EARLY DRAFT FOR CONSIDERATION BY THE WORKING GROUP ON PRE-IMPLEMENTATION ASSESSMENT OF WESTERN NORTH PACIFIC MINKE WHALES

Stock structure of western North Pacific minke whales based on mtDNA *haplogroups* from 'bycatch' and scientific whaling

C. Scott Baker[1], Rebecca M. Hamner[1], R.L. Brownell Jr.[2] and Paul R.Wade[3]
1) Marine Mammal Institute, Oregon State University, Newport, Oregon
2) Southwest Fisheries Science Center, Pacific Grove, California
3) National Marine Mammal Laboratory, Seattle, Washington

Abstract

We investigated differences in mtDNA sequences and sex ratios in western North Pacific minke whales by combining information from samples collected in Korean market surveys (Korean 'bycatch', n=237) with three datasets made available courtesy of the Institute for Cetacean Research (ICR) through the IWC Data Availability Group: Japanese 'bycatch' (n=832), 'coastal whaling' (n=481) and 'offshore whaling' (n=1238). An initial review of the Japanese datasets showed a large number of haplotypes (70 of 157) found in only a single individual (singletons), suggesting some error in sequencing of these samples. Communication with ICR resulted in re-sequencing and revision of the haplotype classification of some of these samples. In an effort to meet the agreed time frame of the Preliminary Implementation Assessment, we chose to focus on the analysis of 4 haplogroups, previously considered to be informative (although not diagnostic) of the 'J' and 'O' stocks. Although collapsing the haplotypes into haplogroups resulted in a substantial loss of resolution in mtDNA differentiation, it avoided the problems of categorical analyses posed by the singleton errors. The 'O-' and 'J-types' defined by the 4 mtDNA haplogroups showed a 93% concordance with samples assigned to the 'O-' and 'J-clusters' in the Structure analysis of microsatellite loci (Kanda *et al.* 2009).

Using the 4 haplogroups and sex, we considered pairwise differences in various strata, including subareas, source (bycatch, coastal whaling, offshore whaling), latitude (1 degree increments) and season (autumn/winter and spring/summer). We found significant differences for either haplogroup frequencies, sex ratios or both for almost all comparisons within these strata. A notable exception was the Korean bycatch (market individuals) vs. subarea 6 bycatch (Japanese coast of Sea of Japan), which did not show significant differences in haplogroup frequencies, but did show a difference in sex ratios and in the haplogroups-by-sex effect. We then focused on subareas 2 and 7W to investigate the potential for one or more coastal stocks along eastern Japan. Haplogroup frequencies of bycatch showed a pronounced change at 33-34^0 N latitude, suggesting this might be a more natural division than the current subarea boundary at 35^0 N latitude. Within subarea 7W, comparisons showed differences in haplogroup frequencies and/or sex ratios for most strata, including 'bycatch (BC)', 'coastal Sanriko', 'coastal Kushiro' and 'offshore' hunting. We further noted that 'coastal' and 'offshore' hunting showed considerable overlap in the reported 'distance from coastline'. By plotting the location of 'offshore' whaling samples we found a number of inconsistencies in the measurement of 'distance from coastline', confounding a combined analysis of this important strata. Plotting the location of haplogroups taken in 'coastal Sanriko' and 'coastal Kushiro' showed no clear demarcation of haplogroups by latitude or distance from coastline.

It is possible that the haplogroup frequencies and sex ratios of coastal whales along eastern Japan could be explained by a complex seasonal mixing of 2 stocks, e.g., a core J and a core O. However, we consider it more plausible that whales in subarea 7W (or from about 33^0 to 42^0) represent a third stock, e.g., O_w, characterized by frequencies of haplotypes that are intermediate, but significantly different from either J_E or O_E.

Introduction

The Government of Japan has requested interim advice from the Scientific Committee of the International Whaling Commission on an *ad hoc* quota for hunting of minke whales with its small-type whaling vessels (Cooke *et al.* 2009). Such advice is critically dependent on an understanding of the complex population structure and migration of minke whales in the western North Pacific, particularly the so-called 'J stock' found in coastal waters of Japan and Korea. Here we propose to compare mtDNA sequences and haplotype frequencies of minke whales from the western North Pacific using samples from 237 'market individuals' sampled in Korean 'whalemeat' markets from 1999-2005, as reported previously (Baker *et al.* 2007; Baker *et al.* 2000; Baker *et al.* 2006), to 2,544 whales taken by Japan as 'bycatch' in both the North Pacific and Sea of Japan from 2001-2007 or in scientific whaling in the North Pacific from 1994-2007 (Goto *et al.* 2009a; Goto *et al.* 2009b). The Korean 'market individuals', including samples transferred to SWFSC (Brownell *et al.* 2000), are assumed to originate from coastal bycatch in Korean waters (representing subareas 5 and 6) and are thus the best available representation of whales from the Yellow Sea, the Korean Strait and Sea of Japan (East Sea), referred to by the IWC as the 'J stock'. The Japanese samples include coastal bycatch, presumed to originate from one or more 'J-like' stocks, as well as scientific hunting targeted at one or more 'O-like' stocks in the western North Pacific. Together, these samples represent the most comprehensive dataset available for minke whales in the western North Pacific, representing subareas 2, 5, 6, 7, 8, 9, 10 and 11.

The intent of the proposed analyses will be to test plausible stock hypotheses as outlined in discussions of the In-depth assessment of western North Pacific common minke whales, with a focus on J stock. Analyses will rely primarily on exact tests to investigate differences in mtDNA haplotype frequencies and sex for various geographic and temporal strata. Such 'hypothesis testing' is considered the most sensitive approach to identifying populations characterized by moderate differences in haplotype frequencies (i.e., moderate levels of gene flow, Waples and Gaggiotti 2006).

Methods

Access to information on the samples from Japanese bycatch and scientific whaling was requested from the Institute of Cetacean Research of Tokyo, through submission of a proposal to the IWC Data Availability Group on 19 November, 2009 (IWC 2004). The requested datasets were described in previous reports to the IWC (Goto *et al.* 2009a; Goto *et al.* 2009b; Kanda *et al.* 2009). Following recommendations from the DAG, the proposal was revised and resubmitted on 25 November 2009, with further minor revisions on 7 January 2010. The 3 datasets from the ICR as a single Excel file, as communicated through the IWC DAG, were received on 7 January 2010.

A fourth dataset was based on information from a subset of the Korean market samples, including the Brownell et al. (2000) samples, collected from 1999-2005 and identified as North Pacific minke whales. DNA profiling by microsatellite genotyping, sex and mtDNA were used to eliminate replicate products, as described by (Baker *et al.* 2007; Dalebout *et al.* 2002). These samples are assumed to represent bycatch from coastal waters of Korea. Although all market products were purchased in Busan, Ulsan and Pohan (or nearby areas), it is likely that whales killed as bycatch along other parts of the Korean coast (including the Yellow Sea) are transported to these markets for sale. The Korean bycatch dataset, including date and location of purchase, sex, and sequence information from 464 bp of the mtDNA control region, was submitted previously to the IWC Data Availability Group, following protocols for the in-depth assessment of the North Pacific minke whales. This submitted dataset was substantially revised in preparation for the analysis reported here and in SC62/BCxx.

Initial review of mtDNA control region sequences in the ICR dataset (text files only, no chromatograms) was conducted with the program McClade (Maddison and Maddison 2000). Statistical analyses relied primarily on binomial tests of frequencies, available in Excel and on the test of differentiation (i.e., modified exact test) as implemented in GENEPOP (Rousset 2008). Locations of catches were plotted in ArcGIS (courtesy of T. Follet).

Results

Haplotype sequence review and haplogroup definitions

The 3 datasets (Coastal, Offshore and Japanese Bycatch) totalled 2,551 samples, of which 2,544 included mtDNA control region sequences. All requested information was included in the datasets, with the exception of latitude and longitude for bycatch, which were reported only by prefecture. A fourth dataset, referred to as 'Korean bycatch' included the mtDNA control region sequences and sex for 237 'market individuals' represented by one or more products purchased on the Korean market between 1999 and 2005. These products are assumed to represent whales taken in Korean coastal waters, including Yellow Sea, Korean Strait and Sea of Japan (East Sea), and thus characteristic of the primary habitat of J stock.

Initial review of the three ICR datasets showed that the mtDNA control region sequences (487-489 bp) defined 157 haplotypes, of which 70 were represented by only one sample (e.g., a 'singleton'). Many of these singletons are defined by only a single nucleotide change relative to more common haplotypes. Some of these nucleotide variants were also tranversions, a change that is otherwise rare in comparisons of control region sequences within species of whales (i.e., within species diversity). The 70 singletons are distributed as follows: 32 haplotypes in Offshore, 14 haplotypes in Coastal and 24 haplotypes in Japanese Bycatch. In an effort to follow the quality control recommendations of Morin et al. (2010), we also reviewed the sequences from the Korean market surveys and compared sequence identity to the ICR datasets. Although sequence quality and length was somewhat variable, given the poor quality of many of the market products, 23 of the 24 haplotypes found in the Korea market dataset, matched a common haplotype in the ICR datasets, and 1 matched a singleton in the ICR dataset. This provided considerable confidence in the identity of these shared haplotypes and the comparability of the combined datasets.

On 8 February, we communicated by email with Pastene and Goto about our concern that some of the 70 singletons in the ICR database were sequencing artefacts. Such errors, if uncorrected, will inflate the haplotype diversity and could distort categorical tests of haplotype frequencies (e.g., tests of differentiation and randomized Chi-squared tests of independence). Pastene agreed to review the sequences and to revise the dataset if errors were found. In

anticipation of the time required for such a review and any subsequent re-sequencing, we chose to redirect our analyses on the 4 haplogroups previously considered to be informative (although not diagnostic) of 'J' and 'O' stocks (Baker *et al.* 2000). Although collapsing the 157 haplotypes into 4 haplogroups resulted in a substantial loss of resolution in mtDNA differentiation, it avoided the problems of distorted categorical analyses posed by the potential singleton errors. See below (*Recommendations*) for communication with Pastene on progress with sequence review and corrections.

Concordance of haplogroups with Structure assignment
Of the 2544 samples with mtDNA sequences included in the 3 ICR datasets, 2541 could be classified into one of the 4 haplogroups defined by variation at positions 298 and 463 (Table 1), as described by Baker et al. (2000). Of this total, 1692 (66.5%) were identified as the common 'AG' haplogroup. This haplogroup is considered representative of the O stock as found in subarea 9 where it is found at a frequency of 96% (Fig 1). The other 3 haplogroups are considered representative of the J stock as found in the Korean market sample, where, together, they are found at a frequency of 91% (i.e., the frequency of the 'AG' haplogroup is 9%). Based on these distinctive frequency differences, we refer to the AG haplogroup as 'O type' and the other 3 haplogroups as 'J types'.

The 'O types' and 'J types' defined by haplogroups showed good concordance with the 'O-' and 'J-clusters' in the Structure analysis of microsatellite loci (Kanda *et al.* 2009). Of the 2297 samples assigned to either 'O-' and 'J-clusters' by Structure, 93% agreed with the classification into 'O types' or 'J types' based on the haplogroups (Table 2). The degree of this concordance varied somewhat in the different source strata and by subarea but remained high across the entire dataset. Of the 240 samples that were not assigned with a high probability to either O or J clusters, 70% were classified as 'O type' by haplogroups. This is similar to the overall frequency of samples assigned to the O cluster.

Differentiation of haplogroups by subareas and source
The frequencies of 'J type' haplogroups were highest in the Korean bycatch (BCK) and subarea 6, declining in subarea 2 and subarea 7W, with a continued decline in subarea 8 and 9 (Fig 1). Haplogroup frequencies differed significantly between almost every subarea and source, with a few exceptions (Table 3): Korean bycatch and subarea 6 bycatch did not differ, although differences were found in sex ratios (see below); offshore hunting in subarea 7W and 7E did not differ, although the sample size for 7E was small; and subareas 8 and 9 did not differ. Although we calculated F_{ST} values as a standard measure of genetic effect size, the categorization into the 4 informative haplogroups results in an inflation of this index by removing the within-haplogroup diversity. This is particularly true for the large number of haplotypes that are collapsed into the 'O type' haplogroup. Consequently, these values should not be considered comparable to F_{ST} calculated from haplotype frequencies (Goto *et al.* 2009a).

Comparisons between sources of catches, i.e., bycatch or hunting, could only be conducted for subarea 7W. Here we found significant differences between all 4 sources of catches: bycatch (BC7), coastal Sanriko (CS), coastal Kushiro (CK) and 'offshore' hunting. The largest effect was between BC7 and offshore hunt (F_{ST} =0.223; $p<0.0001$). The smallest effected was between CK and CS (F_{ST} =0.0003; p=0.012).

Sex ratios by subarea and source
Significant biases in sex ratios of samples were found in each stratum of subarea and source (Table 4). Bycatch in subareas 6, 2 and 7 (BC6, BC2 and BC7) showed a significant female bias, although the bias was small for BC6 and BC7. A male bias was found in BCK and in all coastal and offshore hunting, except for CS, where there was a female bias.

Differentiation of haplogroups by sex - for subarea and source
Within each subarea and source, we tested for differences in haplogroup frequencies by sex. This showed significant differences for BCK, BC2, BC7, CS and 7W offshore (Table 4). However, the F_{ST} values suggested that the effect size of these sex differences were rather small, e.g., F_{ST} = 0.007 for BC7. Surprisingly, there was no significant difference in haplogroup by sex for CK, despite the large male bias in the overall catch.

Haplogroup by season and sex – for bycatch, by subarea
In an effort to understand seasonal differences in distributions of stocks along the two coasts, we stratified haplogroup frequencies by 2 six-month seasons (spring/summer and autumn/winter) and by sex, for bycatch in subareas 6, 2 and 7. For BC6, there was no significant difference by season, or by sex within seasons (data not shown). Sex ratios were also similar in the 2 seasons. For BC2, there was a small but significant effect of season on

haplogroup frequencies (F_{ST} =0.0065; p=0.01), with slightly smaller proportion of 'O types' during autumn/winter, and a larger female bias during autumn/winter (Fig 2). For BC7, there was a large effect of season on haplogroup frequencies (F_{ST} =0.177; $p < 0.0001$), with a smaller proportion of 'O types' during autumn/winter showing, and a larger female bias in spring/summer.

Haplogroup and sex ratios by latitude – for bycatch
In an effort to understand differences in distributions of stocks along the two coasts, we stratified haplogroup frequencies and sex ratios by 1^0 of latitude for bycatch in subareas 6, 10 and 11, and in subareas 2 and 7 combined (Fig 3). Because of the lack of information on longitude and latitude for the bycatch samples, we approximated the latitude based on a midpoint of each coastal prefecture. For BC6, the results showed no obvious latitudinal trend for either haplogroup or sex. For BC2 and BC7, however, the haplogroups showed an increase in 'O types' at about 33-34^0 N, rather than the current boundary between subarea 2 and 7 at 35^0 N. There was no obvious trend in sex ratios.

Haplogroups by distance from coastline – coastal and 'offshore' whaling in subarea 7W
Initial efforts to consider 'distance from coastline' as a stratum for differentiation of haplogroups in subarea 7W proved frustrating. First, we noted that 'coastal' and 'offshore' hunting showed considerable overlap in the reported 'distance from coastline', e.g., 64% of 'offshore' catches in 7W were reported to be <50 nm from shore. Further, by plotting the location of 'offshore' whaling samples we found a number of inconsistencies or inaccuracies in the measurement of 'distance from coastline', e.g., samples reported as >50 nm from shore were intermingled with samples labelled as <50 nm from shore. These inconsistencies confounded an analysis of haplogroup frequencies by 'distance from coastline' for the combined 'coastal' and 'offshore' samples. Instead, we plotted the positions (lat/long) of the two datasets for subareas 7W and 7E, labelled by haplogroup and sex (Fig 4). As seen in the location of individuals assigned by Structure to O- and J-clusters (Kanda *et al.* 2009), the locations of the 4 haplogroups in subarea 7W and 7E showed little demarcation of haplogroups by latitude or distance from coastline, except for a concentration of O-type males in the central longitude and latitudes of the subarea. The locations of samples taken in the two coastal hunting programs, CS and CK, also showed little evidence of stratification of haplogroups within the current range of the hunt (Fig 5).

Conclusions
Western North Pacific minke whales have long been recognized as having a complex pattern of heterogeneity in mtDNA haplogroups and sex ratios. Disentangling the underlying stock structure is confounded by the lack of consistent sampling across strata, e.g., bycatch is represented across all seasons but not considered representative of offshore populations and is presumably biased towards juveniles; market samples are consistent with coastal bycatch but are of questionable seasonality, given delays in distribution; 'coastal' whaling is highly concentrated at two different latitudes and at two different times of year; and, 'offshore whaling' in subarea 7W is not really offshore, but a mix of coastal and offshore. All pelagic samples (subareas 7E, 8 and 9) were collected during a limited season with an extreme sex bias. Given these confounding sampling issues, it is difficult to convincingly corroborate or refute the various stock structure hypotheses under consideration. Despite this, some patterns did emerge from our preliminary analysis of haplogroups:

1) BCK and BC6 are similar in haplogroups frequencies, consistent with a primary influence of one stock, presumably the 'core' J stock, present year-round in the Sea of Japan. In BCK, however, the male biased sex ratio and the haplogroup-by-sex differences could reflect migratory mixing (or mixing in the market) of a second stock, perhaps from the Yellow Sea. Although the majority of Korean bycatch is reported from the Sea of Japan (East Sea), some proportion of whales killed in the Yellow Sea are probably transported for sale to Busan, Ulsan and Pohang, where our samples were collected. No sex bias or haplogroup-by-sex differences were found for BC6, suggesting a year-round presence of a non-migratory coastal stock.
2) BC2 differs from BCK and BC6, and from BC7, suggesting the potential for an eastern coastal stock (J_E) with characteristics of the 'core' J stock in the Sea of Japan (J_W).
3) BC7, CS and CK differ from 'offshore' hunting, particularly in SA8 and 9, suggesting the potential for a second coastal stock (O_W) along eastern Japan, with some (perhaps seasonal) mixing of J_E and O_E. Stocks characterized by intermediate haplotype frequencies are well described in humpback whales, where stock divisions are supported by multiple lines of evidence (e.g., photo-ID records).
4) Although it is possible that the haplotype frequencies of subarea 7W could be explained by a complex seasonal, sex- and age-biased mixing of 2 stocks, e.g., a 'core J' and a 'core O', we think this is less parsimonious than the hypothesis of an O_W stock with intermediate haplogroup frequencies. We also note that the absence of a strong haplogroup-by-sex interaction in BC7, CS and CK is inconsistent with the prediction of a sex-biased mixing of two stocks.

Recommendations

1) Complete quality control review and error correction of mtDNA sequences. An email message from Pastene on 23 March 2010, included corrected sequences that are now being reviewed for inclusion into a revised dataset.
2) Repeat analyses with the full dataset of corrected haplotypes. Unfortunately, other constraints on time between now and the SC meeting, may limit our progress on analyses. Review bycatch records in an effort to include latitude and longitude in BC database.
3) Review 'distance from coastline' calculations for 'offshore whaling' in subarea 7W to improve analysis of this important stratum. An email message noting these apparent inaccuracies was sent to Pastene on 31 March 2010.

Acknowledgement

This report was prepared in accordance with the conditions established in the SC data access protocol for Procedure A and the agreement to use the data only for the investigation of stock structure for pre-Implementation Assessment of NP minke whales. We thank the people that collected these samples during JARPN/JARPN II and those that conducted the laboratory work, particularly L. Pastene, N. Kanda and M. Goto. Thanks also to members of the IWC Data Availability Group, T. Kitakado, G. Donovan, and D. Palka.

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Table 1. Summary of minke whale samples and haplogroups for each of the three ICR datasets (Offshore, Coastal and Bycatch) and individuals identified from Korean market samples.

	n	With mtDNA	J-type			O-type	?
			AA	GA	GG	AG	
ICR Total	2551	2544	164	415	271	1691	3
Offshore	1238	1232	41	39	28	1123	1
Coastal	481	480	29	48	26	376	1
Bycatch	832	832	94	328	217	192	1
Korean Market	237	237	28	108	80	21	

Table 2. Concordance of mitochondrial DNA haplogroups with microsatellite assignment to O- or J-clusters by Structure (? = membership coefficient <0.9 for either J or O). The % agreement was calculated only based on samples assigned as J or O.

TOTAL		**ICR assignment**		
93% agreement		**J**	**O**	**?**
Haplogroup	**AA**	95	54	13
	GA	354	20	41
	GG	243	11	17
	AG	76	1444	169

COASTAL		**ICR assignment**		
93% agreement		**J**	**O**	**?**
Haplogroup	**AA**	10	16	3
	GA	36	5	7
	GG	24	1	1
	AG	8	331	36

BYCATCH		**ICR assignment**		
91% agreement		**J**	**O**	**?**
Haplogroup	**AA**	81	4	8
	GA	296	1	31
	GG	202	2	13
	AG	62	111	19

OFFSHORE		**ICR assignment**		
94% agreement		**J**	**O**	**?**
Haplogroup	**AA**	4	34	2
	GA	22	14	3
	GG	17	8	3
	AG	6	1002	114

Table 3. Pairwise tests of differentiation for mtDNA haplogroups from western North Pacific minke whales showing (a) p-value and (b) F_{ST}, calculated using Genepop for subareas and sources (BC, bycatch, C, coastal whaling) of samples. Shaded values are not significant.

a)	n	BCKo	BC6	BC2	BC7	CK	CS	7W 'off'	7E	8
BCKo	237	-								
BC6	410	0.123	-							
BC2	184	0.001	<0.0001	-						
BC7	212	<0.0001	<0.0001	<0.0001	-					
CK	253	<0.0001	<0.0001	<0.0001	<0.0001	-				
CS	226	<0.0001	<0.0001	<0.0001	<0.0001	0.012	-			
7W 'off'	415	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	-		
7E	47	<0.0001	<0.0001	<0.0001	<0.0001	0.243	0.320	0.263	-	
8	223	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001	0.005	-
9	446	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.166

b)	n	BCKo	BC6	BC2	BC7	CK	CS	7W 'off'	7E	8
BCKo	237	-								
BC6	410	0.0003	-							
BC2	184	0.007	0.011	-						
BC7	212	0.157	0.146	0.093	-					
CK	253	0.402	0.372	0.327	0.100	-				
CS	226	0.403	0.372	0.330	0.098	0.0003	-			
7W 'off'	415	0.548	0.499	0.487	0.223	0.026	0.025	-		
7E	47	0.404	0.381	0.327	0.117	0	0	0	-	
8	223	0.568	0.514	0.512	0.265	0.063	0.066	0.009	0.034	-
9	446	0.671	0.601	0.631	0.374	0.115	0.118	0.028	0.093	0.002

Table 4. Sex bias in sample of North Pacific minke whale by subareas and sources of samples (Excel Binomial Test). Pairwise F_{ST} and differentiation calculated in GenePop. Shaded values are not significant.

Area	n	Female	Male	Binomial p	F_{ST}	Differentiation by sex, p
BCK	204	0.34	**0.66**	<0.0001	0.0143	<0.0001
BC6	410	**0.52**	0.48	0.0242	0	0.653
BC2	184	**0.64**	0.36	<0.0001	0.0115	0.012
BC7	212	**0.57**	0.43	0.0086	0.0071	0.017
CK	254	0.28	**0.72**	<0.0001	0	0.419
CS	227	**0.60**	0.40	0.0006	0.0159	0.044
7W 'offshore'	415	0.14	**0.86**	<0.0001	0.0033	0.001
7E	47	0.11	**0.89**	<0.0001	0	0.383
8	224	0.08	**0.92**	<0.0001	0	0.239
9	472	0.09	**0.91**	<0.0001	0	0.151

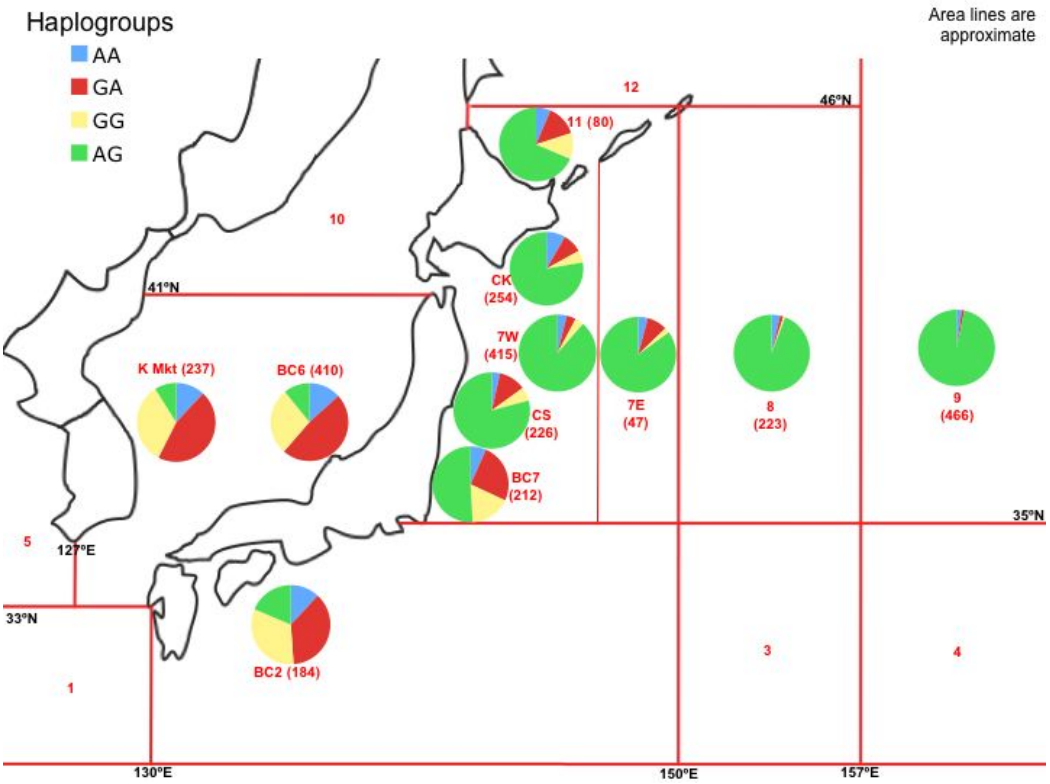

Figure 1. Frequencies of 4 'stock-informative' mtDNA haplogroups for western North Pacific minke whales.

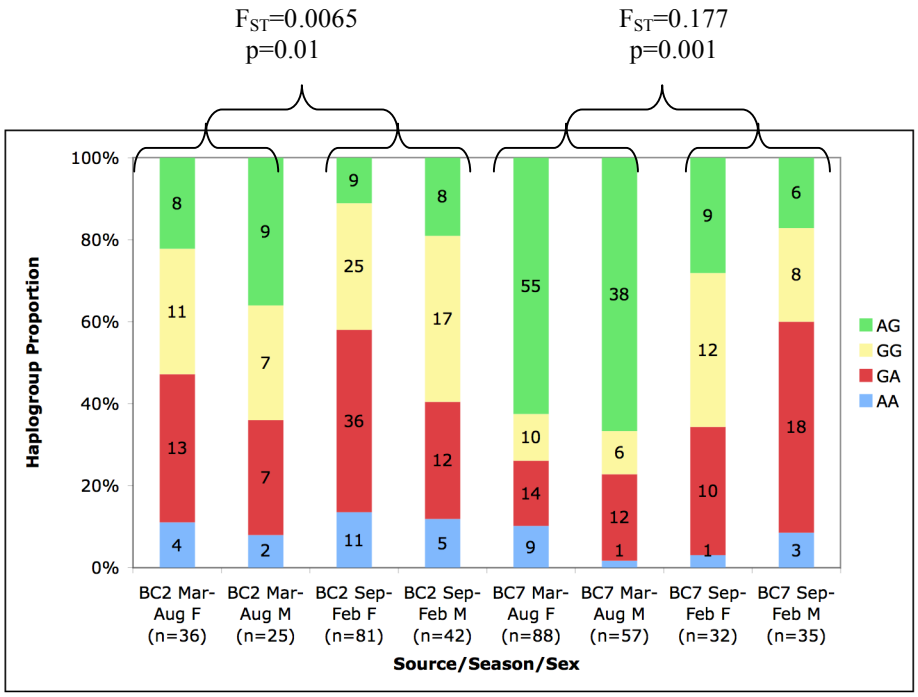

Figure 2. Haplogroup frequencies for bycatch from areas 2 and 7 stratified by season and sex. Pairwise F_{ST} and test of differentiation calculated using Genepop.

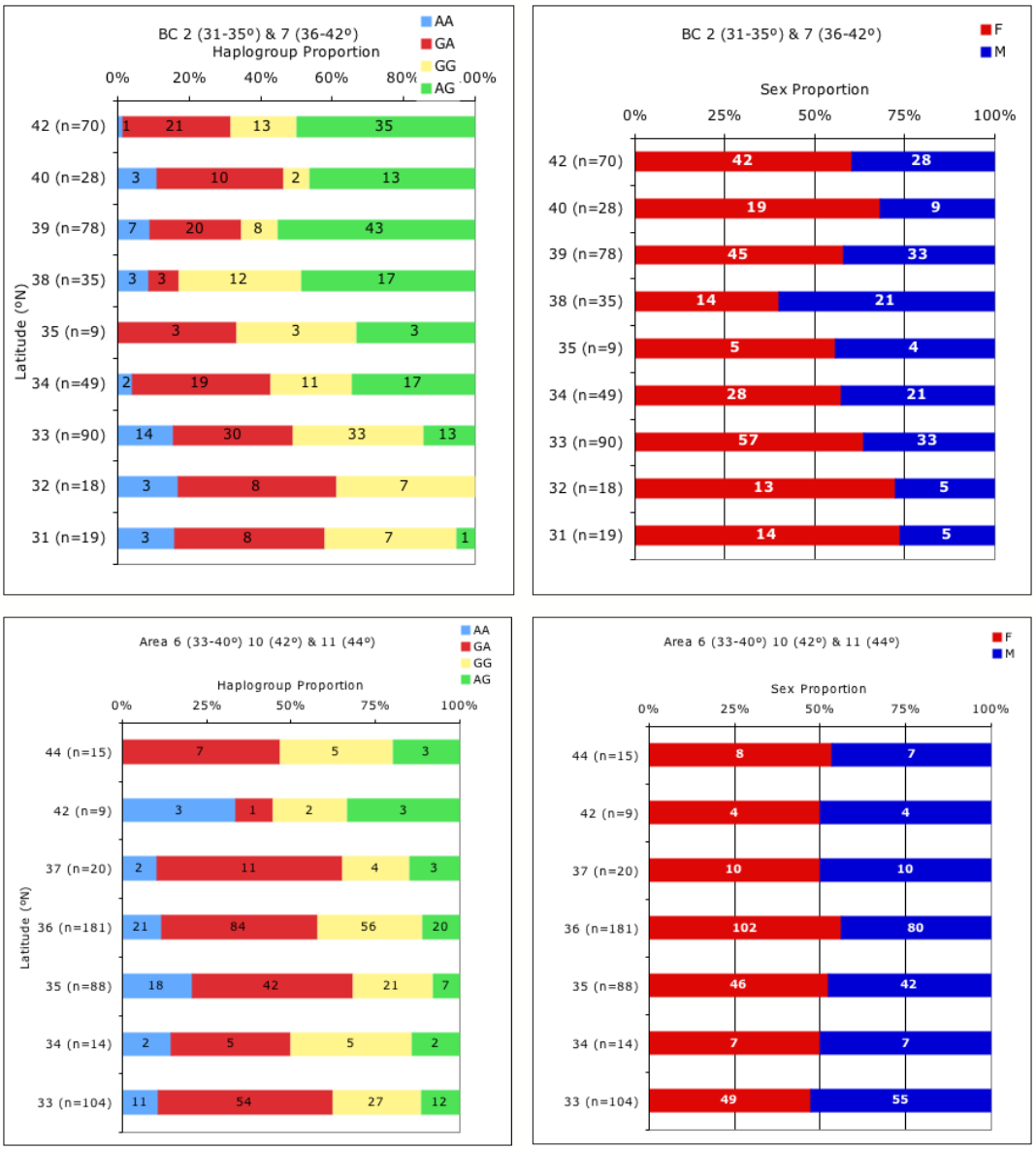

Figure 3. Latitudinal stratification of haplogroup frequencies (left) and sex ratios (right) for bycaught minke whales from subareas 2 and 7 (upper) and 6, 10, & 11 (below).

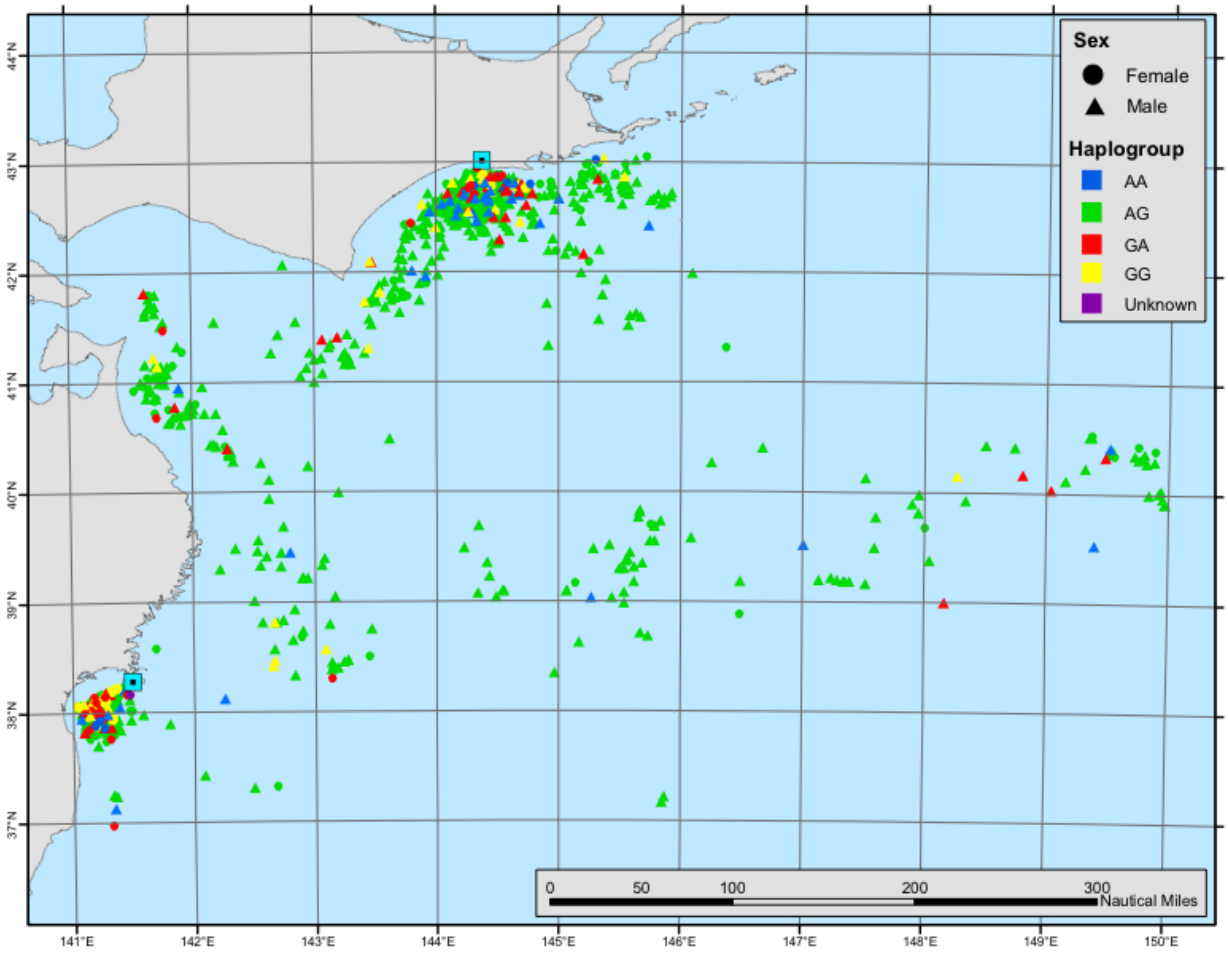

Figure 4. Distribution of 'coastal' and 'offshore' minke whale samples labelled by sex and mitochondrial DNA haplogroup.

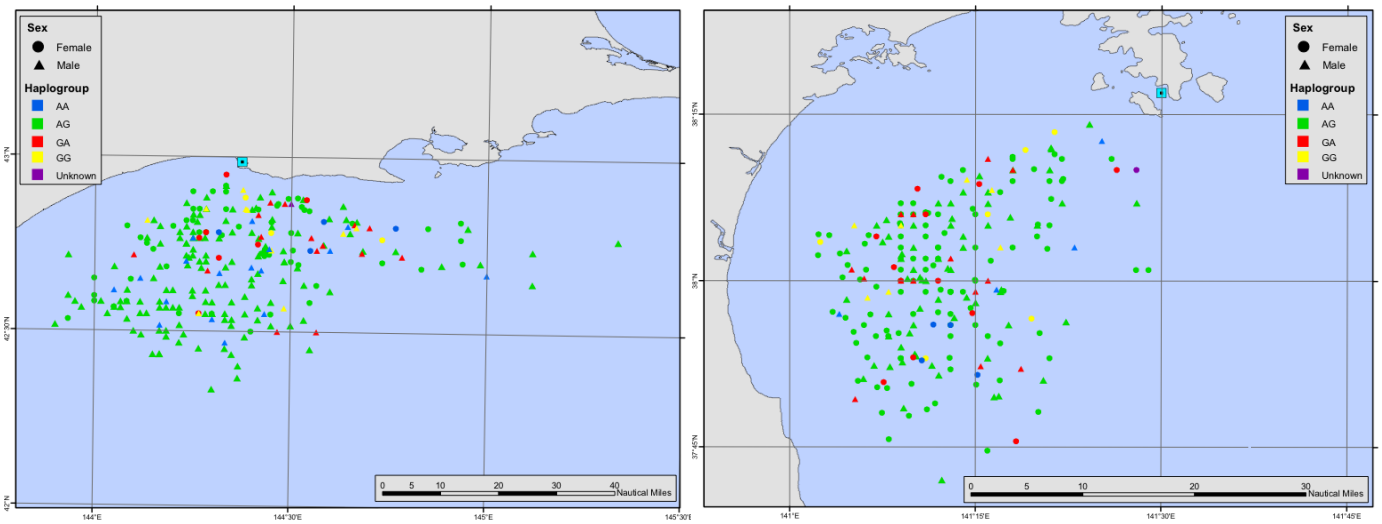

Figure 5. Distribution of 'coastal' minke whale samples from (a)Kushiro and (b) Sanriku labelled by sex and mitochondrial DNA haplogroup.