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Preliminary analyses of population structure of North Pacific minke whales based on Japanese 'bycatch' and scientific whaling

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Summary
Here we update results of previous analyses of population structure in the Western North Pacific minke whale (Baker *et al.* 2011), using information from Japanese 'bycatch' and scientific whaling made available courtesy of the Institute for Cetacean Research of Tokyo (ICR) through the IWC Data Availability Group. The dataset included 16 microsatellite loci, sex and mtDNA haplotypes from 4,707 samples collected from 1994 to 2016. After quality control review, 41 samples representing mtDNA haplotypes found only once (i.e., singletons) were removed for these initial analyses, reducing the sample size to 4,666. The records, representing 11 subareas, were sorted into 9 strata by pooling samples from some adjacent subareas (e.g., subareas 8 and 9) and by delineating bycatch (7bc) from scientific whaling in subareas 7CS and 7CN. Conventional analyses of differentiation (F_{ST}) and modified exact tests showed significant differences between most strata for both microsatellite allele frequencies and mtDNA haplotypes frequencies. In general, the results of these initial analyses were consistent with the predictions of Hypothesis C (formerly III), providing evidence for JE and JW stocks based on significant differences between subareas 6E and 2C, and evidence for OW from OE stocks based on significant differences between 7bc and other subarea 7 strata. A comprehensive analysis of Hypothesis C will require inclusion of standardised genetic markers (microsatellite genotypes and mtDNA haplotypes) from samples around the Korean Peninsula, including the Yellow Sea and Sea of Japan (East Sea).

Introduction

Here we report on preliminary analyses of the population structure of western North Pacific minke whales using records of mtDNA haplotypes and microsatellite genotypes from both bycatch and scientific hunting, made available courtesy of the Institute for Cetacean Research of Tokyo (ICR). The intent of these initial analyses is to contribute to information on plausible stock hypotheses as outlined in preparation for a revised Implementation Review (IR). This is intended to update and extend the findings of the previous IR (IWC 2012). Our analyses rely primarily on exact tests to investigate differences in microsatellite genotypes and mtDNA haplotype frequencies for various strata identified previously as informative of stock structure, as proposed in Hypothesis C (formerly Hypothesis III, Wade and Baker 2012).

Such 'hypothesis testing' is considered the most sensitive approach to identifying populations characterized by moderate differences in haplotype or allele frequencies (i.e., moderate levels of gene flow (Waples and Gaggiotti 2006). However, we note that the power of hypothesis testing is dependent primarily on a sampling design appropriate for the range of plausible hypotheses, as well as sample size and number of genetic markers. This is particularly important for migratory whales, many of which assort annually onto breeding and feeding grounds, with potential for mixing on either seasonal habitat as well as on migratory corridors (Baker *et al.* 2013). Samples available for genetic analysis of the Western North Pacific minke whale have not been collected with such an experimental design. Increasingly, scientific whaling samples have been collected secondarily to other priorities, including operational constraints for

'small-type coastal whaling' programs (IWC 2012). Samples collected from fisheries 'bycatch' are more representative geographically and temporally than those from scientific whaling but are restricted to inshore waters and, as opportunistic samples, not necessarily representative of all age/sex classes. To date, no genetic samples are available from the putative breeding grounds of any stock of North Pacific minke whale and only a small number from the presumed feeding grounds of western stocks in the Sea of Okhotsk. As a consequence, hypothesis testing has been limited, unsatisfactorily, to the nomination of sampling strata from migratory corridors as proxies for 'pure' stocks (Wade and Baker 2012).

Procedural Background of Data Request

We note that a Data Availability request under Procedure A was first submitted to the Data Availability Group (DAG) on 16 January 2018. The DAG deferred action on this Data Availability request at that time, pending a formal initiation of the Implementation Review (IR) at the annual meeting in April 2018. We were then notified by the DAG on 2 October 2018 in regards to our original proposal and asked to resubmit a revised proposal. The renewed request was submitted on 3 October 2018. Further procedural requests and responses carried on until December 2018, after which the data were transmitted on 5 December 2018 with revisions sent on 12 December. Although the initial delay in the procedure was understandable given the delay in the initiation of the IR, we did not expect to have to resubmit the data request after the IR was formally engaged at the annual meeting. This resulted in further delay that has limited our time for analysis.

Methods

Information from Japanese 'bycatch' and scientific whaling was made available electronically in a single dataset on 5 December 2018, courtesy of the Institute for Cetacean Research of Tokyo (ICR) through the IWC Data Availability Group (DAG). The dataset was in a CSV format and titled, 'DataSet_NPminke_WS2018_120518'. A secondary dataset with genetic information on foetuses was also made available but time has not allowed for any review or analyses of these records. Following an initial review of the primary dataset, we noted that the following requested variables were missing: 1) the source of the catch as bycatch (bc), Coastal Kushiro, Coastal Sanriko, or Offshore hunting; 2) prefecture of landing for BC; 3) assignment to 'J', 'O' or 'unknown' stock based on STRUCTURE analysis of genotypes; and 4) probability of assignment to either 'J' or 'O' based on STRUCTURE analysis of genotypes. After notifying the DAG of the missing variables, a supplemental dataset including these requested variables (except for prefecture of landing) was received on 12 December 2018. Given the delays in the procedure for Data Availability requests and the requirement to submit reports of conventional analyses by 3 January 2019, (IWC 2018a), there was no time to communicate with scientists from ICR for any further clarification of quality control of haplotype or genotype data (see below). Consequently, the conventional analyses presented here are considered preliminary.

The CSV file was imported into Excel and then formatted using GenAlex (Peakall and Smouse 2006). The binomial test of frequencies, available in Excel, was used for sex ratios. The program GENEPOP (Rousset 2008) was used for calculation of indices of differentiation (F_{ST}) and test of differentiation (i.e., modified exact test) for mtDNA haplotypes and microsatellite allele frequencies. The program ArcGIS Version 10 was used to plot point locations of bycatch and to generate a 'heat map of the relative density of locations.

Results

Haplotype codes and quality control review

The revised ICR datasets totalled 4,707 samples of North Pacific minke whales collected from scientific whaling or coastal bycatch from 1994 to 2016. All but one of the records included mtDNA control region sequences, haplotype codes, sex and microsatellite genotypes for 16 loci. An initial review indicated that haplotype codes have been entirely revised in the current database, relative to the database made available for the previous Implementation Review (Baker *et al.* 2011). Although this may be internally consistent,

the recoding complicated reference back to the previous analyses and will need to be considered in any effort to reconcile or integrate haplotype information from Korean samples.

As in the previous IR and data availability request, we reviewed the mtDNA haplotypes for potential sequencing error by searching for haplotypes reported for only one individual. In total, the 4,707 samples represented 132 haplotypes, of which 41 were reported for only a single sample. Such a large number of 'singletons' is surprising given the very large sample size and could reflect minor sequencing error, similar to that found in quality control review for the previous IR (Steel *et al.* 2011). Given the limited time available for further review and revision, we chose to delete the records of singletons, reducing the total samples size to 4,666 (Table 1).

Distribution of bycatch samples
Samples provided from the bycatch of whales destined for the commercial market now account for 1,963 (894 males, 1069 females) individuals in the database. To better understand the spatial distribution of the bycatch, we plotted the individual locations of each record (Fig. 2a). As many of the point locations overlap, presumably because of multiple bycatch samples from fixed set nets, we also included a heat map to provide a quantitative view of the bycatch distribution (Fig. 2b). Several 'hot spots' are apparent, especially: 1) Toyama Bay and the Noto Peninsula of the Ishikawa Prefecture (Sea of Japan); the border of the Miyagi and Iwate Prefecture (on the Pacific Coast of northeast Honshu); 3) the coast of Kyoto Prefecture (Sea of Japan); and 4) near Hakodate (southwestern Hokkaido). There are also several apparent gaps in the distribution, particularly the coast of the Ibaraki Prefecture. Further analyses of spatial and temporal patterns of genetic differentiation of bycatch samples could be useful in resolving stock identity.

Genetic differentiation by strata
Review of previous analyses and preliminary investigations of pairwise tests of differentiation from all subareas and sources allowed us to reduce the overall comparison to 9 sample strata (Table 1). For this, we combined adjacent subareas that previously showed no significant differences or were limited by small samples sizes: 6E with 10E, 7WR with 7E, and 8 with 9. Given evidence of previous heterogeneity in coastal samples, we also delineated samples collected from scientific whaling in 7CS and 7CN from those taken as bycatch in these subareas, referred to as 7bc. Note, that the 9 strata used here differ somewhat from the 9 strata used in analysis for the previous IR in that no samples were included from subareas 5 (i.e., Yellow Sea) or 6W (i.e., the eastern coast of the Korean Peninsula).

Overall, the patterns of differentiation for both microsatellites and mtDNA were similar to that reported for the previous IR (Baker *et al.* 2011; Wade and Baker 2012). Of the 36 pairwise comparisons all but 4 were significant for microsatellite alleles frequencies (Table 2) and all but 7 were significant for mtDNA haplotypes (Table 3). Notably, the non-significant differences were for strata that were adjacent geographically (e.g., 7WR/7E with 8/9), or represented by small sample sizes (e.g., 1E with 6E and 2C) or both (e.g., 11 with 7bc). Although we did not formally impose a Bonferroni correction, almost all p values were less than the critical value of 0.0014. There was a strong linear relationship of the F_{ST} values from the pairwise analysis of microsatellites with the pairwise analysis of mtDNA haplotypes ($r^2 = 0.964$, $p < 0.0001$). This suggests that the biparentally inherited microsatellite loci and the maternally inherited mtDNA haplotypes are reflecting the same pattern of differentiation.

Preliminary conclusions
Overall, results of the analyses presented here for the 1994 to 2016 dataset and previously for the subset of samples collected from 1994 to 2007 (Baker *et al.* 2011; Slikas and Baker 2011; Wade and Baker 2012) are consistent with the proposal to recognise 5 stocks, as described in Hypothesis C of the previous Implementation Review,

1) Bycatch from subareas 6E/10E represents the best proxy for the 'JW' stock, a year-round or partially migratory stock in the Sea of Japan. **Genetic Evidence:** Subareas 6E/10E differed for microsatellite and mtDNA haplotype frequencies from all other strata except the adjacent subarea 1E, which is also represented by only a small sample size. Further. Further comparison of subarea 6W with 6E/10E is planned, pending a data request for DNA profiles of Korean bycatch from the Cetacean Research Center (CRC), Korea. The timeline of such a request will depend on progress with standardisation of mtDNA haplotypes and microsatellite allele sizes (see below).
2) Bycatch from subarea 2C represents the best proxy for the 'JE' stock, a year-round or partially migratory stock along the eastern coast of Japan. **Genetic Evidence**: Subarea 2C differs for mtDNA and microsatellite loci from all other strata except the adjacent subarea 1E, which is also represented by only a small sample size. Further analyses are planned to evaluate differences in the frequencies of common shared haplotypes. As previously, such difference would be consistent with the hypothesis of 2 related stocks (e.g., JE and JW), rather than a mixing of two stocks with many unshared haplotypes (e.g., J and O).
3) Bycatch from subarea 7 represents the best proxy for the 'OW' stock. **Genetic Evidence**: The 7bc strata differed from all other strata in both microsatellite and mtDNA haplotypes, except for the adjacent subarea 11, which is represented by a relatively small sample size of mixed sources (i.e., bycatch and scientific whaling).
4) Scientific whaling from subareas 8 and 9 represent the best proxy for the 'OE' stock. **Genetic Evidence:** These two subareas did not differ from each other at either mtDNA haplotypes or microsatellite loci, but do differ from all other subareas or strata, except for the relatively small sample from the adjacent subareas 7E/7WR.
5) Scientific whaling samples from the revised subareas 7CS and 7CN require further analysis as a complex area of mixing of OW and OE stocks. **Genetic Evidence**: With the larger samples sizes from the 1994-2016 dataset, the scientific whaling from the two coastal subareas now differ from each other as well as from 7bc at both microsatellite allele and mtDNA haplotype frequencies. Further analyses are planned for Hardy-Weinberg expectations for potential mixing (Waples 2015) and approximate boundaries between OW and OE.

We cannot yet add any new evidence to the hypothesis of a Yellow Sea stock that is resident year-round in subarea 5. Previous genetic analyses were conflicting, with significant differences in microsatellite loci but not with mtDNA haplotypes (Steel *et al.* 2011). Progress with further genetic analyses of minke whales in Korean waters will require comparison with the Japanese database to standardise codes for mtDNA haplotypes and bin sizes for microsatellite allele frequencies (IWC 2018a). Such a standardised 'DNA register' is a necessary requirement for the comprehensive analyses of plausible stock hypotheses under consideration for the Implementation Review.

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Table 1. Sample strata used in the analyses reported in Tables 2 and 3, including corresponding subareas and sources, and Data Availability Agreement (DAA). Note, 'Without singletons' refers to sample sizes after removal of records representing a mtDNA haplotype found only once in the dataset.

Sample Strata	Subarea	n	without singletons	Source	DAA
1E	1E	76	76	Japanese Pacific coastal bycatch	ICR
2C	2C	372	371	Japanese Pacific coastal bycatch	ICR
6E	6E+10E	1020	1017	Japanese Sea of Japan bycatch	ICR
7(bc)	7CS+7CN	446	443	Japanese Pacific coastal bycatch	ICR
7CS	7CS	684	678	Japanese Pacific coastal and offshore whaling	ICR
7CN	7CN	1049	1037	Japanese Pacific coastal and offshore whaling	ICR
7WR/7E	7WR+7E	138	135	Japanese Pacific offshore whaling	ICR
9/8	9+8	793	782	Japanese Pacific offshore whaling	ICR
11	11	129	127	Japanese Pacific coastal bycatch and whaling	ICR

Table 2: Analysis of population differentiation among subareas (or combined subareas) for the western North Pacific minke whale based on 16 microsatellite loci. Panel A shows the fixation indices (F_{ST}) of each pairwise comparison and panel B shows the probability value based on the exact G test with 10,000 iterations, as calculate with the program Genepop. Bold values are NOT significant. Gray shading shows pairwise comparisons of bycatch from subarea 7bc to all other subareas, including whaling in 7CS and 7CN.

A)

subarea	8/9	11	1E	2C	6E	7CN	7bc	7CS
8/9	----							
11	0.0096	----						
1E	0.0396	0.0097	----					
2C	0.0297	0.0052	**0.0000**	----				
6E	0.0419	0.0105	**0.0002**	0.0019	----			
7CN	0.0010	0.0044	0.0279	0.0198	0.0298	----		
7bc	0.0154	**0.0002**	0.0066	0.0028	0.0067	0.0085	----	
7CS	0.0021	0.003	0.0258	0.0174	0.0261	0.0003	0.0062	----
7E/7W	**0.0000**	0.0118	0.0462	0.0349	0.0484	0.0017	0.0184	0.003

B)

	8/9	11	1E	2C	6E	7CN	7bc	7CS
8/9	----							
11	Highly	----						
1E	Highly	Highly	----					
2C	Highly	Highly	**0.1415**	----				
6E	Highly	Highly	**0.1021**	Highly	----			
7CN	Highly	Highly	Highly	Highly	Highly	----		
7bc	Highly	**0.2702**	Highly	Highly	Highly	Highly	----	
7CS	Highly	Highly	Highly	Highly	Highly	Highly	Highly	----
7E/7W	**0.5937**	Highly	Highly	Highly	Highly	Highly	Highly	Highly

Table 3: Analysis of population differentiation among subareas (or combined subareas) for the western North Pacific minke whale based on mtDNA haplotypes. Panel A shows the fixation indices (F_{ST}) of each pairwise comparison and panel B shows the probability value based on the exact G test with 10,000 iterations, as calculate with the program Genepop. Bold values are NOT significant. Gray shading shows pairwise comparisons of bycatch from subarea 7 to all other subareas, including whaling in 7CS and 7CN.

A)

subarea	8/9	11	1E	2C	6E	7CN	7bc	7CS
8/9	----							
11	0.0272	----						
1E	0.0868	0.0152	----					
2C	0.0664	0.0081	**0.0000**	----				
6E	0.0953	0.0201	**0.0005**	0.0040	----			
7CN	0.0027	0.0117	0.0584	0.0417	0.0661	----		
7bc	0.0393	**0.0000**	0.0093	0.0044	0.0134	0.0209	----	
7CS	0.0064	**0.0065**	0.0490	0.0347	0.0566	0.0008	0.0147	----
7E/7W	**0.0000**	0.0199	0.0772	0.0572	0.0853	**0.0000**	0.0309	**0.0022**

B)

	8/9	11	1E	2C	6E	7CN	7bc	7CS
8/9	----							
11	Highly	----						
1E	Highly	Highly	----					
2C	Highly	Highly	**0.3918**	----				
6E	Highly	Highly	**0.0853**	Highly	----			
7CN	Highly	0.0038	Highly	Highly	Highly	----		
7bc	Highly	**0.9791**	0.0070	Highly	Highly	Highly	----	
7CS	Highly	**0.1526**	Highly	Highly	Highly	Highly	Highly	----
7E/7W	**0.6699**	Highly	Highly	Highly	Highly	**0.3764**	Highly	**0.0683**

Figure 1. Subareas used by IWC for management of western North Pacific minke whales, as currently recognized for Implementation Review (IWC 2018b).

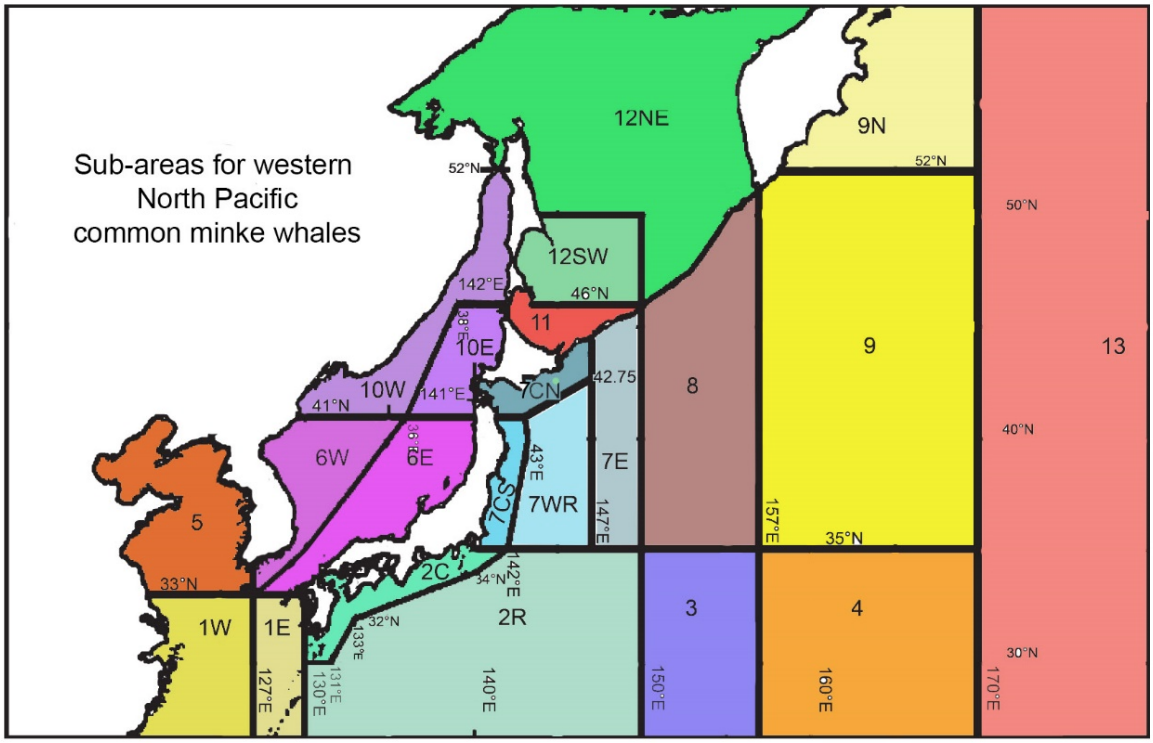

Figure 2. Point locations (Panel A) and raster kernel densities (Panel B) of Japanese bycatch of minke whales plotted in ArcGIS Version 10 using latitude and longitude of available data. Note that many point locations are overlapping. A heat map is included to better reflect relative density of reported bycatch.

Panel A) Panel B)