistical significance. In contrast, for the only J stock individuals, no statistically significant difference was observed among the samples from the different subareas. No pair-wise comparison was thus conducted for the only J stock group.

Assessment of statistical power for the tests of homogeneity

Table 6 shows the input parameters used and the results of simulation analysis to assess the statistical power for the homogeneity tests. Our simulation attempted to test the statistical power for very small genetic divergence between two samples from two genetically different sub-stocks, if they exist, in the SA2 and SA6. With our sample sizes, there was enough power to detect the genetic difference between the two stocks that were separated with the migration rate of 0.01 and 0.02.

DISCUSSION

Kanda *et al.* (2009) found that the J stock appeared to distribute all around the Japanese waters with different proportion among the area. In some area, such as the SA6, mostly the J stock individuals exist, in the other areas, such as SA7, mostly the O stock individuals exist. That study also indicated that the SA2 was mainly occupied by the J stock but not by the O stock, which was different from what we had been thought.

The results of the heterogeneity tests reflected these observations. Among all of the heterogeneity tests we conducted, significant differences were detected only from the tests that included all individuals. The samples of all individuals contained not only the J stock individuals but also the O stock individuals and the individuals of unknown origins. The differences in the proportion of the J and O stock individuals among the subareas, but not the possibility of different stocks in the different subareas, should have been responsible for the heterogeneity we observed. The proportional difference of the two stocks was also seen within the SA7. Seasonal difference observed in the SA7 was consistent to the observation that the O stock migrate along the coastline of the SA7 in spring (Hatanaka and Miyashita, 1997). In addition to the difference between the Early and Late samples, the difference observed between the Early and JARPN/JARPNII in the SA7W was likely due to the difference in the proportion of the J stock between the samples. The whales in the JARPN/JARPNII sample were collected further offshore than those in the bycatch sample and the proportion of the J stock was larger in coastal than in offshore (e.g., Hakamada *et al.*, 2009).

In contrast, no significant difference at all was observed from the tests that used only the J stock individuals. If the assignment of the whales to the J and O stocks conducted in Kanda *et al.* (2009) had been unreliable, we could have detected statistically significant differences between some of the samples even in the only J stock individuals group. In this study, in order to support the result of the homogeneity we assessed the statistical power by generating the genetic data from a simulation. In the final generation of each replicate of the simulation, a sample of 140 was taken from each population for genetic analysis. The sample size of 140 was come from the number of the J stock individuals in the SA2. We thus attempted to examine the power to see whether or not there were two genetically different sub-stocks, one in the SA6 and the other in the SA2. This simulation study indicated that from genetics standpoint the statistical power for our data set was quite high. We believe from these results that there is only the single J stock around the Japan.

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.

In this paper, we used only the Japanese samples because the new Korean dataset was incomplete for the analyses. In the new data set from Korea, quite a few individuals lack the data at some of the loci and we also recognized errors occurred during the recent electrophoresis. Probably due to these factors, substantial genetic difference observed between the 1999 and other year samples in the previous studies (Kanda *et al*., 2006; Park *et al*., 2006; Goto *et al*., (2007) disappeared when we examined the same individuals using the new genotype scores at the same sets of microsatellite loci to the previous papers. Unfortunately, we were not able to fix them by the 61SC. We therefore should await complete Korean dataset to conclude stock structure of common minke whales around the Japanese and Korean waters.

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Table 1. Samples used for this study.

	Bycatch					JARPN/JARPNII	
	SA2	SA6	SA7	SA10	SA11	SA7W	SA11
1994	0	0	0	0	0	0	0
1995	0	0	0	0	0	0	0
1996	0	0	0	0	0	31	30
1997	0	0	0	0	0	2	0
1998	0	0	0	0	0	25	0
1999	0	0	0	0	0	50	50
2000	0	0	0	0	0	24	0
2001	10	26	12	4	2	43	0
2002	22	49	34	2	2	110	0
2003	21	61	35	0	7	67	0
2004	23	66	26	0	0	73	0
2005	32	59	31	3	2	152	0
2006	29	78	39	0	1	131	0
2007	46	72	35	0	2	186	0
Total	183	411	212	9	16	894	80

Table 2. Number of the individuals assigned to either the J stock, O stock, or unknown origins based on genetic variation at the 16 microsatellite loci and a Bayesian clustering analysis (see Kanda *et al.*, 2009 for details).

	Bycatch					JARPN/JARPNII	
	SA2	SA6	SA7	SA10	SA11	SA7W*	SA11
J	144	383	93	7	15	103	23
freq.	0.787	0.932	0.439	0.778	0.938	0.115	0.288
O	24	4	90	0	0	701	49
freq.	0.131	0.010	0.425	0.000	0.000	0.784	0.613
unknown	15	24	29	2	1	90	8
freq.	0.082	0.058	0.137	0.222	0.063	0.101	0.100

Table 3. Results (p-value) of the tests for genetic difference among the bycatch samples within the same subarea (SA2, SA6, SA7) collected from different years (Total), collected from different years of the same season (Early, Late), and from different seasons of the year (E x L). Tests were conducted respectively for all individuals and for the only J stock individuals. Early: April - September, Late: October - March.

		All individuals				Only J			
		Total	Early	Late	E x L	Total	Early	Late	E x L
Bycatch SA2	N	183	46	137		144	34	110	
	hypo. test.	0.190	0.284	0.265	0.400	0.309	0.289	0.215	0.871
	F_{ST}	0.001	0.002	0.001	0.000	0.000	0.001	0.001	-0.002
Bycatch SA6	N	411	188	223		383	178	205	
	hypo. test.	0.502	0.856	0.494	0.717	0.556	0.818	0.473	0.700
	F_{ST}	0.000	-0.002	0.002	0.000	0.000	-0.001	0.003	0.000
Bycatch SA7	N	212	143	69		93	43	50	
	hypo. test.	0.391	0.209	0.938	p<0.001	0.888	0.789	0.875	0.530
	F_{ST}	-0.001	0.001	-0.005	0.010*	-0.003	-0.003	-0.004	-0.002

* Statistically significant F_{ST}.

Table 4. Results (p-value) of the tests for genetic difference between the bycatch and JARPN/JARPNII samples in the SA7W and SA11. Tests were conducted respectively for all individuals and for only the J stock individuals. Total indicates that individuals in the bycatch sample were not divided by their collected seasons. Only the individuals in the bycatch sample were divided into the Early and Late samples by the collected seasons and each of the samples was compared to the total JARPN/JARPNII sample. Early: April - September, Late: October - March.

		All individuals			Only J
		Total	Early x	Late x	
SA7W	hypo. test.	p<0.001	p<0.001	p<0.001	0.976
	F_{ST}	0.007*	0.002*	0.021*	-0.002
SA11	hypo. test.	p<0.001			0.632
	F_{ST}	0.020*			-0.004

* Statistically significant F_{ST}.

Table 5. Results (p-value) of the tests for genetic difference among the samples collected from the different subareas. Tests were conducted for all individuals and for only the J stock individuals. SA2, SA6, SA7, SA10, and SA11: bycatch sample, NP7 and NP11: JARPN/JARPNII sample.

			hypo. test.	F_{ST}
All individuals			p<0.001	0.018*
SA2	x	SA6	p<0.001	0.001*
	x	SA7	p<0.001	0.005*
	x	SA10	0.596	-0.002
	x	SA11	0.302	0.001
	x	NP7	p<0.001	0.023*
	x	NP11	p<0.001	0.010*
SA6	x	SA7	p<0.001	0.010*
	x	SA10	0.782	-0.005
	x	SA11	0.311	-0.001
	x	NP7	p<0.001	0.033*
	x	NP11	p<0.001	0.019*
SA7	x	SA10	0.388	0.004
	x	SA11	p<0.01	0.011*
	x	NP7	p<0.001	0.007*
	x	NP11	0.076	0.001
SA10	x	SA11	0.892	-0.013
	x	NP7	p<0.001	0.028*
	x	NP11	p<0.05	0.010*
SA11	x	NP7	p<0.001	0.035*
	x	NP11	p<0.001	0.020*
NP7	x	NP11	p<0.01	0.002*
Only J			0.876	-0.001

* Statistically significant F_{ST}.

Table 6. Input parameter sets for generating simulated data set using EASYPOP to assess statistical power for testing genetic difference between the SA2 and SA6 with our samples and results of the homogeneity tests with the simulated data using FSTAT. The following were fixed in all sets other than those shown in the table: diploid, random mating, equal sex ratio, all subpopulations of constant Ne, mutation rate of 0.005, 16 nuclear gene loci, 18 maximum allelic states, and 100 replicates each with 5000 generations.

	Input parameters							% rejecting panmixia
	n	N	Ne	m	Nem	F_{ST}	S	
N=3Ne	2	9000	3000	0.01	30	0.0083	140	100
	2	9000	3000	0.02	60	0.0041	140	100
	2	9000	3000	0.05	150	0.0017	140	54
	2	9000	3000	0.1	300	0.0008	140	27
	2	9000	3000	0.2	600	0.0004	140	6
N=4Ne	2	9000	2250	0.01	23	0.0110	140	100
	2	9000	2250	0.02	45	0.0055	140	100
	2	9000	2250	0.05	113	0.0022	140	70
	2	9000	2250	0.1	225	0.0011	140	29
	2	9000	2250	0.2	450	0.0006	140	8

n = the number of populations, N = census population size, Ne = effective population size, m = migration rate, Nem = number of the migrants, and S = sample size.

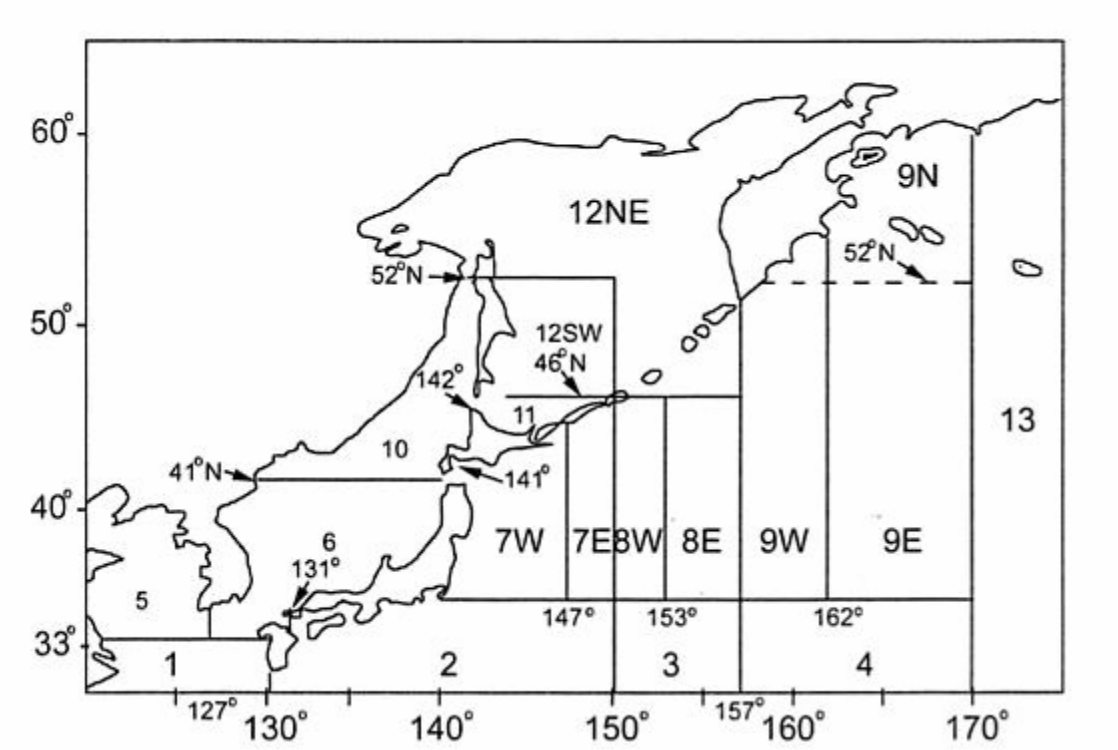

Fig. 1. Eighteen sub-areas for the North Pacific common minke whale management.