Genetic analyses based on mtDNA control region sequencing and microsatellite DNA confirmed the occurrence of a single stock of sei whales in oceanic regions of the North Pacific

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ABSTRACT

A total of 1,554 sei whales were examined genetically (mtDNA control region sequencing, 487bp and microsatellite DNA at 17 loci) to investigate population genetic structure of this species in the North Pacific. Samples were available from different sources, JARPNII (catches) (2002-2014), POWER (biopsy) (2010-2012) and past commercial (catches) (1972-73). For the heterogeneity test two longitudinal sectors were defined in the North Pacific: Western and Eastern at 180°, which covered this ocean basin widely from approximately 145°E to 135°W. No significant spatial genetic heterogeneity was found by the two genetic markers. A phylogenetic tree of 82 mtDNA haplotypes showed several clusters, but none was supported by high bootstrap values. Whales from both Western and Eastern sectors were widely distributed through the clusters. Taken as a whole, the genetic information in this study is consistent with the view that the oceanic regions of the North Pacific is occupied by a single stock of sei whale.

INTRODUCTION

The most comprehensive studies conducted so far with regard to the stock structure of the North Pacific sei whales were those presented at the previous JARPNII Review workshop in 2009 (Kanda *et al*. 2009) as well as those presented at more recent IWC/SC meeting (Kanda *et al*. 2013). These studies, using multiple sets of microsatellite DNA loci and mtDNA markers, examined sei whales samples collected from almost the entire range of North Pacific.

Kanda *et al*. (2009), on the one hand, analyzed genetic variation at 17 microsatellite DNA loci and 487bp of mitochondrial DNA (mtDNA) control region sequences in the JARPNII samples (n=489) from 2002 to 2007 in the area between 143°E and 170°E as well as in the commercial whaling samples (n=301) from 1972 and 1973 conducted in the area between 165°E and 139°W. The results indicated no evidence of significant genetic differences within as well as between the JARPNII and commercial whaling samples. Both females and males showed same pattern of the stock structure. Sequencing and phylogenetic analysis of the mtDNA control region also showed no evidence of the genetic heterogeneity in the JARPNII samples as well as no spatially or temporally unique phylogenetic clusters.

Kanda *et al*. (2013), on the other hand, examined genetic variations at 14 microsatellite DNA loci in the North Pacific sei whale using biopsy samples obtained from the IWC/POWER (International Whaling Commission/Pacific Ocean Whale and Ecosystem Research; hereafter POWER) surveys that covered the 173°E - 172°W area of the central North Pacific in 2010 (n=13), 170°W - 150°W area of the central North Pacific in 2011 (n=29), and 150°W - 135°W area of the eastern North Pacific in 2012 (n=35), and these obtained data was analyzed with those in Kanda *et al*. (2009). This study allowed the authors to examine temporal (40 years apart between the POWER and commercial whaling data) and spatial (143°E to 135°W area divided into western, central and eastern) genetic differences of the North Pacific sei whales. Similarly to Kanda *et al*. (2009), the results showed no evidence of the temporal genetic differences between the recent POWER and past commercial whaling samples collected from the same area and no evidence of the spatial genetic differences among the western, central and eastern samples.

One drawback to these two studies was that there was no direct comparison among samples collected at the same time of the year from the different areas over the North Pacific. Considering that sei whales conduct seasonal migration from their breeding ground to feeding ground every year, development of stock structure hypothesis should test the genetic differentiation in the samples collected in the same year that eliminate temporal negative biases. If no genetic difference is found, it should strongly indicate no evidence of multiple stocks in the area. Kanda *et al*. (2015a) (SC/F16/JR47) looked at genetic variation at the microsatellite DNA loci to analyze the JARPNII and POWER samples collected from the same time of years in 2010, 2011 and 2012, respectively. Again the study failed to demonstrate evidence of multiple stocks of sei whales in the North Pacific.

The in-depth assessment of North Pacific sei whale started at the 2015 IWC SC meeting. The IWC SC agreed to proceed with two initial alternative stock structure hypotheses: i) a single stock in the entire North Pacific as proposed by Kanda *et al*. (2015a;b), based on several pieces of evidences included the genetics; and ii) a five-stock hypothesis proposed in Mizroch (2015), based mainly on the interpretation of mark-recapture data: Japan coastal; North Pacific pelagic; Aleutian Islands and Gulf of Alaska; eastern North Pacific migratory; and Southern North American coastal stock (coastal California) (IWC, 2015). The IWC SC agreed that discriminating between these two hypotheses is difficult in the absence of genetic data from the potentially extirpated stocks, and thus both hypotheses are plausible (IWC, 2015). The IWC SC agreed that the oceanic regions of the North Pacific are composed by a single stock (IWC, 2015).

In this study the total samples obtained by the surveys of JARPNII between 2002 and 2014, IWC/POWER in 2010-2012 and from past commercial whaling in 1972 and 1973 are analyzed to further examine the spatial population genetic structure of sei whale in the North Pacific, in particular to examine the plausibility of the 'North Pacific pelagic' stock.

MATERIALS AND METHODS

Samples

JARPNII samples (n=1174) of sei whales obtained from 2002 to 2014 were collected from the western North Pacific (Table 1 and Figure 1). Although sampling dates and locations of the surveys slightly differed year by year depending on the sampling plan of a given year, samples were taken in the in the northwestern North Pacific in the range 35°05'N-49°35'N, and 143°19'E-169°58'E.

Skin biopsy samples of sei whales were obtained during the POWER surveys. POWER survey covered the area north of 40°N from 173°E to 172°W in 2010 (10POWER), from 170°W to 150°W in 2011 (11POWER), and from 150°W to 135°W in 2012 (12POWER) (Matsuoka *et al*., 2011, 2012, 2013). A total of 78 POWER biopsy samples were used (Table 1 and Figure 1).

A total of 302 genetic samples from past commercial whaling in 1972 and 1973 were included in the analyses. Individual samples were captured in the sector comprised approximately between 165°E and 139°W (Table 1 and Figure 1).

DNA extraction

The IWC guidelines for DNA data quality (IWC, 2009) were followed as much as possible (see Kanda *et al*., 2014).

Total DNA from each of the whales was extracted from 0.05 g of skin tissue in the JARPNII samples, skin biopsy in the POWER samples and from blood tissues in the commercial samples using the protocol of Sambrook *et al*. (1989). Extracted DNA was stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Microsatellite analysis

Genetic variation at microsatellite loci were analyzed using 17 sets of primers, none of which was designed specifically from sei whales: EV1, EV14, EV21, EV94, EV104 (Valsecchi and Amos, 1996), GT011 (Bérubé *et al*., 1998), GT23, GT211, GT271, GT310, GT575 (Bérubé *et al*., 2000), GATA28,

GATA53, GATA98, GATA417, GGAA520 (Palsbøll *et al.*, 1997), and DlrFCB17 (Buchanan *et al.*, 1996). Primer sequences and PCR cycling profiles generally followed those of the original authors.

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). Amplified products with internal size standard (GENESCAN400HD, Applied Biosystems Japan) were run on a 6% polyacrylamide denaturating gel (Long Ranger) using BaseStation100 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 sei whale's DNA of known size that were rerun on each gel.

Data analysis

Levels of polymorphisms

The number of alleles per locus, expected heterozygosity per locus and inbreeding coefficient was calculated using FSTAT 2.9.3 (Goudet, 1995). Statistical tests for the deviations from expected Hardy-Weinberg genotypic proportions were conducted using GENEPOP 4.0 (Rousset, 2008).

Homogeneity test

In order to detect genetic differences, conventional hypothesis testing procedure was conducted using heterogeneity test in frequencies of the microsatellite alleles among sectors. Null hypothesis to be tested was if the samples came from a genetically same group of sei whales. If statistically significant allele frequency differences exist, then it could indicate these samples came from genetically different stocks of sei whales. Probability test (or Fisher's exact test) implemented in GENEPOP 4.0 (Rousset, 2008) was used to conduct the heterogeneity test. Statistical significance was determined using the chi-square value obtained from summing the negative logarithm of *p*-values over the 17 microsatellite loci (Sokal and Rohlf, 1995). The False Discovery Rate (FDR) approach (Benjamini and Yekutieli, 2001) was used for adjustment of *p*-value in case of multiple comparisons. F_{ST} was calculated using FSTAT 2.9.3 (Goudet, 1995).

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

Level of polymorphisms

The number of haplotypes and haplotype diversity were calculated following Nei (1987). The nucleotide diversity (Nei, 1987: equation 10.5) and its standard error for population sampling and stochastic processes were calculated from the pair-wise differences between the mtDNA sequences using the Kimura's 2- parameter adjustment (Kimura, 1980).

Homogeneity test

Conventional hypothesis testing procedure was conducted using heterogeneity test in mtDNA haplotype frequencies among the samples. The randomized chi-square Test of Independence (Roff and Bentzen, 1989) and the conventional F_{ST} were used to investigate the spatial differentiation of mtDNA variation. In each test a total of 10,000 permutations of the original data were performed. Tests were conducted for all samples combined as well for males and females separately. A p-value smaller than 0.05 was used as a criterion to reject the null hypothesis of panmixia. The FDR approach was used for adjustment of *p*-value in case of multiple comparisons. F_{ST} for mtDNA was calculated based on the analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992).

Phylogenetic analyses

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 Bryde's and Omura's whales. To estimate support for each node a total of 1,000 bootstrap simulations were conducted and the majority-rule consensus genealogy estimated.

RESULTS

Microsatellites

Level of polymorphisms

All 17 loci analyzed were polymorphic (Table 2). The total number of alleles per locus ranged from three at the GATA53 to 23 at the EV14 with an average of 9.71. Expected heterozygosity at each of the loci ranged from 0.14 at GT271 to 0.87 at DlrFCB with an average of 0.64. No significant departure from the expected Hardy-Weinberg genotypic proportions was found.

Homogeneity test

No significant genetic heterogeneity was found between Western and Eastern sectors for each locus as well for all loci combined. Results were the same for male, female and both sexes combined (Table 3).

Mitochondrial DNA

Level of polymorphism

Levels of mtDNA diversity were relatively high in the North Pacific sei whale (Table 4).

Homogeneity test

No significant genetic heterogeneity was found between Western and Eastern sectors. Results were the same for male, female and both sexes combined (Table 5).

DISCUSSION

As informed earlier the in-depth assessment of North Pacific sei whale started at the 2015 IWC SC with two initial alternative stock structure hypotheses: i) a single stock in the entire North Pacific as proposed by Kanda *et al*. (2015a;b), based on several pieces of evidences included the genetics; and ii) a five-stock hypothesis proposed in Mizroch (2015), based mainly on the interpretation of mark-recapture data: Japan coastal; North Pacific pelagic; Aleutian Islands and Gulf of Alaska; eastern North Pacific migratory; and Southern North American coastal stock (coastal California) (IWC, 2015). As noted earlier the IWC SC agreed that discriminating between these two hypotheses is difficult in the absence of genetic data from the potentially extirpated stocks, and thus both hypotheses are plausible (IWC, 2015).

The main purpose of this study was to examine genetically all the available genetic samples of the sei whale in the North Pacific in order to confirm the hypothesis of a single stock in the oceanic regions of the North Pacific (the 'North Pacific pelagic' in the second stock structure hypothesis indicated above). As noted by the IWC SC it is not possible to check the plausibility of other putative stocks under the second hypothesis using genetics analyses because no genetic samples are available from the regions where these putative stocks are proposed.

Results of the present analysis are in agreement with previous genetic analyses based on smaller sample sizes (Kanda *et al*., 2009; 2015a: SC/F16/JR47). Kanda *et al*. (2015b: SC/F16/JR48) analyzed all non-genetic evidences that were consistent with the single stock in oceanic regions. In fact, the genetic and non-genetic evidences summarized by Kanda *et al*. (2015b: SC/F16/JR48) were more consistent with the first hypothesis above, a single stock of sei whales in the whole North Pacific.

It is considered that the evidences for proposing the second stock structure hypothesis (five stocks) are weak and that the IWC SC should evaluate objectively the plausibility of the additional putative stock other than the 'North Pacific pelagic'.

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Table 1. Sample sizes of sei whales used in the present study for the microsatellite and mtDNA (figures in parenthesis) analyses, by longitudinal sector, sex, and source of samples. Only the samples genotyped for the complete microsatellite loci set were included in the analysis. Western and Eastern sectors were divided at 180°.

Data source	Sex	Sector		Total
		Western	Eastern	
JARPNII	Male	551 (551)		551 (551)
	Female	623 (622)		623 (622)
POWER	Male	1 (1)	36 (36)	37 (37)
	Female	1 (1)	40 (39)	41 (40)
Commercial	Male	88 (87)	67 (71)	155 (158)
	Female	92 (88)	55 (57)	147 (145)
Total		1356 (1350)	198 (203)	1554 (1553)

Table 2. The number of alleles (A), expected heterozygosity (He), test results for expected Hardy-Weinberg genotypic proportion (HW) and inbreeding coefficient (F_{IS}) in North Pacific sei whale, at 17 microsatellite loci.

Microsatellite loci	A	H_E	HW	F_{IS}
EV21	6	0,65	0,714	-0,008
GGAA52	9	0,80	0,871	0,015
GATA98	7	0,74	0,228	0,009
GT211	6	0,31	0,020	0,010
EV14	23	0,86	0,422	0,005
GATA53	3	0,49	0,816	0,011
EV1	17	0,84	0,750	0,016
EV94	8	0,69	0,520	-0,030
GT23	14	0,60	0,674	0,026
GT575	6	0,59	0,561	0,032
GATA41	9	0,78	0,771	0,010
GT310	5	0,49	0,574	-0,032
EV104	10	0,72	0,839	-0,008
GATA28	11	0,81	0,858	0,011
GT271	4	0,14	0,727	0,004
GT011	6	0,45	0,920	0,008
DlrFCB	21	0,87	0,471	0,007
Average	9,71	0,64	0,939	0,005

Table 3. Results of the heterogeneity test for sei whales between Western and Eastern longitudinal sectors, by sex and locus.

Microsatellite loci	Both sex combined		Male		Female	
	P -values	F_{ST}	*P* -values	F_{ST}	*P* -values	F_{ST}
EV21	0,221	0,001	0,072	0,001	0,930	-0,002
GGAA520	0,759	-0,001	0,834	-0,001	0,831	-0,001
GATA98	0,774	-0,001	0,543	0,000	0,932	-0,003
GT211	0,394	-0,001	0,739	-0,001	0,416	-0,001
EV14	0,623	-0,001	0,607	-0,004	0,723	-0,001
GATA53	0,798	-0,001	0,772	-0,002	0,897	-0,003
EV1	0,100	-0,001	0,088	0,000	0,026	-0,001
EV94	0,638	0,001	0,353	0,004	0,981	-0,002
GT23	0,673	-0,001	0,430	0,001	0,140	-0,002
GT575	0,182	-0,001	0,175	0,000	0,245	0,005
GATA417	0,329	0,000	0,359	-0,004	0,511	-0,001
GT310	0,144	0,000	0,216	0,000	0,795	-0,002
EV104	0,223	0,002	0,707	-0,001	0,092	0,003
GATA28	0,130	0,000	0,407	-0,001	0,199	0,000
GT271	0,829	-0,001	0,749	-0,001	0,820	-0,003
GT011	0,456	-0,001	0,805	-0,002	0,263	-0,002
DlrFCB17	0,181	0,000	0,067	0,000	0,331	-0,001
Overall	0,393	0,000	0,415	-0,001	0,543	-0,001

Table 4. Results of mtDNA diversity for North Pacific sei whale.

Number of haplotypes	Nucleotide diversity (SE)	Haplotype diversity
82	0.00794 (0.00008)	0.9275

Table 5. Results of the mtDNA heterogeneity test between Western and Eastern sectors, by sex.

Western × Eastern					
Chi-square *p*-value			F_{ST}		
Sex combined	Male	Female	Sex combined	Male	Female
0.1071	0.3549	0.0991	0.00028	-0.00021	0.00111

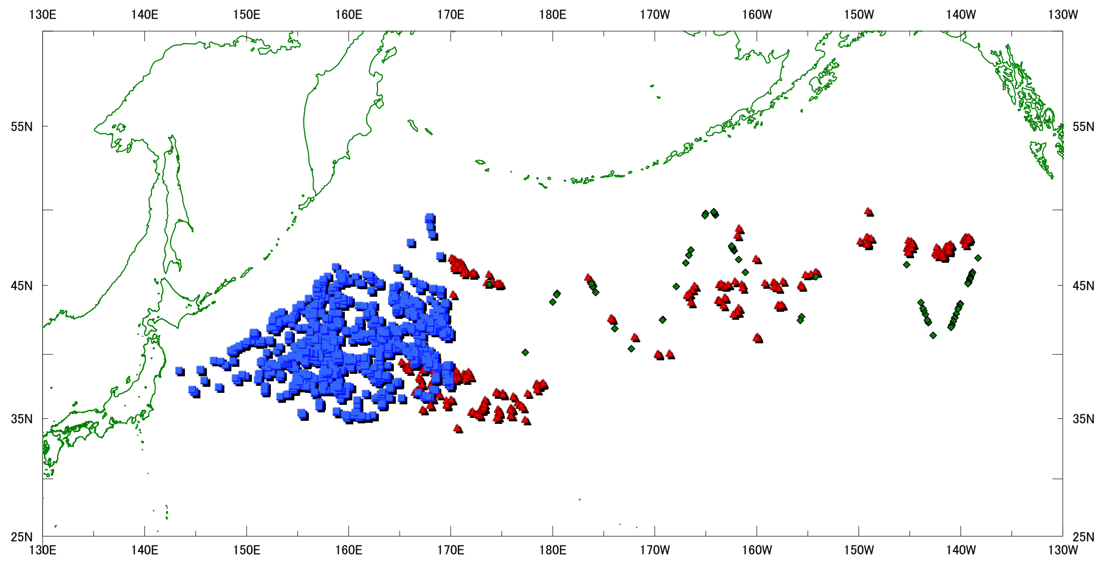
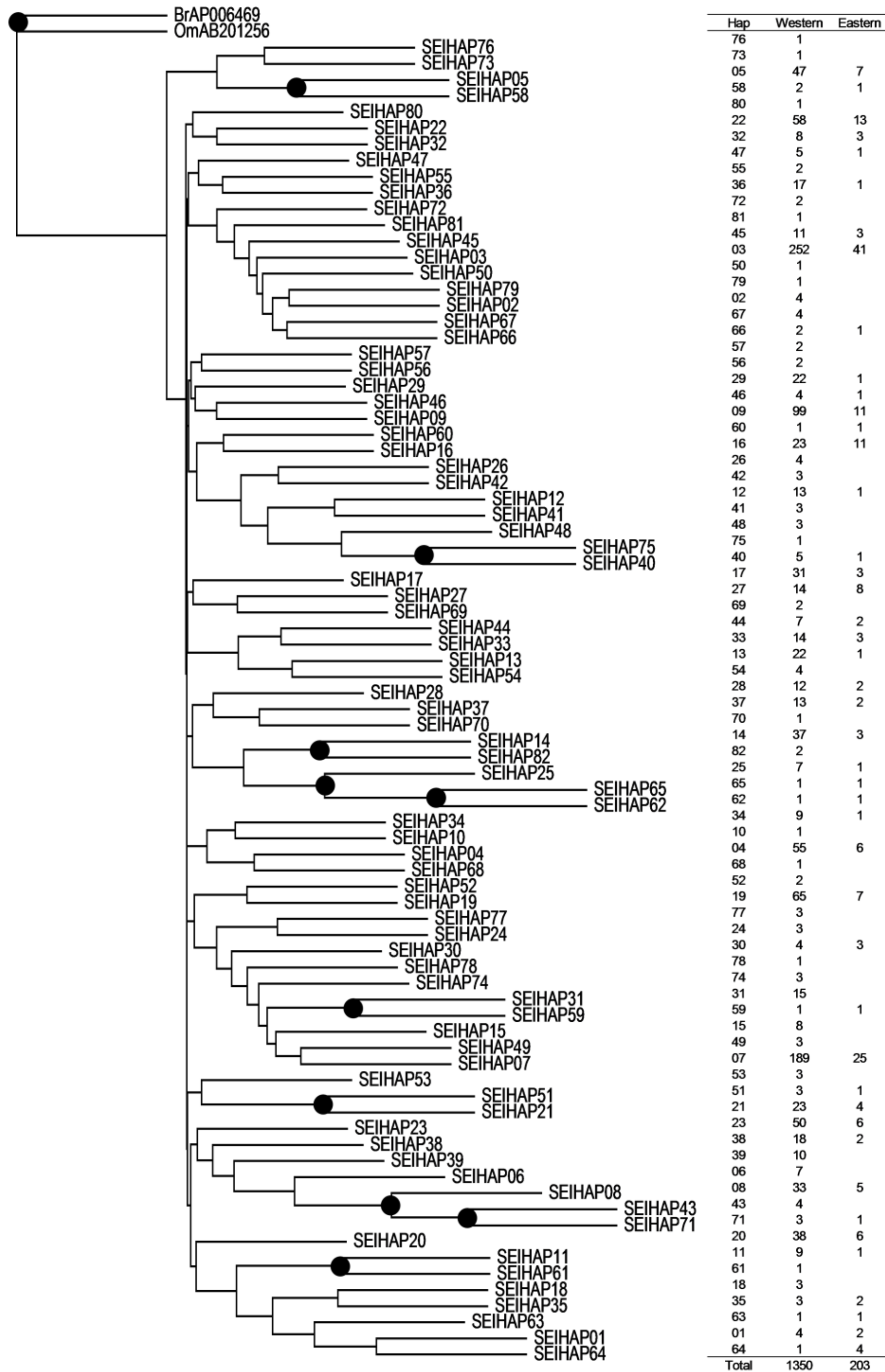


Figure 1. Sampling locations of sei whales used in the present genetic study. Blue: JARPNII samples; Red: past commercial whaling samples; Green: IWC/POWER samples. For the statistical analyses samples were arbitrarily divided into two sectors: Western and Eastern at 180°.



Hap	Western	Eastern
76	1	
73	1	
05	47	7
58	2	1
80	1	
22	58	13
32	8	3
47	5	1
55	2	
36	17	1
72	2	
81	1	
45	11	3
03	252	41
50	1	
79	1	
02	4	
67	4	
66	2	1
57	2	
56	2	
29	22	1
46	4	1
09	99	11
60	1	1
16	23	11
26	4	
42	3	
12	13	1
41	3	
48	3	
75	1	
40	5	1
17	31	3
27	14	8
69	2	
44	7	2
33	14	3
13	22	1
54	4	
28	12	2
37	13	2
70	1	
14	37	3
82	2	
25	7	1
65	1	1
62	1	1
34	9	1
10	1	
04	55	6
68	1	
52	2	
19	65	7
77	3	
24	3	
30	4	3
78	1	
74	3	
31	15	
59	1	1
15	8	
49	3	
07	189	25
53	3	
51	3	1
21	23	4
23	50	6
38	18	2
39	10	
06	7	
08	33	5
43	4	
71	3	1
20	38	6
11	9	1
61	1	
18	3	
35	3	2
63	1	1
01	4	2
64	1	4
Total	1350	203

Figure 2. Phylogenetic relationships among North Pacific sei whale mtDNA haplotypes and frequencies of haplotypes in two longitudinal sectors.