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ABSTRACT

A total of 4,275 western North Pacific common minke whales were examined with a set of 16 microsatellite DNA loci and the program STRUCTURE to assign individual to either J or O stocks. Samples were available from JARPN/JARPNII (1994-2014; n= 2,637)), and by-catches (2001-2014; n= 1,638), from different management sub-areas (SA) around Japan. Results of the Bayesian clustering analysis confirmed that the whales came from two genetically differentiated stocks, J and O stocks. The number of unassigned individuals ('unknown') decreased with the increase in the number of microsatellite loci used, and they were widely distributed. By using 16 loci, more than 90% of the individual whales were assigned to either stocks. Almost all of the individuals collected from the Sea of Japan side (SA6 and SA10E) belonged to the J Stock, whereas almost all of the individuals from the offshore North Pacific (east of SA7WR) belonged to the O stock. Intermediate areas (SA7CN, 7CS and SA11) contained individuals from both stocks. The SA2 was mainly occupied by the J stock. In SA2 the J stock was predominant (around 80% in proportion) around the year. In SA7CS and SA7CN the proportion of the J stock increase in autumn/winter and decrease in spring/summer. A phylogenetic tree of mtDNA haplotypes showed several clades but none supported by high bootstrap values. There was no stock-specific clade although most of the individuals assigned to the J stock shared a same clade. Most of the individuals assigned to the O stocks share clades where the J stock individuals were less frequent. The unknown samples were widely distributed through the clades.

INTRODUCTION

At least two different stocks of common minke whales are known to occur around the Japanese coast: one stock distributes in the western North Pacific ('O' stock) and the other in the Sea of Japan ('J' stock) (Omura and Sakiura, 1956; Ohsumi, 1977; Kato, 1992; Wada and Numachi, 1991; Goto and Pastene, 1997; Hatanaka and Miyashita, 1997; Pastene *et al*., 2007). Whales of both stocks migrate to the Okhotsk Sea in spring and stay there until the end of summer. Although they share feeding ground in the Okhotsk, their temporal distribution in the area slightly differ (Goto and Pastene, 1997). Recent genetic analyses suggested that the J stock distribute in the Pacific side of Japan (Kanda *et al*., 2009a). These two stocks differ from each other in body size, conception dates, allozyme allele frequencies, microsatellite allele frequencies and mitochondrial DNA (mtDNA) haplotype frequencies, suggesting their reproductive isolation.

One of the sub-objectives of JARPNII under Objective 3 (Stock structure) is a systematic monitoring of the occurrence of J and O stock around the Japanese coast to determine spatial and temporal dynamics of its occurrence. In a situation of geographical overlap of multiple stocks, stock identification at an individual base will allows the direct estimate of mixing rates and the pattern of temporal and spatial distribution of the stocks. The effect size between the O and J stocks is large so that Bayesian-based program like STRUCTURE have been able to assign individuals to either stocks in the past (Kanda *et al*., 2009a; b).

The objective of this study is to update the work started by Kanda *et al*. (2009a) on the assignment of individual whales to the O and J stocks, to gain further understanding of the spatial and temporal distribution of these stocks around Japan. The study by Kanda *et al*. (2009a) was presented originally at the 2009 JARPNII review workshop (IWC, 2010a), and updated in subsequent studies (Kanda *et al*., 2009b; 2010).

Discussions at the IWC SC have focused on the well-documented difficulty that STRUCTURE has in detecting weakly differentiated populations and on the significance of unassigned individuals (e.g. IWC, 2010b) (see Discussion section). On the first point, it should be noted here that the objective of the present study is not the use of STRUCTURE to resolve the number of stocks involved in the available samples from by-catches and JARPN/JARPNII, but to use this program to assign individuals to the recognized J and O stocks in order to monitoring their distribution around the Japanese coast.

The question of whether or not additional stock structure occur within the 'O' stock is addressed in Pastene *et al.* (2016: SC/F16/JR40) and Bando and Hakamada (2016: SC/F16/JR41) where other methods usually used at the IWC SC for this purpose (e.g. hypothesis testing and PCA), are applied to the individuals assigned to the 'O' stock by the STRUCTURE analysis in this study.

MATERIALS AND METHODS

Sample collections

A total of 22 sub-areas were set for management purpose of the western North Pacific common minke whale during the RMP *Implementation Review* conducted in 2013 (Figure 1). JARPN and JARPNII surveys were conducted in Sub-areas 7, 8, 9, and 11.

Offshore samples of common minke whales from the western North Pacific were from JARPN/JARPNII surveys from 1994 to 2013 at SA7, SA8, SA9, and SA11 (Table 1). Common minke whale samples obtained from the coastal JARPNII survey between 2002 and 2014 were also used in this study (Table 1), Kushiro in sub-area SA7CN and Sanriku in sub-area SA7CS. Samples from common minke whales that were bycaught on set net fishery along the Japanese coast from 2001 to 2014 were also used (bycatches) (Table 1). The by-catches used were from the SA2, SA6, SA7, SA10, and SA11 year-round.

DNA extraction

We followed the IWC guidelines for DNA data quality (IWC, 2009) as much as possible (see Kanda *et al*., 2014). Genomic DNA was extracted from 0.05g of skin or muscle tissues using standard proteinase K, phenol-chloroform procedure described by Sambrook *et al*. (1989). Extracted DNA was stored in the TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Microsatellites

Microsatellite polymorphisms were analyzed using 16 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), DlrFCB14 (Buchanan *et al*., 1996). EV1, EV14, EV21 were developed from sperm whale, EV37, EV94, GT23, GT310, GT575, GATA28, GATA98, GATA417, TAA31 from humpback whale, and DlrFCB14 from beluga whale. All GT, EV and DlrFCB primers are dinucleotide repeats, TAA31 trinucleotide repeats, and all GATA primers tetranucleotide repeats. Primer sequences and PCR profiles follows those of the original authors with slight modifications.

PCR amplifications were performed in 15μl reaction mixtures containing 10-100ng of DNA, 5 pmole of each primer, 0.625 units of Ex Taq DNA polymerase (Takara Shuzo), and 2mM of each dNTP, and 10x reaction buffer containing 20mM $MgCl_2$ (Takara Shuzo). PCR amplifications followed the manufacturer's instructions for the use of Ex *Taq* DNA polymerase (Takara Shuzo). Amplified products with internal size standard (GENESCAN400HD, Applied Biosystems Japan) were run on a 6% polyacrylamide denaturating gel (Long Ranger™) using a BaseStation TM100 DNA fragment analyzer (Bio-Rad). Although alleles were visualized using Cartographer™ software specifically designed for the BaseStation, allelic sizes were determined manually in relation to the internal size standard and common minke whale DNA of known size that were rerun on each gel.

Data analysis
The number of alleles per locus, expected heterozygosity per locus, and inbreeding coefficient per locus were calculated using the FSTAT 2.9.3 (Goudet, 1995). Statistical tests for the deviations from expected Hardy-Weinberg genotypic proportions were conducted using the GENEPOP 4.0 (Rousset, 2008). The False Discovery Rate (FDR) approach (Benjamini and Yekutieli, 2001) was used for adjustment of *p*-value in case of multiple comparisons.

The Bayesian clustering approach was implemented with the microsatellite data in the STRUCTURE version 2.0 (Pritchard *et al*., 2000) to determine the most likely number of genetically distinct stocks present in our samples. The program is a model-based clustering method for inferring stock structure (K, the number of stocks in the model) using multilocus genotype data with and without information on sampling locations. STRUCTURE allowed for the analyses of the samples without choosing sample units that did not necessarily correspond to real biological stock boundaries. Posterior probabilities for K were estimating from ten independent runs for each value of K from one to five with only genetic information. These data were calculated based on burn-in period of 10,000 iterations and runs of 100,000 iterations. Individual assignment was then conducted for the most plausible K using estimated individual proportion of membership probability (90%). The ancestry model 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 stocks are likely to be similar due to migration or shared ancestry.

More details of the application of STRUCTURE to western North Pacific common minke whales are available in Kanda *et al*. (2009b).

Mitochondrial DNA
Sequencing analysis of the 487bp control region of mtDNA was conducted using the primers light-strand MT4 (Árnason *et al*., 1993) and heavy-strand P2 (5'-GAAGAGGGATCCCTGCCAAGCGG-*3'*; Hori *et al*., unpublished). 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™ or ABI3100 Automated DNA Sequencer (Applied Biosystems, Inc), following the protocols of the manufacture. For each sample both strands were sequenced.

Data analysis
The genealogy of the mtDNA haplotypes was estimated using the Neighbor-Joining method (Saitou and Nei 1987) as implemented in the program PHYLIP (Felsenstein 1993). Genetic distances among haplotypes were estimated using the program DNADIST of PHYLIP, based on Kimura's 2-parameter model (Kimura 1980). A transition-transversion ratio of 5:1was used. The genealogy was rooted using the homologous sequence from North Atlantic common and Antarctic minke whales. To estimate support for each node a total of 1,000 bootstrap simulations were conducted and the majority-rule consensus genealogy estimated.

RESULTS

Genetic variations
All 16 loci analyzed were polymorphic (Table 2). The total number of alleles per locus ranged from two at the EV21 to 29 at the EV1 with an average of 12.7. Expected heterozygosity at each of the loci ranged from 0.330 at TAA31 to 0.890 at GT23 with an average of 0.690. Twelve out of 16 loci showed significant deviation from the expected Hardy-Weinberg genotypic proportions even after correction for the multiple tests. For those loci showing significant departure from the Hardy-Weinberg genotype proportion, the F_{IS} were all positive suggesting a homozygote excess. This deviation suggested existence of individuals from multiple stocks in the sample.

Bayesian clustering analyses conducted on the total samples (4,275 individuals) without information on their geographic origins presented the highest likelihood probability at K=2 (Table 3). These results confirmed that the samples came from two genetically distinct stocks of common minke whales (J and O stocks) (but see Discussion section). In this study, the individuals with the membership probability of over 90% for either of the two stocks at each of the runs were assigned as pure individuals. All other individuals with the membership probability less than 90% to the either groups were assigned as individuals of unknown origin ('unknown').

Spatial distribution of J and O stocks along the Japanese coast
Both of the assigned and unassigned individuals ('unknown') were grouped based on their sampling origins (offshore, coastal, and bycatch) as well as locations (IWC sub-areas) (Figure 2). In this way, distribution of the pure individuals that were genetically assigned to the different stock was clearly separated geographically. Almost all of the individuals collected from the Sea of Japan side belonged to the J Stock, whereas almost all of the individuals from the offshore North Pacific (east of the SA7WR) belonged to the O stock.

Intermediate areas (SA7CN, 7CS and SA11) contained individuals from both stocks. The SA2 was mainly occupied by the J stock. Locations of the assigned and 'unknown' individuals were plotted in Figures 3a and coastal area was closed up in Figure 3b. The individuals of unknown origins distributed widely through the sub-areas.

Temporal distribution of J and O stocks along the Pacific coast of Japan
Figure 4 shows the temporal distribution of the J and O stocks and unknown individuals in SA2, SA7CN and SA7CS, expressed as three months moving average. In SA2 the J stock is predominant (around 80% in proportion) around the year. In SA7CS and SA7CN the proportion of the J stock increase in autumn/winter and decrease in spring/summer. Conversely the proportion of O stock decrease in autumn/winter and increase in spring summer.

Phylogenetic relationship of mtDNA control region haplotypes
Figure 5 shows the phylogenetic relationship of mtDNA haplotypes. The figure also shows the haplotype frequencies in the O and J stock as well in the unknown. Several clades were observed in the figure but none was supported by high bootstrap values. There was no stock-specific clade although most of the individuals assigned to the J stock shared a same clade. Most of the individuals assigned to the O stocks shared clades where the J stock individuals were less frequent. The unknown samples were widely distributed through the clades.

DISCUSSION

Issues on the program STRUCTURE
After reviewing the results of Kanda *et al*. (2009a) on the use of STRUCTURE to assign individuals to the O and J stocks, the 2009 JARPNII review panel made a recommendation to provide more details on the analyses involving this program (IWC, 2010a). Most of those details were presented in a revised version of the document discussed at the annual meeting of the IWC SC in 2009 (Kanda *et al*., 2009b). Furthermore analyses that followed recommendations by the IWC SC in 2009 were conducted and presented to the 2010 IWC SC meeting (Kanda *et al*., 2010). Those details on the application of STRUCTURE are not repeated here.

As noted previously, one of the major concerns was the well-documented difficulty that STRUCTURE has in detecting weakly differentiated populations (IWC, 2010b). Regarding the results from JARPNII, the IWC SC agreed that the STRUCTURE results provided clear evidence for two populations/stocks, and that these generally conform to what have been referred to as O and J stocks (IWC, 2010b). It also recognized the difficulty to determine under what circumstances the failure to find evidence for additional stocks might simply be an inability to detect presence of an additional gene pool (s) that is genetically similar to one of the two detected stocks (IWC, 2010b). In this context the JARPNII review workshop recommended to conduct simulations to evaluate the power of STRUCTURE to detect various mixture fractions of closely related stocks, although recognized this was a challenging recommendation (IWC, 2010a). Such simulations have not been conducted but it is believed that this was not relevant for the

objective of this study, which was not the investigation of the number of stocks involved but the investigation of the spatial and temporal distribution of what have been called J and O stocks around Japan.

Of primary interest for management is whether or not additional structure occur within the O stock, and this topic was treated in Pastene *et al.* (2016: SC/F16/JR40) and Bando and Hakamada (2016: SC/F16/JR41), using alternative approaches. Regarding the J stock, the genetic evidence for additional structure within this stock was considered low (IWC, 2013).

Other issue discussed in 2009 was on the significance of the unassigned ('unknown') individuals that could not reliably be assigned to either J or O stocks (IWC, 2010b). Some IWC SC members have argued that some if not all of the unknown individuals, may belong to a different stock. Alternatively, these unknown individuals could be the product of low statistical power of the analysis. Kanda *et al*. (2010) showed that the second explanation was more feasible. Here this issue is further elaborated.

The effect of using different number of microsatellite loci on the proportion of unknown individuals, was investigated (Figure 6). This figure shows the proportion of unknown individuals obtained for different sub-areas and sources of samples, for the case of six, nine, twelve and 16 loci. In all cases, the proportion of unknown individuals decrease with the increase of the number of loci used. Thus even for the case of the J and O stocks where the effect size is considered high, the application of STRUCTURE will require a larger number of loci to minimize the number of unassigned individuals to those stocks.

These results demonstrate that the 'unknown' individuals are not related to the occurrence of a different stock but they are derived by the low power of the analyses (in term of number of loci). If more loci are used then all individuals will ultimately be assigned to either J or O stocks. This result, summed to the wide and random nature in the temporal and spatial distribution of the unknown samples (Figures 3a, b), provide strong validation for the application of additional analytical approaches to investigate additional stock structure within the O and J individuals assigned by STRUCTURE (see Pastene *et al.*, 2016: SC/F16/JR40).

Temporal and spatial distribution of J and O stocks

Results of the present analyses on the total available samples of common minke whale are similar to the results provided by Kanda *et al*. (2009a; b). J stock animals distribute mainly in the Sea of Japan and in SA2 in the Pacific side of Japan. O stock animals distribute mainly in the Pacific side of Japan and mix with J stock animals in SA7CS, SA7CN and SA11. The pattern of mixing between the two stocks in these intermediate sub-areas has a strong temporal component with J stock predominating in autumn and winter and the O stock in spring and summer.

The fact that the J stock distribute in SA2 through the year suggests that the Kuroshio Current, which is one of the strongest west-boundary currents of the subtropical gyre, is working as the stock boundary between J and O stocks.

It is important to note that the individuals from the JARPN/JARPNII and those from the bycatch samples differ in their body length. Average body length of the JARPN and JARPNII samples including both the offshore and coastal components was 6.67m (SD= 1.13) and that of the all bycatch sample was 4.94m (SD=0.985). Kato (1992) estimated mean body length at the sexual maturity of the North Pacific minke whales to be 6.3m for males and 7.1m for females, so that the bycatch sample in the present study consisted mostly, if not all, of immature whales. The observation that the number of immature O stock individuals increased in spring along the Pacific coast of Japan is well consistent to that illustrated by Hatanaka and Miyashita (1997). The observed difference in the maturity status between the individuals from the bycatch and JARPN/JARPNII samples, however, could indicate that the patterns of the temporal and spatial distributions illustrated with the bycatches for the SA2 and SA7 in this study may be different at some extent from those of adults. In regard to SA2, common minke whales from the offshore area have not been available yet. Related concern can be also seen in the SA11. The number of the J stock individuals in the SA11 differed between the bycatch and JARPN samples. This difference we observed between the bycatch and JARPN samples could be due to the immature/mature, temporal, or both factors, but we were not able to distinguish which one accounted for at this moment. Although we definitely

gained our understanding of common minke whales distribution around the Japanese water substantially from this study, our samples are still missing some pieces to depict the whole picture of distribution and movement.

The series of studies on J and O stock (Kanda *et al*., 2009a; b; 2010; this study) are the first that shed light on the dynamics of geographic overlap between the two stocks at the individual base. It is believed that the results of these studies are also quite useful for the effective management of the two stocks. Another usefulness of the individual identification by the genetic markers is that it can be used to look for stock differences in other traits, such as morphometry, pollutant levels, and biological parameters (see Nakamura *et al.*, 2016: SC/F16/JR39; Bando and Hakamada 2016: SC/F16/JR41).

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Table 1. Sample size used in the microsatellite analyses. This involves the number of whales genotyped for all 16 loci successfully.

By-catch (2001-2014)						JARPN/JARPNII Coastal (2002-2014)		JARPN/JARPNII Offshore (1994-2014)						
BC2	BC6	BC7CN	BC7CS	BC10	BC11	K7CN	S7CS	7CN	7CS	7E	7WR	8	9	11
487	717	90	282	13	49	656	514	320	125	49	100	252	541	80

Table 2. The number of alleles (A), expected heterozygosity (H_E), test result for the expected Hardy-Weinberg genotypic proportions (HW) and inbreeding coefficient (F_{IS}) at 16 microsatellite loci of western North Pacific common minke whales.

Microsatellite loci	A	H_E	HW	F_{IS}
EV37	12	0,70	0,084	-0,005
EV1	29	0,77	**<0.001**	0,028
GT310	14	0,83	**<0.001**	0,041
GATA28	21	0,83	**<0.001**	0,011
GT575	13	0,80	**<0.001**	0,026
EV94	8	0,63	0,125	0,026
GT23	15	0,89	**<0.001**	0,020
GT509	23	0,88	**<0.001**	0,040
GATA98	7	0,60	0,921	0,005
GATA417	14	0,73	**<0.001**	0,005
GT211	16	0,87	**<0.001**	0,031
EV21	2	0,34	0,741	-0,006
DlrFB14	6	0,41	**<0.001**	0,030
EV14	6	0,52	**<0.001**	0,067
GT195	12	0,86	**<0.001**	0,057
TAA31	5	0,33	**<0.001**	0,037
Overall	12,7	0,69	**High. sign.**	0,027

Table 3. Results of the Bayesian clustering method analyzed for overall samples.

K	Log P(k/x)	variance	Pr(k/x)
1	-210753.0	85.2	~0.0
2	-202085.7	873.1	~1.0
3	-203026.1	3693.0	~0.0
4	-202960.7	4761.0	~0.0
5	-204283.6	8075.9	~0.0

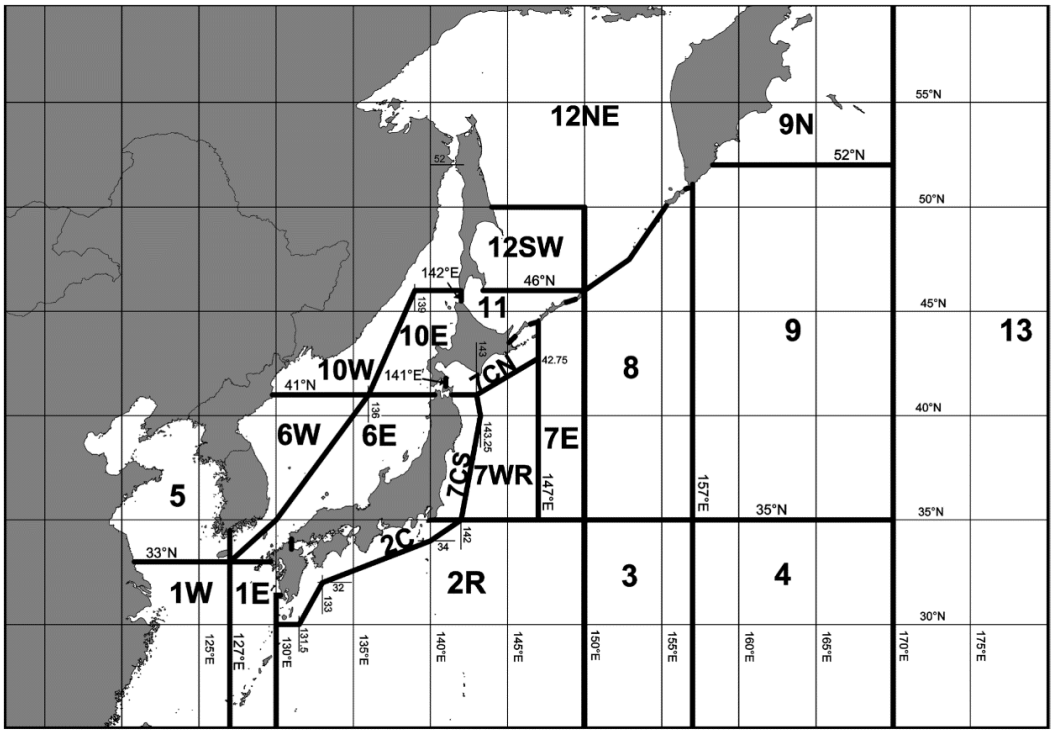


Figure 1. Sub-areas used for the management of common minke whale under the RMP.

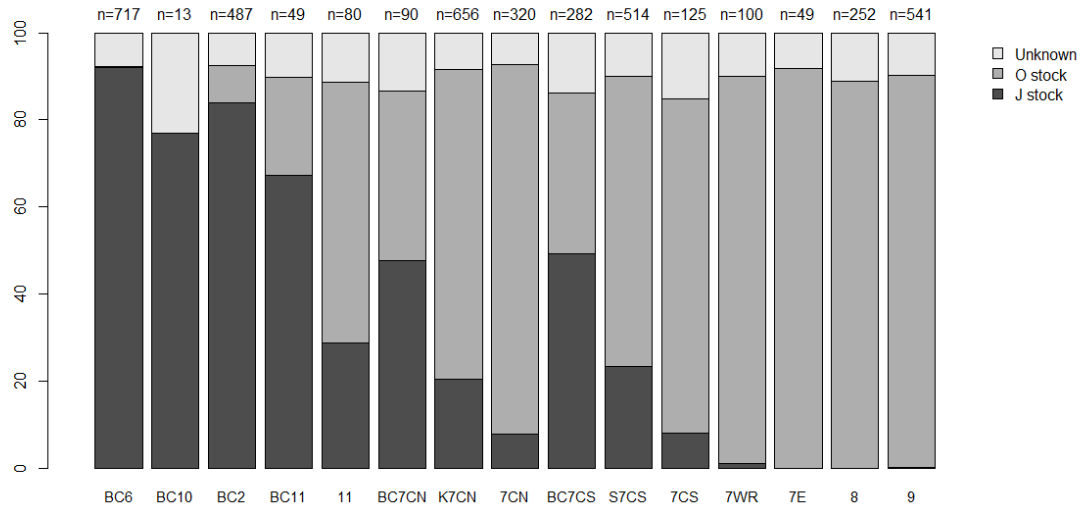


Figure 2. Spatial occurrence of O and J stocks in waters around Japan. BC2, BC6, BC7CS, BC7CN, BC10, BC11= bycatches from sub-areas 2, 6, 7CS, 7CN, 10 and 11. K7CN= coastal survey at Kushiro. S7CS= coastal survey at Sanriku. 7CS, 7CN, 7WR, 7E, 8, 9 and 11= offshore survey of JARPN and JARPNII. Sample size is on the top of each bar.

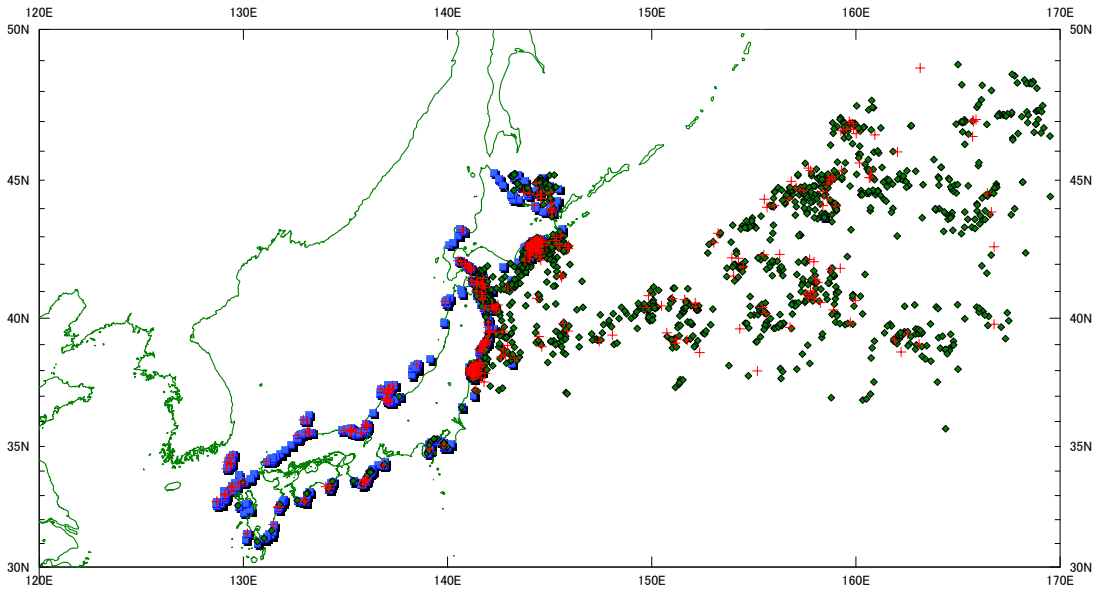


Figure 3a. Locations of the common minke whales that were assigned to O stock (green), J stock (blue), and unknown (red), based on STRUCTURE.

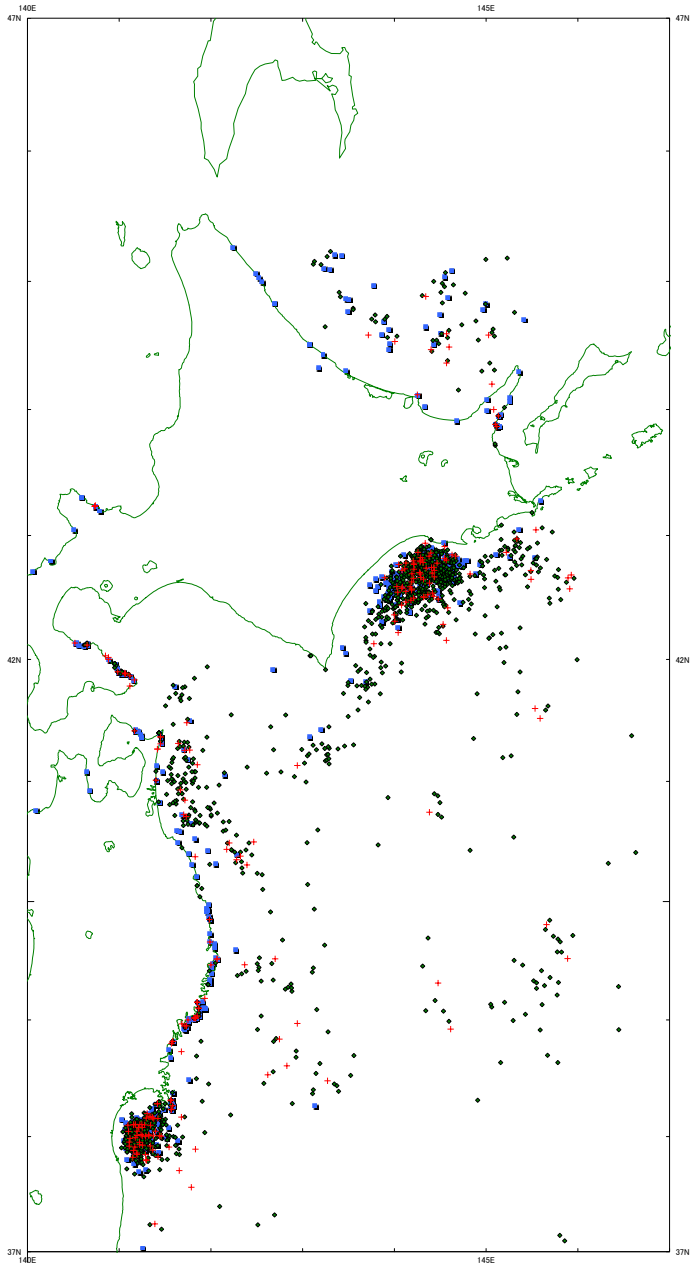


Figure 3b. Zoomed into the coastal area of the Figure 3a. O stock (green), J stock (blue), and unknown (red), based on STRUCTURE.

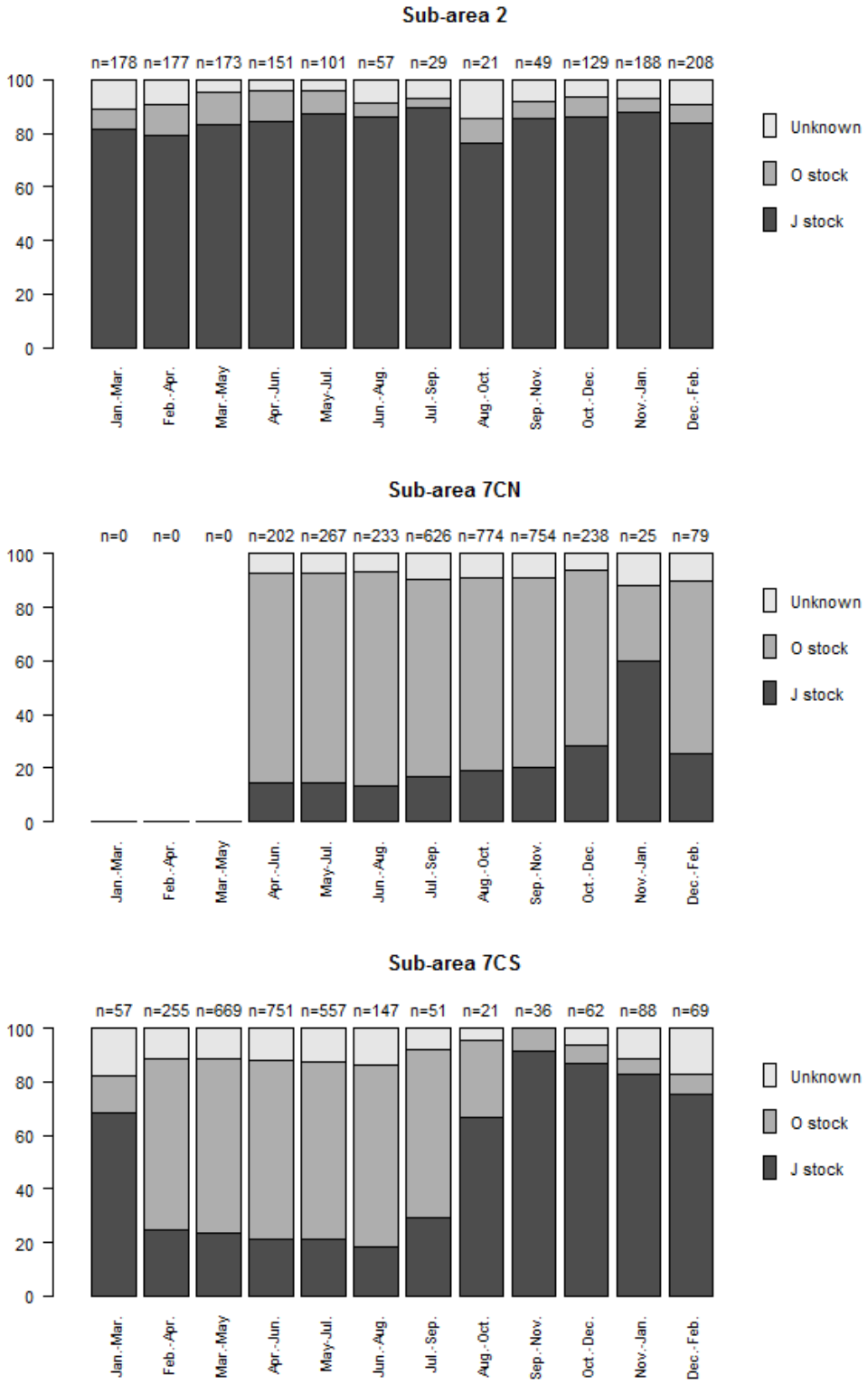
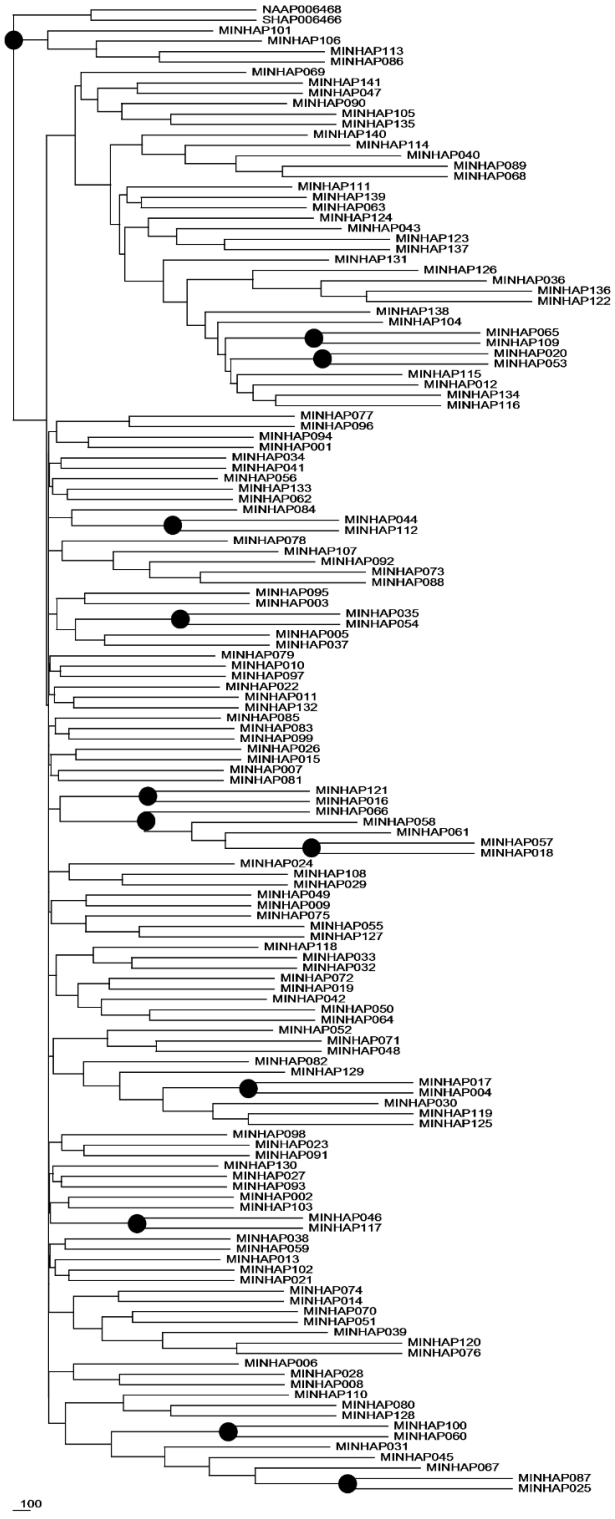


Figure 4. Monthly occurrence of O and J stocks in sub-areas 2, 7CS and 7CN. Each bar is expressed as three months moving average. Sample size is on the top of each bar. The sampling years in SA2 was 2001-2014; in SA7CN and SA7CS was 1994-2014.



Hap	J	O	UK
101	12		2
106	9		1
113	6		1
086	38		4
069		1	1
141	1		
047	101		12
090	85		14
105	42		2
135	2		
140	1		
114	13		
040	311	5	25
089	4		
068		1	
111	8		
139	1		
063	78		7
124	1		
043	24		10
123	17		1
137	1		
131	1		
126	1		
036	68	11	12
136	1		
122			1
138	1		
104	3		1
065	14		2
109	17		1
020		10	1
053		1	
115	11		3
012	416	10	30
134	3		
116	3		
077		8	2
096		1	
094		1	
001	1	34	5
034		1	1
041	61		7
056	68	9	5
133	5		
062	119		10
084		3	
044		4	1
112		2	
078		2	
107		1	
092		1	
073		17	1
088		1	
095		5	
003	1	308	40
035		4	1
054		10	
005		122	24
037		7	2
079		2	
010	35	97	18
097			1
022		36	7
011		81	6
132		1	
085		5	1
083		4	
099		1	
026		1	
015	1	245	26
007	1	43	6
081	9	1	
121		1	
016		13	3
066		1	
058		15	5
061		12	
057		1	
018		60	2
024		17	1
108		1	
029		1	
049		3	
009		43	1
075		1	1
055	1	30	1
127		1	
118		1	
033		4	1
032		3	
072		3	
019	1	14	2
042		14	1
050		14	2
064		13	
052		3	
071		5	4
048		5	
082			1
129			1
017		64	10
004		25	
030	3	99	8
119	4		
125		1	
098		2	
023		61	8
091		2	
130		1	
027		124	12
093		1	
002		112	7
103		1	
046		11	2
117		1	
038		1	
059		1	
013	1	24	2
102		1	
021		15	1
074		3	
014		108	10
070		3	
051		9	
039		19	1
120		1	
076		4	
006	3	99	8
028		1	
008		23	1
110		1	
080		1	
128		1	
100		2	
060		6	1
031		7	2
045		25	2
067		2	1
087		1	
025		25	5
Total	1608	2264	402

Figure 5. Phylogenetic relationship of mtDNA control region haplotypes and frequencies of whales assigned to O stock, J stock and unknown by the microsatellite analysis. North Atlantic common and Antarctic minke whale haplotypes are used as outgroups.

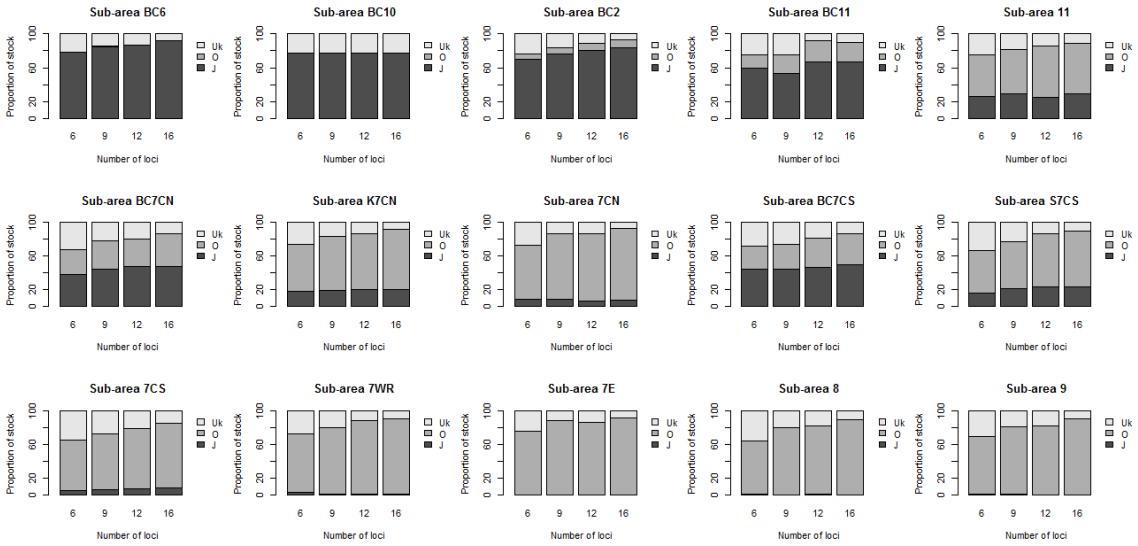


Figure 6. Proportion of J, O and 'unknown' individuals in each sub-area in relation to the number of microsatellite loci used in the assignment test.