SC/61/JR7

Further mitochondrial DNA analysis on stock structure in the western North Pacific common minke whales

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ABSTRACT

This paper is the revised version of the mitochondrial DNA analysis presented in SC/J09/JR29 to cover the recommendations from the Expert Workshop to review the JARPNII Programme. Genetic variation at the mtDNA control region in the western North Pacific common minke whales was analyzed to examine the plausibility of four stock structure baseline scenarios adopted at the final stage of the *Implementation Simulation Trials (ISTs)* process under the Revised Management Procedure by the IWC Scientific Committee in 2003. A total of 1,639 whales collected during JARPN and JARPNII surveys from 1994 to 2007 in the area from the Japanese coast to the offshore waters (to 170°E) on the Pacific side, was used for the analyses. The samples from 2002 to 2007 (n= 1,124) were not used during the previous *ISTs* process. Heterogeneity tests were based on the randomized chi-square test and Fst as recommended by the review Workshop. Significant mtDNA heterogeneity was found in sub-areas 7W (for both chi-square test and Fst in the sample of 2007) and 9W (for Fst in the sample of 1995). Additional analysis in sub-area 7W suggested that the source of heterogeneity was due to the occurrence of J stock animals in this sub-area. Our updated analysis based on a larger data set confirmed that 1) whales from the J stock existed in the 7W with low but large enough number to cause genetic heterogeneity observed in the 7W samples as well as between the 7W and other samples, 2) except the J stock whales, the survey area was mainly occupied by O stock, and 3) the baselines C and D were not supported because no other genetically distinct stock was observed in the survey area, however the genetic heterogeneity found in sub-area 9 by the *Fst* analysis in a single year should be further investigated in the context of baseline scenario A.

KEY WORDS: COMMON MINKE WHALE, MTDNA, STOCK STRUCTURE, JARPN, JARPNII, NORTH PACIFIC

INTRODUCTION

The *Implementation Simulation Trials* (*ISTs*) for North Pacific common minke whale under the Revised Management Procedure (RMP) was completed during the 2003 International Whaling Commission Scientific Committee (IWC SC) meeting. At the final stage of the *ISTs* process the SC adopted the following four stock structure baseline scenarios (IWC, 2004):

(1) Baseline A: three-stock scenario ('J', 'O', 'W') with the 'W' stock found only in part of sub-area 9 and only sporadically.
(2) Baseline B: two stock scenario ('J' and 'O') with no W stock as a limiting case of Baseline A.
(3) Baseline C : four-stock scenario overall, with 'O_W', 'O_E' and 'W' to the east of Japan. Boundaries are fixed at 147°E and 157°E and there is no mixing between the stocks.
(4) Baseline D : three-stock scenario ('J', 'O', 'W'), with 'O' and 'W' mixing over 147°E and 162°E, O being dominant to the west and W to the east.

Unfortunately the SC did not examine plausibility of the stock structure scenarios. In order to get agreement among SC members it gave the same 'high' plausibility to the four scenarios (IWC, 2004).

In this study the plausibility of the four stock structure scenarios is examined through genetic analysis based on mitochondrial DNA (mtDNA) and samples collected by JARPN and JARPNII from 1994 to 2007. The samples from 2002 to 2007 were not used during the previous *IST*s process.

This paper is a revised version of Document SC/J09/JR29 presented to the JARPN II review workshop, which covers some of the recommendations of the Expert Workshop to review the JARPNII Programme held at Tokyo from 26-30 of January. Regarding to the mtDNA analysis, one of the recommendations by the panel was that a revised paper should include estimates of genetic divergence in addition to probabilities of homogeneity tests.

MATERIALS AND METHODS

Samples

Table 1 shows the number of samples used in the present mtDNA analysis by year, sub-area and the offshore and coastal components of JARPN II. 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). Sub-areas 7, 8 and 9 were divided into western and eastern strata by 147°E, 157°E and 162°E, 7W (140.01°E -147.00°E), 7E (147.01°E -150.00°E), 8W (150.01°E -153.00°E), 8E (153.01°E -157.00°E), 9W (157.01°E -162.00°E), and 9E (162.01°E -170.00°E). The sighting position of common minke whales used in this study is shown in Figure 2. Whales were sampled by JARPN and JARPNII surveys during 1994-2007

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 (Kanda *et al*., 2009b) 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 evolutionary distance between two nucleotide sequences was calculated according to Kimura's two parameters method (Kimura, 1980). The degree of genetic diversity within each locality was estimated using the nucleotide diversity (Nei, 1987). 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 5 individuals were excluded from the genetic divergence 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 conducted: a) analysis of genetic differences between the coastal and offshore samples collected in the same year from sub-area 7W; b) analysis of genetic differences among whales collected in different years from a same sub-area; and c) analysis of genetic differences among whales grouped according to the geographic divisions defining the four stock structure baseline scenarios used in previous *IST*s.

Assignment for J or O stock types

Analyses were conducted with and without suspected J stock animals in the case of sub-area 7W. Individual assignment to O and J stocks was based on analysis of microsatellite and the computer program STRUCTURE (Kanda *et al*., 2009a).

RESULTS

Kanda *et al.* (2009a) showed that there were suspected J stock individuals in the samples of common minke whales from the Pacific side of Japan. On the basis of the individual identifications to the stocks in Kanda *et al*. (2009a), we

conducted the tests on two different grouping: 1) one that included all the individuals and 2) one that excluded the suspected J stock individuals (samples contained individuals of unknown origin and the O stock).

Genetic diversity within samples
Sequence variations in a 487bp segment of the mtDNA control region resulted in 123 unique haplotypes in the total sample of 1,632 whales. Forty-one polymorphic sites were detected, 37 of which were transitions, three transversion and one insertion/deletion event.

Table 2 shows the nucleotide (π) and haplotype (H) diversities in each sample. Nucleotide diversities and its standard error of JARPN (1994-1999), JARPNII offshore component (2000-2007) and JARPNII coastal component (2002-2007) were 0.0079 (0.0002), 0.0081(0.0002) and 0.0089 (0.0002), respectively. Haplotype diversities were 0.9522, 0.9529 and 0.9624, respectively. For both indices these values are higher in the JARPNII coastal whales. For nucleotide diversity the difference was statistically significant between JARPN and JARPNII coastal component.

Genetic divergence between samples
Genetic differences between offshore and coastal samples in sub-area 7W
Table 3 shows the results of the heterogeneity tests for the comparison between samples taken in 7W by coastal and offshore components of JARPN II, by year and with and without suspected J stock animals. None of the comparison showed significant mtDNA differences except the 2007 samples for the chi-square analysis. We conducted the heterogeneity tests for this year excluding suspected J stock animals. In these cases no significant differences were found. Therefore significant differences found were most likely due to the inclusion of whales from the J stock in the sample. For the subsequent analyses, we conducted the heterogeneity tests with and without suspected J stock animals in the 7W sample.

Yearly genetic differences within sub-areas
Table 4 shows the results of the heterogeneity tests for yearly differentiation in each sub-area. No significant yearly differences were found in the sub-areas. In subsequent analyses samples from different years were combined in the sub-areas.

Plausibility of hypotheses
Baseline A
In order to test the heterogeneity within sub-area 9, we compared the samples collected in the western and eastern sides of sub-area 9, by year (Table 5). Regarding to the Chi-square statistics, no significant differences were found and there was no significant difference between western and eastern sides of sub-area 9 using total samples. Significant difference was detected in the 1995 samples in the analysis of *Fst*, and also significant difference was detected between western and eastern sides of sub-area 9 using total samples. However this significant difference for total samples was not detected when samples from western side of sub-area 9 in 1995 were excluded (Table 5).

Baseline B
We examined the genetic differences among all sub-areas (7W, 7E, 8W, 8E, 9W and 9E). No significant differences were found among all sub-areas with (P= 0.0595) and without (P=0.6106) suspected J stock animals in the sub-area 7W. Statistically tests in the sub-area 9 for the scenario B are same as those for the baseline A shown above.

Baseline C
Table 6 shows the results of heterogeneity tests for samples divided according to scenario C (e.g. samples divided by the longitudinal boundaries at 147°E and 157°E). We conducted the heterogeneity tests with and without the western samples in sub-area 9 in 1995. First heterogeneity test was conducted among samples from 7E, 8W and 8E, and no significant differences were found on both statistics. Therefore these samples were combined into one as 7E-8E for the following analyses. For the chi-square analysis no significant differences were found among samples from 7W, 7E-8E and 9W-9E with and without suspected J stock animals in the 7W samples and with or without samples from 9W in 1995. For the *Fst* analysis no significant differences were observed when the samples from the western side of sub-area 9 in 1995 were excluded from the analysis.

Baseline D
We examined the genetic differences among three groups divided by the longitudinal boundaries at 147°E and 162°E Table 7). We also conducted the heterogeneity tests with and without the western samples in sub-area 9 in 1995. Regarding to the Chi-square statistics no significant differences were found among samples from 7E, 8W, 8E and 9W, and. These samples were combined into one as 7E-9W for the following analyses. We then conducted the heterogeneity

tests among 7W, 7E-9W, and 9E with and without suspected J stock individuals in the 7W sample (7W x 7E-9W x 9E. 7W* x 7E-9W x 9E). For the chi-square test there are no significant differences among these samples with and without suspected J stock individuals in the 7W sample and with or without samples in 9W in 1995. For the *Fst* analysis no significant differences were found when the samples from sub-area 9 in 1995 were excluded from the analysis.

DISCUSSION

Level of genetic diversity
Genetic diversity at the mtDNA control region in common minke whales used in this study was similar to that of other large baleen whales in the North Pacific, such as Bryde's (Kanda *et al*., 2009c) and sei whales (Kanda *et al.*, 2009d). Diversities in the common minke whale in the JARPNII coastal component samples are slightly higher than in the other samples. This could be caused by the occurrence of J stock animals distributed around the coastal area in the Pacific side of Japan described by Kanda *et al*. (2009a).

Genetic divergence
Overall our study based on a larger data set than used previously failed to find any evidence of significant genetic heterogeneity in sub-areas 7, 8 and 9 other than in sub-areas 7W(2007 sample) and 9W (1995 sample). Significant mtDNA heterogeneity was found in sub-area 7W in the 2007 sample for the chi-square analysis, and in the sub-area 9 in 1995 for the *Fst* analysis. Additional analysis in sub-area 7W suggested that the source of heterogeneity was due to the occurrence of J stock animals. Heterogeneity in sub-area 9W in a particular year should be further investigated in the context of stock structure baseline scenario A (see below).

These results based on mtDNA and a larger data set are valuable to re-evaluate the plausibility of the four stock structure scenarios defined by the IWC SC in 2003. Results constitute a very strong demonstration that the underlying hypothesis of scenario C, e.g. three stocks with hard boundaries at 147°E and 157°E, has no independent support as there are no genetic evidences for multiple O stocks as proposed by this scenario. Genetic differentiation among stocks is facilitated by some geographical or ecological 'barrier', which acts restricting the gene flow among them. Minke whales in sub-areas 7, 8 and 9 have been described as opportunistic feeders. Distribution of these whales is related to the distribution and dynamics of prey species and the distribution of prey species is defined by oceanographic conditions in the area, which are variable. There is no evidence for ecological barriers at 147°E or 157°E.

Further common minke whales were taken by the Japan's small-type coastal whaling in sub-area 7W for more than 40 years. The annual catches of minke whales were in the range 200-300 (IWC, 2004). If the occurrence of a small coastal Ow stock, as proposed by the Baseline C stock scenario, is true, such stock could have been extinct under such catch levels. Kawahara (2002) reviewed the small-type whaling and two types of CPUE analyses. Judging from the corrected CPUE1 trend for about 35 years, the drastic decline of abundance seems unlikely. And CPUE2 analysis in 1977-1987 with operation hours by area also does not suggest that O stock has declined. Consequently no considerable decline was detected in either CPUE series. These results are consistent with result of the present genetic analysis and show that the plausibility of Baseline C is low.

Regarding to the Scenario D, results of our genetic analysis are also difficult to reconcile with the underlying hypothesis of scenario D. This hypothesis establishes that O and W stocks mix with each other between 147°E and 162°E. The O stock is predominant to the west and the W stock to the east of this range. Under this scenario only O stock occur between the Japanese coast and 147°E and only W stock from 162° to the east. One of the inconsistencies is that the mtDNA haplotype frequency in samples from sub-area 9E (scenario D assume that only W stock is distributed in this sector) is genetically similar to those in sub-areas 7 and 8 (O stock). Furthermore analysis of microsatellite indicated no significant departure from the Hardy-Weinberg equilibrium in the sector 147°-162°E (Kanda *et al*., 2009b). A significant departure from equilibrium could indicate a situation of mixing between different stocks as suggested by the scenario D. Scenario D is therefore inconsistent with genetic data.

One of the objectives of the JARPNII was to look for any evidence of existence of the W stock in sub-area 9 (Stock scenario A). We did not find the genetic heterogeneity in 9W in the present study based on the Chi-square test but analysis of *Fst* revealed significant heterogeneity in sub-area 9W in a particular year (1995) same as in past analyses (e.g., Goto *et al*., 2000). The microsatellite analysis found some degree of heterogeneity in sub-area 9E (Kanda *et al*., 2009b).

The situation in sub-area 9 is difficult to interpret from the biological point of view. In the case of 1995 the mtDNA heterogeneity was observed in the western part of sub-area 9. However the eastern part was genetically homogeneous

with sub-area 7 and 8. The mtDNA heterogeneity observed in the western part of sub-area 9 could be due to additional stock structure other than the O stock (e.g. W stock) or could be due to sampling bias.

If the first explanation is correct then we have a scenario in where a different stock distributes in part of sub-area 9 at least in one or some year(s) (stock scenario A). The sub-area 9 has been surveyed in several years (Table 1) and the mtDNA heterogeneity has been detected only in 1995 survey and in only a western part of sub-area 9. For the second explanation further analysis on possible relative in the sample from sub-area 9 should be conducted. This will be done in the near future using microsatellite in the context of a recommendation from the JARPN II review workshop (IWC, 2009)

Therefore we should further evaluate stock scenario A in the context of results from other genetic analysis based on microsatellites (e.g. Kanda *et al*., 2009b) as well from other non-genetic approach (e.g., morphometric) that use the same samples. Continued monitoring of common minke whales migrating to sub-area 9 is also necessary.

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Table 1. Sample sizes used in this study by year, sub-area in offshore (A) and Coastal (B) components.

A) Offshore

Y	7W	7E	8W	8E	9W	9E	Total
1994					7	14	21
1995					78	22	100
1996	31		1	15			47
1997	1	1	1	30	19	48	100
1998	25	31	44				100
1999	50						50
2000	24				16		40
2001	43	7		21	29		100
2002	60			8	32		100
2003	17	7	21	16	25	14	100
2004	16				42	42	100
2005	32		7	7	21	33	100
2006	36	2	10	28	23	1	100
2007	79		2	13	2	4	100
Total	414	48	86	138	294	178	1158

(B) Coastal

Year	7W
2002	50
2003	50
2004	59
2005	120
2006	95
2007	107
Total	481

Table 2. Nucleotide (π) and haplotype (H) diversities in each sample.

samples		N	π	SE	H
JARPN（1994-1999）		418	0.0079	0.0002	0.9522
JARPNII (2000-2007) Offshore component		734	0.0081	0.0002	0.9529
	2000	40	0.0078	0.0007	0.9321
	2001	100	0.0086	0.0004	0.9489
	2002	100	0.0079	0.0004	0.9535
	2003	100	0.0078	0.0004	0.9491
	2004	100	0.0087	0.0004	0.9594
	2005	94	0.0083	0.0005	0.9520
	2006	100	0.0077	0.0004	0.9582
	2007	100	0.0082	0.0004	0.9590
JARPNII (2002-2007) Coastal component		480	0.0089	0.0002	0.9624
	Kushiro	260	0.0091	0.0003	0.9656
	Sanriku	220	0.0086	0.0003	0.9584
Sum		1632	0.0083	0.0001	0.9561

Table 3. Statistical comparison between offshore and coastal samples in sub-area 7W by year, and with and without suspected J stock animals.

	All animals					Without suspected J stock animals				
Year	N		Chi-square P value	Fst	P	N		Chi-square P value	Fst	P
	Offshore	Coastal				Offshore	Coastal			
2002	60	50	0.173	-0.001	0.543	56	43	0.281	-0.003	0.434
2003	17	50	0.137	0.008	0.253	16	44	0.167	0.009	0.187
2004	16	59	0.281	0.000	0.583	13	34	0.127	0.000	0.363
2005	32	119	0.233	0.004	0.148	29	94	0.280	0.005	0.246
2006	36	95	0.382	0.006	0.173	34	78	0.307	0.007	0.101
2007	79	107	0.019	0.004	0.075	75	88	0.124	0.002	0.305

Table 4. Results of the heterogeneity test for yearly differences within each sub-area

Sub-area	Year	All animals				Without suspected J stock animals			
		N	Chi-square P value	Fst	P	N	Chi-square P value	Fst	P
7W	1996	31	0.360	0.002	0.067	30	0.393	0.001	0.201
	1998	25				25			
	1999	50				48			
	2000	24				21			
	2001	43				42			
	2002	110				99			
	2003	67				60			
	2004	75				68			
	2005	151				123			
	2006	131				112			
	2007	186				163			
8W	1998	44	0.181	0.012	0.289				
	2003	21							
	2006	10							
8E	1996	15	0.368	-0.004	0.774				
	1997	30							
	2001	21							
	2003	16							
	2006	28							
	2007	13							
9W	1995	78	0.370	0.004	0.130				
	1997	19							
	2000	16							
	2001	29							
	2002	32							
	2003	24							
	2004	42							
	2005	19							
	2006	23							
9E	1994	14	0.410	0.002	0.287				
	1995	22							
	1997	48							
	2003	14							
	2004	42							
	2005	30							

Table 5.Results of the statistical comparison between 9W and 9E by year and total samples.

	Sample size		Chi-square		
	9W	9E	P value	Fst	P
1994	7	14	0.543	-0.029	0.767
1995	78	22	0.103	0.029	0.013
1997	19	48	0.858	-0.007	0.656
2003	25	14	0.063	0.031	0.114
2004	42	42	0.347	0.002	0.355
2005	19	30	0.456	-0.002	0.456
Total	292*	175*	0.419	0.004	0.016
Total**	214*	175*	0.427	0.003	0.090

*: including 2000, 2001, 2002, 2006 and 2006 samples

**: exclude 1995 samples from the 9W.

Table 6. Results of the heterogeneity tests for the baseline C.

Combination of samples	Chi-square P value	Fst	P
7E x 8W x 8E	0.366	0.003	0.153
9W x 9E	0.419	0.004	0.016
9W** x 9E	0.427	0.003	0.090
7W x 7E-8E x 9W-9E	0.193	0.001	0.023
7W* x 7E-8E x 9W-9E	0.890	0.000	0.778
7W* x 7E-8E x 9W**-9E	0.985	-0.001	0.967

*: suspected J stock individuals were excluded from the 7W sample.

**: exclude 1995 samples from the 9W.

Table 7.Results of the heterogeneity tests for the baseline D.

Combination of samples	Chi-square P value	Fst	P
7E x 8W x 8E x 9W	0.234	0.003	0.022
7E x 8W x 8E x 9W**	0.330	0.002	0.119
7W x 9E	0.357	0.002	0.078
7W* x 9E	0.562	0.000	0.352
7W x 7E-9W x 9E	0.165	0.001	0.007
7W* x 7E-9W x 9E	0.781	0.000	0.595
7W x 7E-9W** x 9E	0.281	0.001	0.075
7W* x 7E-9W** x 9E	0.889	0.000	0.757

*: suspected J stock individuals were excluded from the 7W sample.

**: exclude 1995 samples from the 9W.

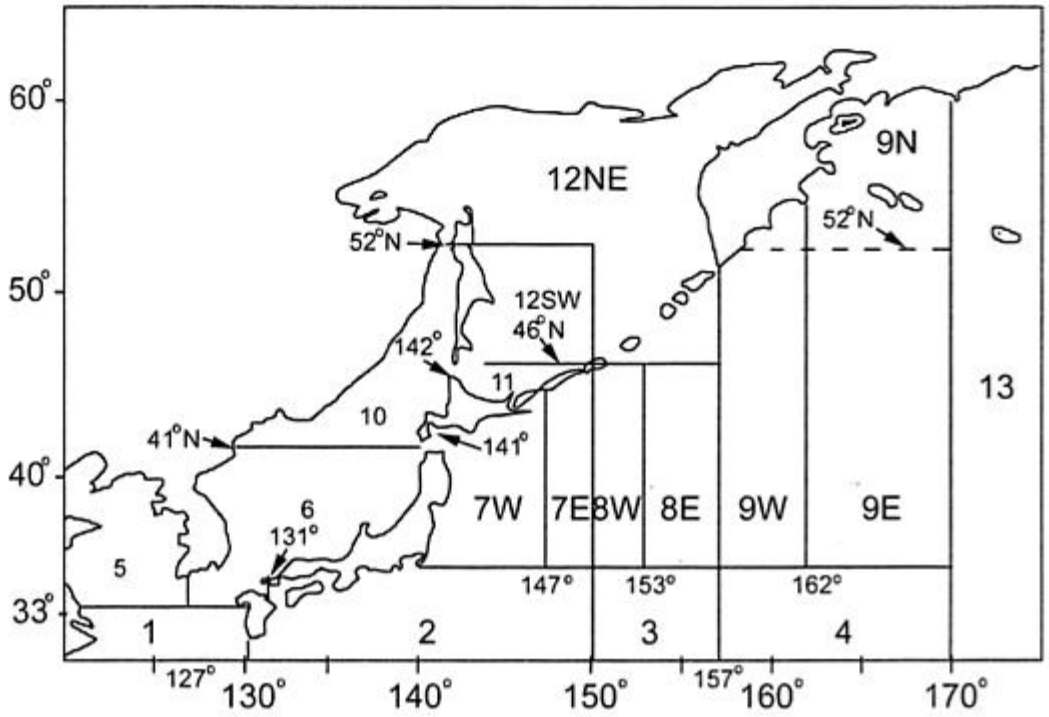

Fig. 1. The 18 sub-areas used for the Implementation Simulation Trials for North Pacific minke whales.

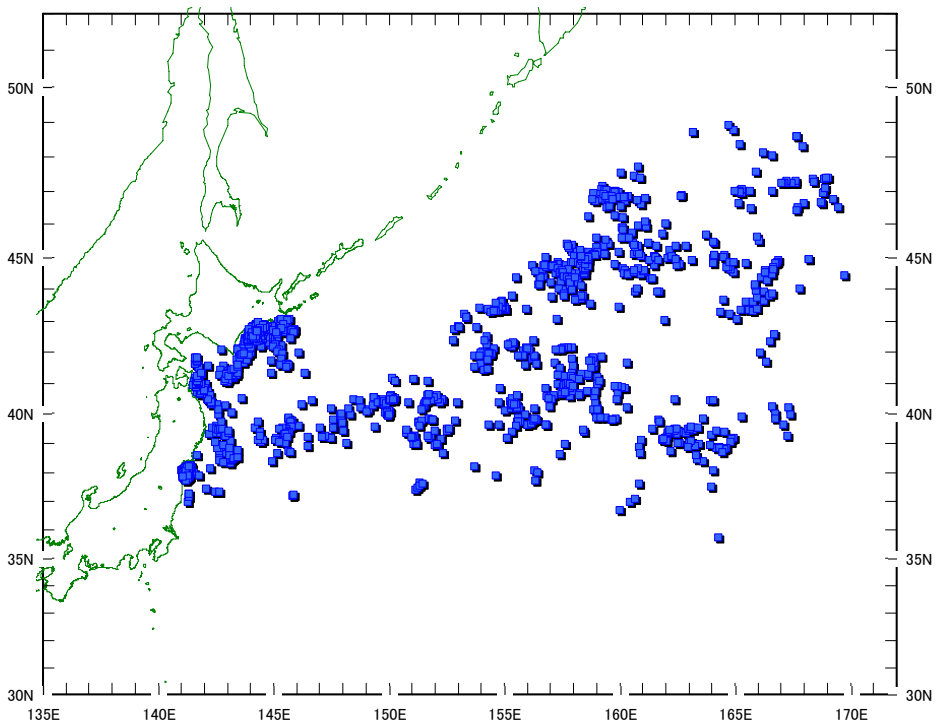

Fig.2. Geographic distribution of sighting position of common minke whale used in this study taken by JARPN and JARPNII surveys during 1994-2007.