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Update of the analyses on individual identification and mixing of J and O stocks of common minke whale around Japanese waters examined by microsatellite analysis

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ABSTRACT
This paper is the revised version of SC/J09/JR26, which described the results of microsatellite analysis of the J and O stocks of common minke whales, presented to the Expert Workshop to review the JARPNII Programme. In this study, we attempted to distinguish sampled common minke whales into genetically distinct stocks using a combination of microsatellite analysis and a Bayesian clustering approach. Past studies indicated that two different stocks of common minke whales existed around the Japanese coast: O stock in the western North Pacific and the J stock in the Sea of Japan. Samples of 2,542 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 Bayesian clustering analysis implemented in the computer program STRUCTURE (Pritchard *et al.*, 2000) indicated that our samples came from two genetically differentiated groups of minke whales. Approximately 91% of the individuals were assigned into the either stocks based on their high membership probability (>90%) obtained from the program. Spatial distribution of these assigned individuals clearly indicated that these two stocks were the J and O stocks. In addition, it was also found that 1) the O stock individuals appeared to migrate, although rarely, to the Sea of Japan, 2) the J stock individuals migrated to the 7W of the North Pacific side and very rarely to further east, and 3) the SA2 (western side of North Pacific coast) was mainly occupied by the J stock. Temporal distribution of the assigned bycatches collected from the SA7 (eastern side of Japan, North Pacific coast) where both the J stock and O stock whales were contained in the samples in about 50:50 indicated seasonal movement of the whales with the number of the O stock increased in spring. This study allowed us to better understand the pattern and dynamics of distribution of the minke whales inhabiting around Japan. The results of this revised version confirmed the main conclusion in SC/J09/JR26.

KEY WORDS: MINKE WHALE, MICROSATELLITE, O STOCK, J STOCK, JARPN, JARPNII, NORTH PACIFIC

INTRODUCTION

Common minke whales, *Balaenoptera acutorostrata*, are the smallest and the most abundant baleen whales inhabiting major open oceans world-wide. Spatial and temporal separations of the minke whale populations have occurred (Wada and Numachi, 1991; Bakke *et al*., 1996; Martinez and Pastene, 1999; Pastene *et al*., 2007). They live up to 50 years in age and the adult size is, on average, 6-7m. They feed on various prey species, such as copepods, Euphausiids, and fish. Their age at first reproduction is five, and they are thought to reproduce every year. Minke whales undergo seasonal movement from winter breeding grounds in low latitude to summer feeding grounds in high latitude although their exact breeding

ground is usually unknown. They feed in the fertile cold waters in summer near the poles, and spend their winter season in warmer oceans to pair, mate, and give a birth.

Around the ocean off the Japanese coast, at least two different stocks of minke whales are known to exist: 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 the 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). These two stocks differ from each other in body size, conception dates, allozyme allele frequencies, and mitochondrial DNA (mtDNA) haplotype frequencies, suggesting their reproductive isolation.

One of the objectives of JARPN II is a systematic monitoring of the occurrence of J stock in sub-area 7 to determine spatial and temporal dynamics of its occurrence. In a situation of geographical overlap of multiple stocks, stock identification at an individual base allows us to directly estimate mixing rates and pattern of temporal and spatial distribution of the stock. The Scientific Committee (SC) has recommended that the J stock individuals should be excluded from the analyses of the North Pacific common minke whales. However, the past genetic studies failed to detect diagnostic markers between the two stocks. In the past genetic studies, the number of the J stock individuals available had been limited and the genetic markers used (mtDNA and allozymes) might have not been suitable for stock identification due to their relatively low resolution power.

The objective of this study was thus to identify stocks of common minke whales collected from around Japan at an individual level and to gain an understanding of spatial and temporal distribution of the stocks. We attempted to distinguish samples of minke whales obtained around Japan into the J and O stocks irrespective their sampling sites by utilizing genetic variation at hypervariable microsatellites with Bayesian clustering approach. A model based Bayesian approach has been recently developed to define clusters of individuals based on their genotypes at multiple loci (Pritchard *et al*., 2000). This approach allows us to treat each individual in the samples as a single operational taxonomic unit in a stock-level analysis, and Bayesian approach provides relative probability to estimate the number of stocks rather than reject one of the two hypotheses. An advantage of Bayesian method implemented in the STRUCTURE (Pritchard *et al.*, 2000) used in this study is thus that the obtained results are not affected by arbitrary chosen sample strata that do not necessarily correspond to real biological stock boundaries.

This paper is a revised version of SC/J09/JR26 to cover the recommendations of the expert panel of the Expert Workshop to review the JARPNII Programme held at Tokyo from 26-30 of January. The panel recommends that SC/J09/JR26 should provide more details on the analysis involving the STRUCTURE (Pritchard *et al.*, 2000) used for genetic assignment of individuals.

MATERIALS AND METHODS

Sample collections

Eighteen sub-areas were set for management purpose of the western North Pacific common minke whale (Figure1). JARPN and JARPNII surveys were conducted in sub-areas 7, 8, 9, and 11. These sub-areas were further divided into western and eastern strata for analyses, 7W (140-147°E), 7E (147-150°E), 8W (150-153°E), 8E (153-157°E), 9W (157-162°E), and 9E (162-170°E).

Offshore samples of common minke whales from the western North Pacific were JARPN and JARPNII samples collected from 1994 to 2007 in SA7, SA8, SA9, and SA11 (Table 1). Each year up to 100 common minke whales were collected. The number of individuals from the SA11 was total 80 collected in 1995 and 1997. Sampling dates of scientific surveys expand from May to September depending on the sampling plan of a given year. Because of other scientific purposes of the survey (e.g., feeding ecology of whales), the sampling locations differed from year by year. Details of offshore component of JARPNII survey can be found in Tamura *et al*. (2009). Minke whales obtained from coastal component of JARPNII were also used in this study (Table 1). Coastal JARPNII surveys were 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. Sample size per survey was maximum 60 common minke whales. Details of the coastal component of JARPNII survey can be found in Kishiro *et al.* (2009). Common minke whales that were bycaught on

set net fishery conducted along the Japanese coast from 2001 to 2007 were also used (bycatches) (Table 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 year-round.

Microsatellites analysis

We followed the IWC guidelines for DNA data quality (IWC, 2009) as much as possible at the moment. Genomic DNA was extracted from 0.05g each of the 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).

Microsatellite polymorphisms were analyzed using 16 sets of primers: EV1, EV14, EV21, EV37, EV94, (Valsecchi & 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 (*Physeter macrocephalus*), EV37, EV94, GT23, GT310, GT575, GATA28, GATA98, GATA417, TAA31 were from humpback whale (*Megaptera novaeanglia*), and DlrFCB14 from beluga whale (*Delphinapterus leucas*). All GT, EV, and DlrFCB primers are dinucleotide repeat, TAA31 trinucleotide repeat, and all GATA primers tetranucleotide repeat. Most of the primers used here were already tested for amplification on common minke whales by these authors. 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 manufacture'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 an BaseStationTM 100 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 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 Weir and Cockerham estimator of F_{ST} (Weir and Cockerham, 1984) was 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 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 us to analyze the samples without choosing sample units that did not necessarily correspond to real biological stock boundaries. Posterior probabilities for K were estimating from three 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. The ancestry model we 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.

RESULTS

Multilocus genetic variations for stock numbers and individual assignment

All 16 loci analyzed were polymorphic in overall samples of minke whales we used (Table 2). Total number of alleles per locus ranged from two at the EV21 to 30 at the EV1 with an average of 13.0. Expected heterozygosity at each of the loci ranged from 0.330 at EV21 to 0.855 at GT23 with an average

of 0.692. Eleven out of 16 loci showed significant deviation from the expected Hardy-Weinberg genotypic proportions even after correction for the multiple tests. This deviation suggested existence of individuals from multiple stocks in our samples.

Bayesian clustering analyses conducted on the total samples (2,542 individuals) without information on their geographic origins presented the highest likelihood probability at K=2 (Table 3). Figure 2 shows estimated stock structure at K=2 and K=3. At K=3, the second and third stocks were indistinguishable. These results strongly indicated that our samples came from two genetically distinct groups of common minke whales. Same individuals were grouped into the same groups over the three runs. Assignment probabilities of the same individuals to the groups were quite consistent at the different runs (Figure 3) with very low CV (average CV =0.014). These observations allowed us to assign the individuals into the different groups on the basis of their membership probabilities at arbitrary chosen level. In this study, the individuals with the membership probability of over 90% for either of the two groups 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. Even with this relatively strict criterion, 2,302 individuals (91%) were assigned as the pure individuals to the either stocks (770 to stock 1 and 1,532 to stock 2). F_{ST} between the two pure groups was 0.049, which was significantly larger than zero.

Spatial distribution of the two genetically different stocks along the Japanese coast

Both of the assigned and unassigned individuals were grouped based on their sampling origins (offshore, coastal, and bycatch) as well as locations (IWC sub-areas) (Table 4; Figure 4). 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 Stock 1, whereas almost all of the individuals from the offshore North Pacific (east of the SA7E) belonged to the stock 2. Intermediate areas (SA7W and SA11) contained the individuals from the both stocks. The results indicated that the Stock 1 and Stock 2 were the J and O stock. Hereafter, we named the Stock 1 and 2 as the J and O stock, respectively. The SA2 was mainly occupied by the J stock, and the SA7W was by the two stocks. Two individuals collected from the 9W (N=260) were assigned as the J sock individual, while four individuals from the SA6 (N=387) was assigned as the O stock individual. Sighting locations of the assigned individuals from the JARPN and JARPNII samples were plotted in Figure 5a and coastal area was closed up in Figure 5b. The individuals of unknown origins also distributed widely.

Temporal distribution of the two genetically different stocks along the Japanese coast of North Pacific side of Japan

The proportion of the J stock and O stock individuals in the bycatches from the SA7 were approximately 50:50 (93 J and 90 O). In addition to that, the sampling dates of the bycatches expand year around. These things allowed us to depict temporal distribution of the J and O stocks along the Japanese coast of the North Pacific side. Because the sample sizes were relatively small in a single month base, we calculated the proportion of the J stock, O stock, and unknowns from a three months moving average (Figure 6). The result revealed that the proportion of the O stock increased in spring and decreased toward winter season although both the J and O stock individuals stayed year round. At the SA2, total of 168 individuals was assigned to 114 J and 24 O individuals, and temporal distribution of these individuals showed similar, but less clear, pattern to that observed at the 7W.

DISCUSSION

Delineation of the samples into geographic groups for analyses is often arbitrary based on cultural, political jurisdictions, or simply sampling locations. The arbitrary predefined groups, however, may not necessarily correspond to real biological stock boundaries. This is especially the case for the geographically widely distributed species that are capable of long distance migration. They may not have clear stock boundaries or may be genetically structured through unidentified barriers of gene flow.

In this study, combined use of the hypervariable microsatellite markers and Bayesian clustering approach successfully distinguished most of the individuals in the samples into the J and O stocks. One notable aspect of this study was that the number of samples used, especially from the Sea of Japan side, was much larger than the previous genetic studies. Past genetic studies dealing with the J and O stocks used only small amount of the samples taken from past commercial whaling operated in coastal areas of Korea in 1982 as the J sock (Wada and Numachi, 1991; Goto and Pastene, 1997). In addition to that, this paper

used the samples of minke whales collected from along the Japanese coast of both sides of Japan and offshore North Pacific at a similar time frame. This allowed us to minimize potential large temporal influence, if it exists, on describing present day stock structure, although no samples had been obtained in winter in the western North Pacific due to logistic constraints.

According to the probability for each of K, our data was not supported at all by K=1, 3, 4, and 5. Very high membership probabilities and great geographic concordance indicated that K=2 was appropriate choice for our data. When we lower our membership probability criteria from 90% to 75%, 97% of the individuals in the samples are assigned to either the two stocks and the additional assigned individuals does not change the general picture of spatial distribution of the two stocks. Status of the unassigned individuals especially those with the membership probability of less than 75% (82 individuals) are difficult to be identified at this moment. They could be either individuals of interbreeding origins or pure individuals simply with low power to be assigned by the current markers and analyses. The clear genetic difference between the two stocks with moderate level of the F_{ST} value and the observation from the past ecological and biological studies of minke whales suggests that the interbreeding between them is probably a rare event.

The individual assignments by the microsatellites had good agreements with that by mtDNA haplotype analysis. It has been known that some of the mtDNA haplotypes were unshared by the individuals from the Sea of Japan and North Pacific and these unshared haplotypes with a few shared ones made an independent cluster in a phylogenetic tree (Goto *et al*., 2000). Based on the specific nucleotide sequences common to the haplotypes in that cluster, Goto *et al*. (2000) identified 25 of the 80 minke whales from the SA11 as the J stock individuals. Our microsatellite assignments of the same SA 11 individuals were very well consistent to those in Goto *et al*. (2000). By our method, 23 of the 80 were assigned as the pure J stock, and 22 of them were identified as the J stock by the method of Goto *et al*. (2000). In the remaining three J stock individuals from the mtDNA identification, two were the J stock individuals with the membership probability over 75%, and one was the O stock individual with the membership probability over 90%. Although the microsatellite markers we used are not complete diagnostic in the common minke whales samples, all of these findings suggest that assignments of the individuals is very reliable.

Spatial and temporal distributions of the assigned individuals depicted a pattern and dynamics of geographic overlap of the two stocks around Japan. As predicted, main distribution areas of the J and O stock were clearly separated. The J stock occupied the Sea of Japan and the O stock offshore did the North Pacific. Other important findings that should be raised are 1) the O stock individuals appeared to occasionally, but rarely, migrate to the Sea of Japan, 2) the J stock individuals migrated to the 7W of the North Pacific side and very rarely to further east, and 3) the SA2 was mainly occupied by the J stock. The third finding 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 the two stocks. Temporal distribution of the assigned whales indicated seasonal differences in the movement of the two stocks along the Japanese coast of North Pacific (SA7 and SA2). The number of the O stocks increased in spring although both stocks appeared to distribute there year around.

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 all JARPN and JARPNII samples including both the offshore and coastal components was 6.95m (s.d.=0.992) and that of the all bycatch sample was 4.92 m (s.d.=0.942). Kato (1992) estimated mean body length at the sexual maturity of the North Pacific common minke whales to be 6.3m for males and 7.1m for females, so that the bycatch sample in this paper consisted of mostly, if not all, immature whales. The observation that the number of the immature O stock individuals increased in spring along the Japanese coast of the North Pacific side was thus 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 we illustrated with the bycatches for the SA2 and SA7 may be different at some extent from those of adults. In regard to the SA2, 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. Average body length of the 14 bycatches from the SA11 was 4.5m and 13 of the 14 were collected from late fall (September to November). 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 whole picture of it.

The IWC SC completed the RMP *Implementation* for the western North Pacific common minke whales during the 2003 Annual Meeting and adopted four stock scenarios in the western North Pacific at the final stage of the *Implementation* process (IWC, 2004). Three of the four baselines assumed the third stock other than the J and O in the SA7, 8, and 9. This study denied that possibility because it showed that the North Pacific side of Japan was occupied by the O stock with some J stock individuals migrated into near coastal line area. Detailed analyses on this matter can be seen in Kanda *et al.* (2009).

This study is the first one that shed the light on the dynamics of geographic overlap between the two stocks at the individual base. We believe that the results of this study are also quite useful for the effective management of the two stocks. Another usefulness of the individual identification by the genetic markers is it can be used to look for stock differences in other traits, such as morphometry, pollutant levels, and biological parameters.

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Table 1. Number of individuals used in this study.

		Survey area									
	Total	2	6	7W	7E	8W	8E	9W	9E	10	11
JARPN and JARPNII											
Offshore (‘94-‘07)	1231			414	47	86	139	291	174		80
Coastal (‘02-‘07)	480			480							
Bycatch (‘01-‘07)	831	183	411	212						9	16

Table 2. The number of alleles (A), expected heterozygosity (He), and test result for the expected Hardy-Weinberg genotypic proportions (HW) at 16 microsatellite loci analyzed in the JARPNII samples of minke whales. n.s. = not significant

Marker	A	He	HW
DlrFCB14	5	0.405	p<0.01
EV1	30	0.785	p<0.01
EV14	6	0.543	p<0.001
EV21	2	0.330	n.s.
EV37	12	0.709	n.s.
EV94	8	0.638	n.s.
GATA28	24	0.830	p<0.001
GATA417	13	0.737	n.s.
GATA98	7	0.610	n.s.
GT195	13	0.857	p<0.001
GT211	17	0.874	p<0.001
GT23	16	0.885	p<0.001
GT310	14	0.833	p<0.001
GT509	23	0.881	p<0.001
GT575	13	0.809	p<0.001
TAA31	5	0.349	p<0.001

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

K	Log P(k/x)*	variance*	Pr(k/x)**
1	-128869.8	95.3	~0.0
2	-123973.8	613.1	~1.0
3	-124376.2	2243.8	~0.0
4	-125643.1	5312.3	~0.0
5	-127277.0	9061.4	~0.0

* log likelihood of the data for different values of K and the variance.
** Probability for each of K.

Table 4. Number of individuals assigned to each of the categories based on the assignment probabilities obtained from STRUCTURE.

Source	Bycatch					Coastal*		Offshore						
Area	2	6	7	10	11	CK	CS	11	7W	7E	8W	8E	9W	9E
J	144	383	93	7	15	38	41	23	24	0	0	0	2	0
O	24	4	90	0	0	190	164	49	346	46	75	125	258	161
Unknown	15	24	29	2	1	25	22	8	43	2	11	13	32	13
Total	183	411	212	9	16	253	227	80	413	48	86	138	292	174

* CK7=Kushiro coastal component, CS7=Sanriku coastal component

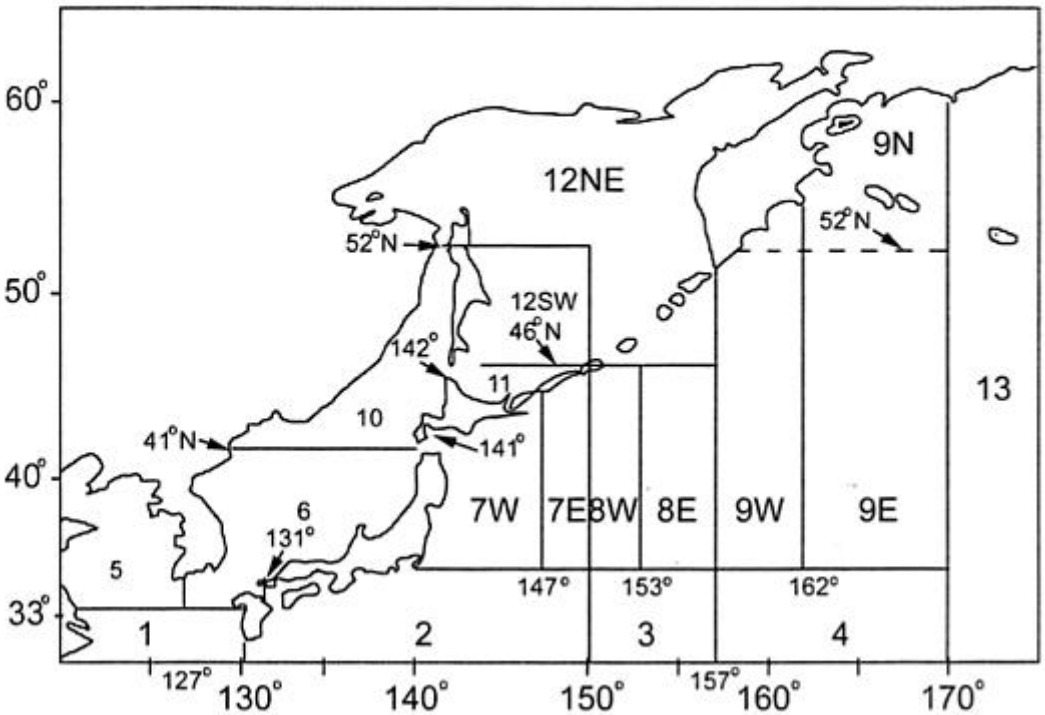

Figure 1. The 18 sub-areas designated by the IWC for North Pacific common minke whales.

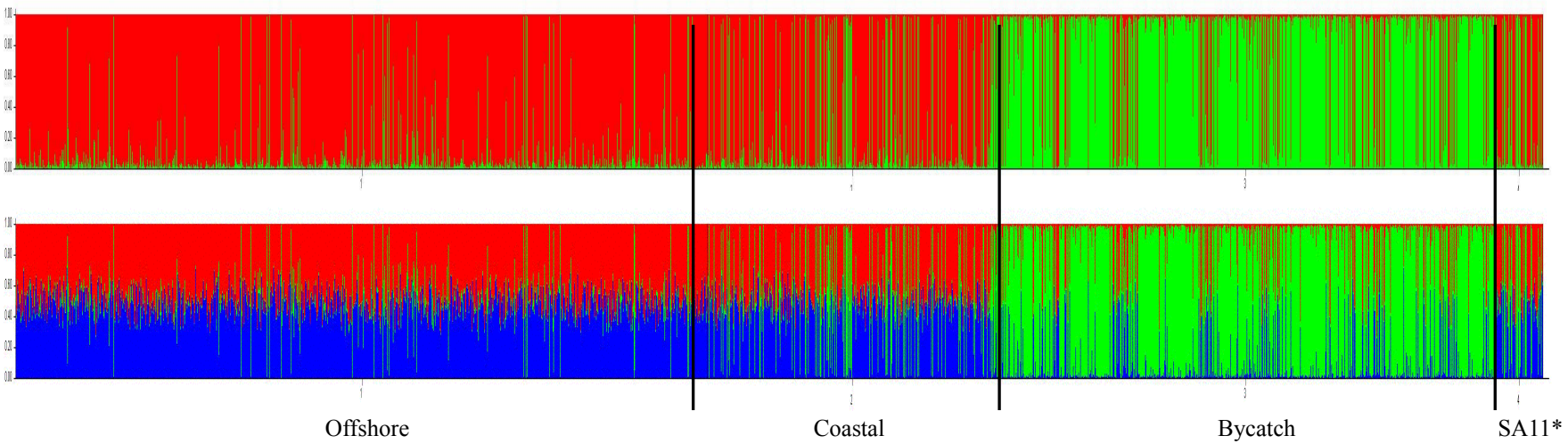

Figure 2. Plot of membership probabilities at K=2 (above) and K=3 (blow). Each individuals is characterized by a thin vertical line, which is divided into K colored segments on the basis of the individual's membership fractions in K clusters. *: Samples only from the JARPN.

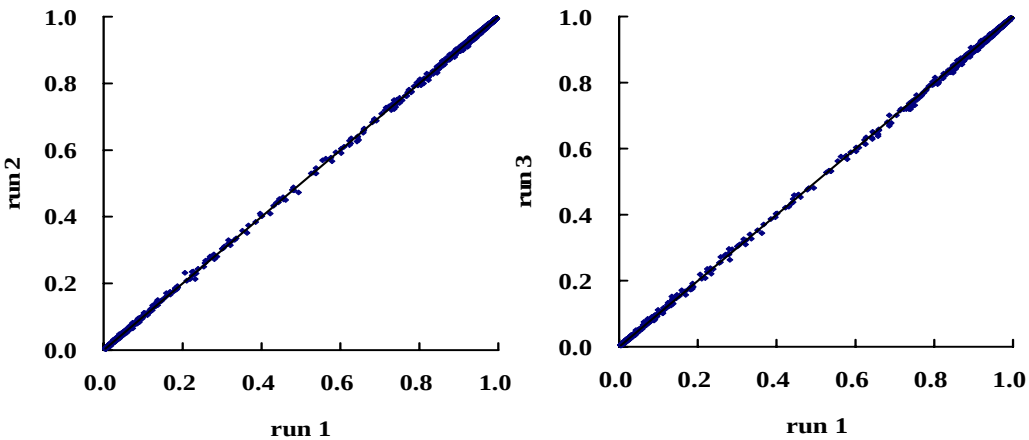

Figure 3. Relationship between the membership probabilities from different runs (run 1~3) for each of the individuals at K=2.

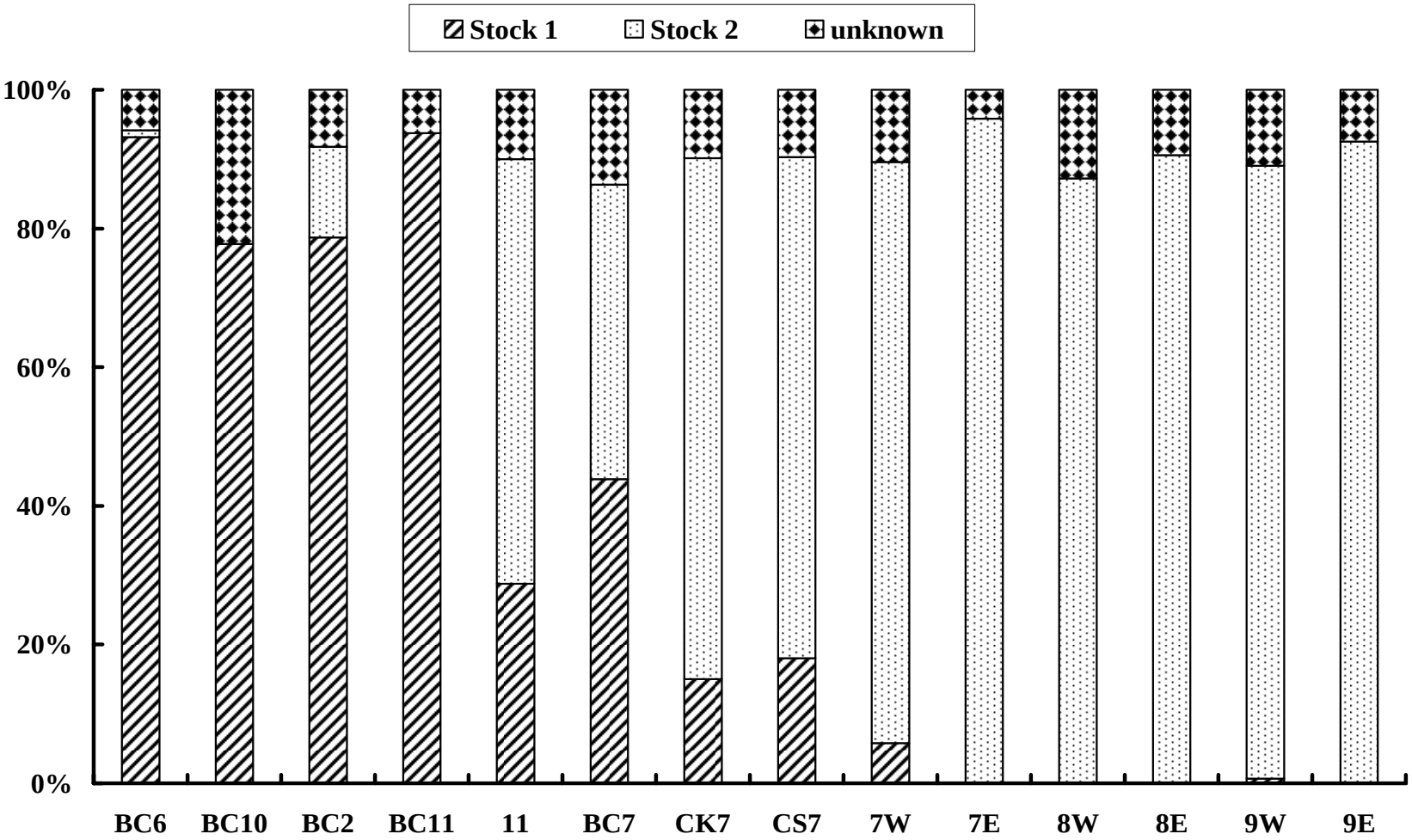

Figure 4. Spatial distribution of the whales assigned to the Stock 1, Stock 2, and unknown categories. See text for assignment criteria. BC2, BC6, BC7, BC10, BC11 = bycatches from the SA2, SA6, SA7, SA10, and SA11. CK7 and CS7 = individuals from coastal survey of JARPNII at Kushiro and Sanriku region, respectively. 7W, 7E, 8W, 8E, 9W, 9E and 11 = individuals collected from offshore survey of JARPN and JARPNII.

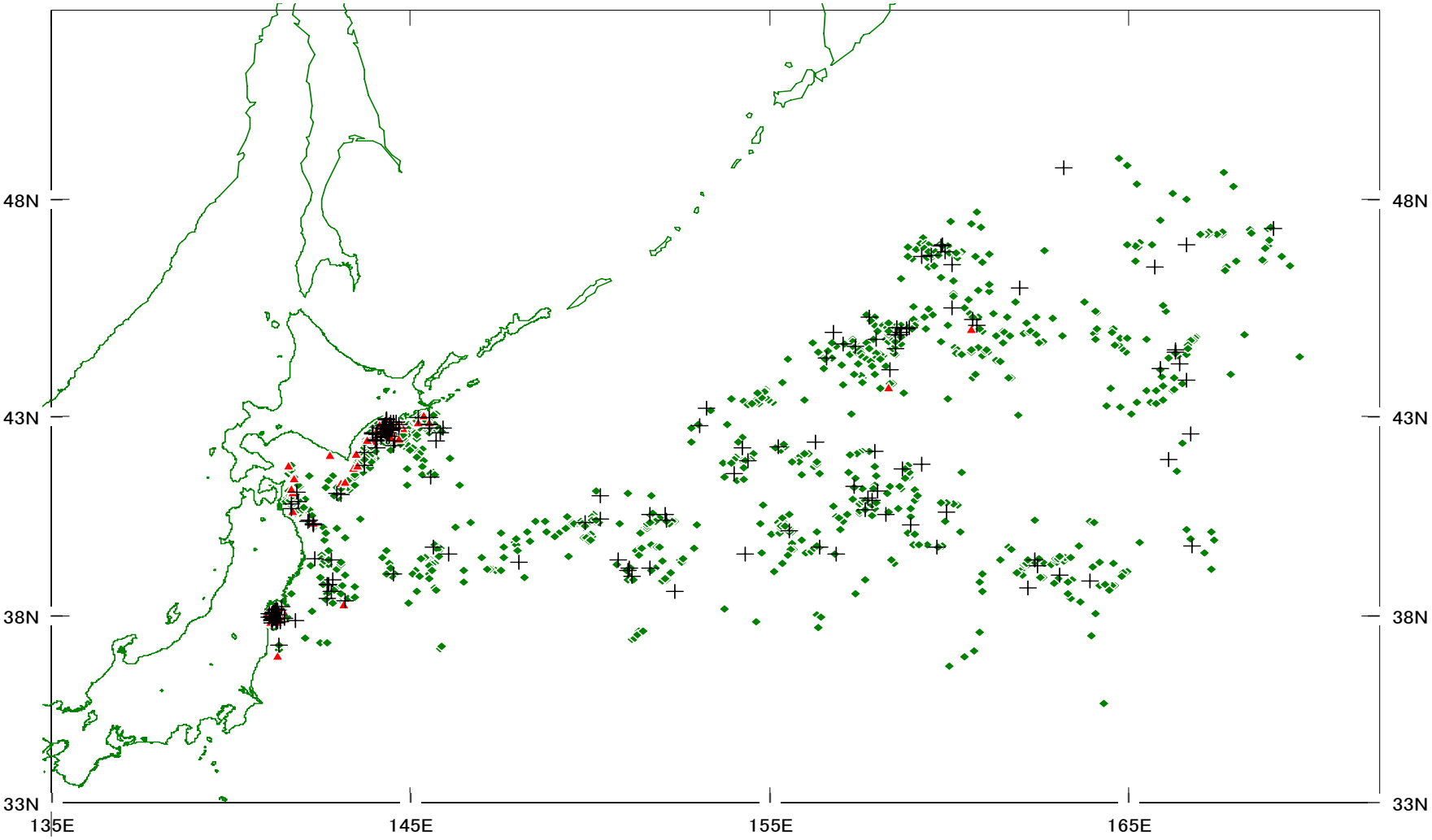

Figure 5a. Locations of the minke whales that were assigned to O stock (◆), J stock (▲), and unknown origin (+) based on STRUCTURE.

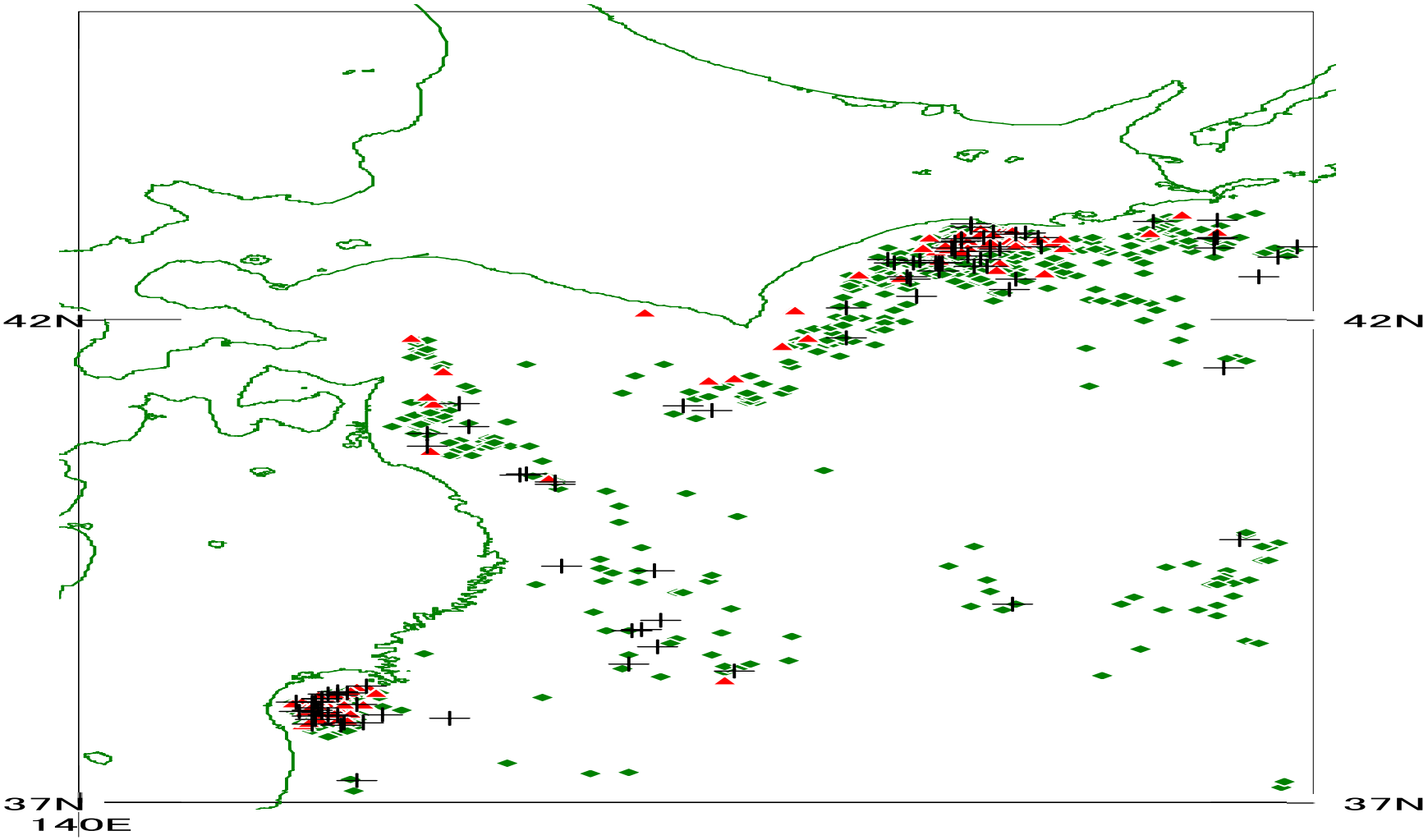

Figure 5b. Zoomed into the coastal area of the Fig. 2a. ◆: O stock, ▲: J stock, +: unknown

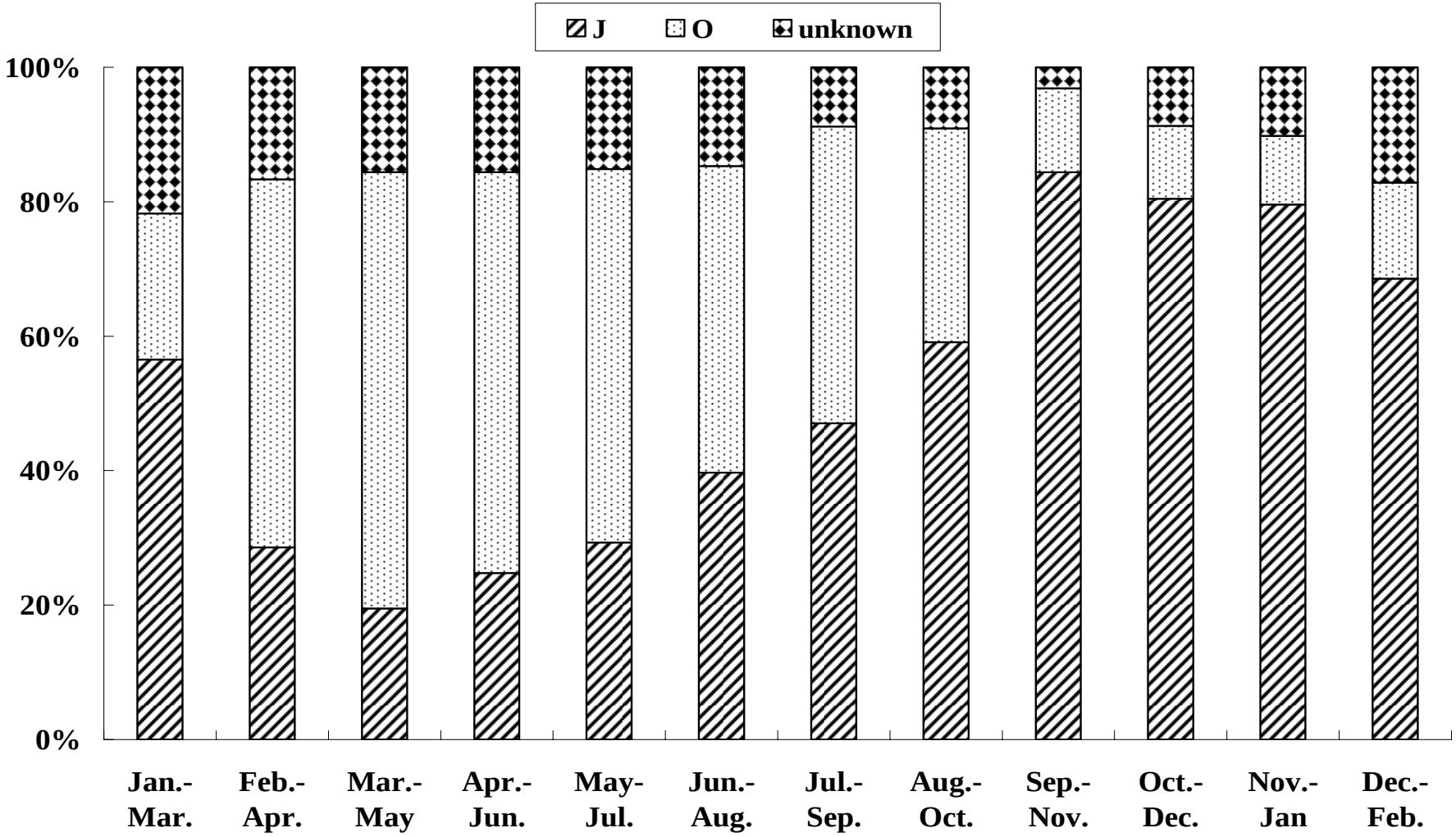

Figure 6. Temporal distribution of the bycatches assigned to the J, O, and unkwon categories in the SA7. Each bar was expressed as three months moving average.