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Cultural Transmission of Fine-Scale Fidelity to Feeding Sites May Shape Humpback Whale Genetic Diversity in Russian Pacific Waters

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Abstract

Mitochondrial DNA (mtDNA) differences between humpback whales on different feeding grounds can reflect the cultural transmission of migration destinations over generations, and therefore represent one of the very few cases of gene-culture coevolution identified in the animal kingdom. In Russian Pacific waters, photo-identification (photo-ID) studies have shown minimal interchange between whales feeding off the Commander Islands and those feeding in the Karaginsky Gulf, regions that are separated by only 500 km and have previously been lumped together as a single Russian feeding ground. Here, we assessed whether genetic differentiation exists between these 2 groups of humpback whales. We discovered a strong mtDNA differentiation between the 2 feeding sites (F_{ST} = 0.18, Φ_{ST} = 0.14, P < 0.001). In contrast, nuclear DNA (nuDNA) polymorphisms, determined at 8 microsatellite loci, did not reveal any differentiation. Comparing our mtDNA results with those from a previous ocean-basin study reinforced the differences between the 2 feeding sites. Humpback whales from the Commanders appeared most similar to those of the western Gulf of Alaska and the Aleutian feeding grounds, whereas Karaginsky differed from all other North Pacific feeding grounds. Comparison to breeding grounds suggests mixed origins for the 2 feeding sites; there are likely connections between Karaginsky and the Philippines and to a lesser extent to Okinawa, Japan, whereas the Commanders are linked to the Mexican breeding grounds. The

mtDNA differentiation between the Commander Islands and Karaginsky Gulf suggests a case of gene-culture coevolution, correlated to fidelity to a specific feeding site within a particular feeding ground. From a conservation perspective, our findings emphasize the importance of considering these 2 feeding sites as separate management units.

Keywords: cultural transmission, Commander Islands, DNA polymorphisms, feeding grounds, humpback whales, Karaginsky Gulf

The marine realm is generally thought to present few barriers to gene flow (Palumbi 1994), yet there are numerous examples of highly mobile marine mammal species presenting strong intraspecific structures (e.g., Hoffman *et al.* 2009; Mendez *et al.* 2011; Alfonsi *et al.* 2012; Fontaine *et al.* 2014; Louis *et al.* 2014; Decker *et al.* 2017). Among other factors, behavioral traits, including habitat preference, diet specialization, and fidelity to birthplace (philopatry) have all been shown to lead to genetic differences among populations of various marine mammal species (Engelhaupt *et al.* 2009; Foote *et al.* 2011; Louis *et al.* 2014; Alexander *et al.* 2016). Maternal experience can be passed to the offspring during the first months of life, thus leading to specific behaviors, socially transmitted over generations and possibly reflected in the distribution of particular mitochondrial DNA (mtDNA) haplotypes (Kopps *et al.* 2014). Maternally directed fidelity to migration routes, which may include specific breeding sites as well as specific feeding sites, has been demonstrated in various species of whales (Baker *et al.* 1990; Carroll *et al.* 2015). For these whales, calves typically stay with their mothers through the migration to feeding grounds and likely learn the migration route and the feeding ground location (e.g., Baker *et al.* 2013; Carroll *et al.* 2015). This cultural inheritance of migration routes may shape a particular geographical distribution of mtDNA haplotypes, and therefore represents one of the very few cases of gene-culture coevolution identified in the animal kingdom (Whitehead 2017).

The population structure of the humpback whale, *Megaptera novaeangliae*, is certainly complex. The humpback whale is a migratory species with worldwide distribution. Humpback whales breed in warm waters at low latitudes during winter and migrate to high latitudes in order to feed in summer. Within each ocean basin and hemisphere, the great dispersal capabilities of humpback whales (Stone *et al.* 1990; Pomilla and Rosenbaum 2005) could lead to a uniform population. But, in fact, other parameters shape the highly complex genetic structuring of humpback whale populations (Baker *et al.* 2013). In each ocean basin, patchworks of genetically differentiated groups which sometimes geographically overlap can be found. Bettridge *et al.* (2015) recently proposed 15 "distinct population segments" (DPSs) worldwide focusing on breeding grounds but considering also migration pathways, on the basis of genetic and ecological traits and range differences. In the Northern Hemisphere, these DPSs appear to be more relevant and consistent with current knowledge than stock areas defined by the International Whaling Commission (IWC). But in some places an even smaller scale could be envisaged, because some of the DPSs are comprised of whales which feed on different feeding grounds, and that in turn feeding grounds can be comprised of whales from multiple DPSs.

In the North Pacific, and in the framework of the international program SPLASH (Structure of Populations, Levels of Abundance and Status of Humpback Whales, Calambokidis *et al.* 2008), more than 2000 humpback whale skin samples were collected from 10 feeding grounds and 8 breeding grounds distinguished on the basis of previous photo-identification (photo-ID) captures and abundance estimations (Calambokidis *et al.* 2008; Barlow *et al.* 2011). This widespread sample allowed an extensive genetic study, based on nuclear microsatellite and mtDNA polymorphisms, of population structure among both breeding and feeding regions (Baker *et al.* 2013). A highly complex situation was revealed. Tests of differentiation based on mtDNA haplotype frequencies highlighted significant differences among almost all feeding and almost all breeding grounds. Differences were also detected by most pairwise comparisons among feeding and breeding grounds, even in some cases where previous photo-ID studies proved strong migration connections (Baker *et al.* 2013).

These significantly divergent distributions of mtDNA between grounds most probably reflect the maternal fidelity for migration routes (Baker et al. 1993, 2013; Palumbi and Baker 1994). However, the majority of samples in this study came from the eastern and central North Pacific, and only a few were obtained from the western North Pacific in Russian waters. Due to the small number of samples available from these areas, Baker *et al.* (2013) analyzed all samples collected in eastern Russia as a single stratum and did not distinguish between the Commander Islands waters off eastern Kamchatka and the waters around Karaginsky Island, located in northern Kamchatka (Figure 1), separated by approximately 500 km. Working at a finer geographic scale and combining visual observations and stable isotope analysis, Filatova *et al.* (2013) found that feeding behaviors differ between whales from the Commander Islands, feeding on zooplankton, and those in Karaginsky Gulf, feeding on fish. It is notable that similar contrasting feeding behaviors on 2 different trophic levels were also recently found around Kodiak Island in 2 geographically proximate groups of humpback whales (Wright *et al.* 2015). But it is unknown whether these 2 feeding strategies are due to a culturally acquired behavior that may be linked to a genetic divergence or simply to different opportunistic use of the 2 habitats. Photo-ID matches of Karaginsky whales were exclusively found on Asian (Philippines; Okinawa, Japan) breeding grounds, whereas whales from the Commander Islands were matched not only to Asia, but to Hawaii and Mexico (Titova *et al.* 2018). However, it remains unknown whether the divergence identified between these breeding regions (Baker *et al.* 2013) results in genetic differentiation between the 2 eastern Russian feeding areas.

In order to decipher the genetic characteristics of humpback whale populations in the Russian waters of the western Pacific, we have undertaken a fine-scale genetic characterization of humpback whales sampled around the Commander Islands and Karaginsky Gulf. We assess nuclear and mitochondrial DNA differentiation between these 2 feeding sites. We also take advantage of the previous results from the SPLASH program to evaluate at the level of the mtDNA the differentiation or relationships among other North Pacific feeding and breeding ground origins.

Materials and Methods

Sample Collection

Skin biopsy samples were collected from humpback whales on the feeding grounds near the Commander Islands (55°08'N, 166°10'E) and in Karaginsky Gulf (58°55'N, 164°20'E) from 2008 to 2016

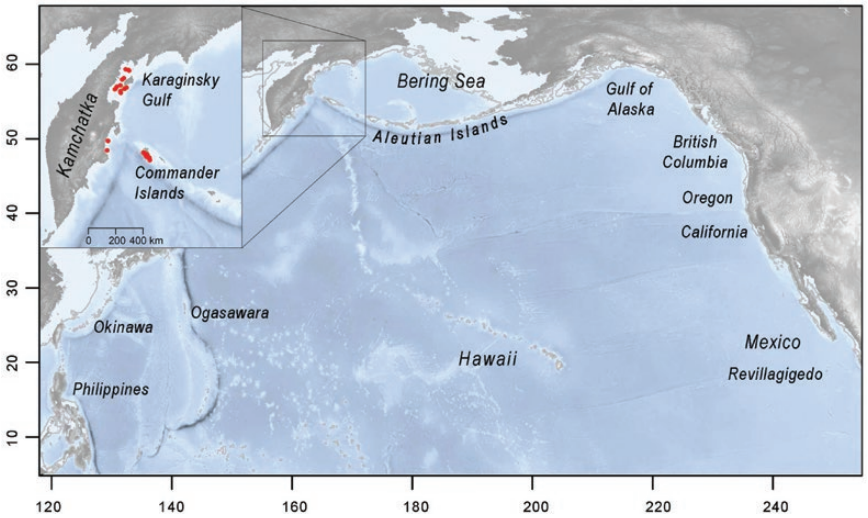

Figure 1. Major feeding and breeding grounds of humpback whales in the North Pacific. Red (dark) points indicate the places where humpback whales were sampled during this study.

(Figure 1). Fieldwork took place each year between May and September in both areas. Biopsies were collected with a crossbow (43 kg strength), which fired a hollow-tipped biopsy dart with a floatable head (Lambertsen 1987). The steel tip on the dart was designed to take a cylinder 0.93 cm^3 and up to 2.4 cm long containing skin and external blubber layers (Lambertsen 1987). During each biopsy, a photograph was taken with a Canon EOS 7D equipped with a 400-mm Canon lens for later individual identification and to prevent repeat sampling. Whales that were evasive were not subjected to repeated attempts to take biopsies. Samples were preserved in ethanol (90%). For each sampling, records were made of the frame number of the picture, the date, location (latitude and longitude), and the whale's reaction to the sampling. Information about the sex of the whale and group composition was also recorded when determined either in the field or later when studying the photo-ID shots.

DNA Extraction

Genomic DNA was isolated from the 2008–2013 biopsy samples using the standard protocol of the *DNeasy Blood and Tissue Kit* (QIAGEN) as described in Alfonsi *et al.* (2012). The genomic DNA concentration and quality were estimated for each extraction by agarose gel electrophoresis and by spectrophotometry using a Nanodrop 1000 (Thermo Scientific). All DNA extracts were then diluted to 5 ng/µL for further amplification. For the 2014–2016 samples, genomic DNA was isolated using KingFisher Flex (Thermo Scientific) magnetic particle processor and InviMag Tissue DNA Kit/KF96 reagent kit (Stratec Molecular) according to the manufacturer's instructions. The overnight lysis of samples was followed by 2 h of additional lysis with extra 25 mL Proteinase K added to each sample. The DNA extracts were directly used for PCR.

Sex Determination

Sex for each sampled whale was identified with a Polymerase Chain Reaction (PCR) co-amplification of the male specific *SRY* region and the *ZFY/ZFX* regions as positive controls, as described in Alfonsi *et al.* (2012).

Microsatellite Genotyping, Descriptive Statistics, and Genotype Analysis

We amplified 8 microsatellite loci: Ev37, Ev96 (Valsecchi and Amos 1996), GATA028, GATA053, GATA417 (Palsbøll *et al.* 1997), GT023, GT211, and GT575 (Bérubé *et al.* 2000). Amplified fragment sizes were determined on an Applied Biosystems 3130 Genetic Analyzer, and analyzed using the GeneMarker software v2.6 (SoftGenetics).

We checked microsatellite data for the presence of null alleles using the program FreeNA (Chapuis and Estoup 2007). Identical genotypes and probabilities of identity (P_{ID}) were determined with the program CERVUS (Kalinowski *et al.* 2007) with mismatches allowed at up to 2 loci out of 8 to avoid false exclusion due to genotyping errors.

Linkage disequilibrium among loci was tested using the program Fstat, V2.9.3.2 (Goudet 2001). The program Fstat (Goudet 2001) was also used to calculate the inbreeding coefficient, F_{IS}, which estimated the departure from the Hardy–Weinberg equilibrium (excess or deficit of heterozygotes). The program Arlequin (Excoffier and Lischer 2010) was used to estimate the level and significance of the genetic differentiation between groups defined *a priori* by calculating F_{ST} (Weir and Cockerham 1984) and R_{ST}. R_{ST} was introduced by Slatkin (1995) as an F_{ST} analogue adapted to microsatellite loci by assuming a high-rate stepwise mutation model. We ran the tests assuming random mating within samples and based on 10 000 randomizations to detect significance.

We used the Bayesian approach implemented in the program Structure, V2.3.4 (Pritchard *et al.* 2000), which partitions multilocus genotypes into clusters, while minimizing departure from the Hardy–Weinberg equilibrium. It allows us to determine if the samples are structured in different genetic groups, without any a priori knowledge of the existence of the groups. We ran the Structure program with 1 to 5 cluster numbers (K = 1–5) and at least 3 independent runs for each K value, using a burn-in period of 100 000 iterations and 1 000 000 Markov Chain Monte Carlo repetitions. We tested ancestry models with and without admixure, and, for both models, correlated or independent alleles.

mtDNA Control Region Sequencing and Sequence Polymorphism Analysis

A section of the mtDNA control region (MCR) of 744 base pairs (bp) ranging from position 15 449 to 16 192 of the complete humpback whale mitogenome (Genbank: AP006467.1) was amplified by PCR using the primers: *L_Dlp1.5/H_Dlp8G* (Garrigue *et al.* 2004).

The PCR products were then purified and sequenced by a commercial sequencing facility (Beckman Coulter Genomics, UK or GATC, Germany). Electropherogrammes were analyzed and edited using SequenceScanner, V1.0 (Applied Biosystem, Foster City, CA, USA). Final sequences were aligned using CLUSTAL W algorithm (Thompson *et al.* 1994) on BioEdit Software (Hall 1999).

The mtDNA sequence data we generated were then combined with the mtDNA sequence data generated in the SPLASH program by Baker *et al.* (2013). These haplotypes overlapped on a 472 bp common region with our data. In a first step, 53 sequences determined on humpback whales sampled near the Commander Islands (n = 17) and in Karaginsky Gulf (n = 36) were added to our data, producing a global dataset of n = 170 sequences (102 from the Commander Islands and 68 from Karaginsky Gulf), sampled over a 12 year period (from 2004 to 2016). A second step consisted of including our sequences into the whole North Pacific dataset determined by Baker *et al.* (2013), thus allowing comparisons between our samples and all the North Pacific breeding and feeding grounds.

Because we were not able to directly compare photo-IDs or microsatellite data between the SPLASH dataset and the 2008–2016 dataset, it is possible that some replicate samples are present between the 2 datasets. We calculated the probability of replicates between the 2 datasets using photo-ID recapture data as follows. During SPLASH, 85 whales were sighted and identified in the Commanders and Karaginsky Gulf, and 57 of these were biopsied. Therefore the probability that a sighted whale was biopsied was 57/85 = 0.67. In 2008–2016, a total of 1448 whales were sighted, including 40 whales identified during SPLASH. Therefore the probability that a whale sighted in 2008–2016 was also sighted in SPLASH was 40/1448 = 0.028, and the probability that a whale sighted in 2008–2016 was biopsied during SPLASH was 0.028*0.67=0.019. A total of 128 whales were biopsied in 2008–2016; assuming a re-sampling rate equal to 0.019, this indicates that approximately 0.019*128=2.4 samples are expected to be replicates between the 2 datasets. This small number of replicates is unlikely to substantially influence the population genetics statistics in this paper.

Descriptive statistics were calculated using the program DnaSP, V.5.10.01 (Librado and Rozas 2009) and Arlequin (Excoffier and Lischer 2010). We determined the numbers of polymorphic sites (S), haplotypes (h), haplotype diversity (H_d), and nucleotide diversity (π). We then compared predefined groups by conducting an χ^2 test, relevant for samples for which $H_d < 1 - \frac{1}{\min(n1;n2)}$ (n_1 and n_2 are the number of individuals in the compared group 1 and group 2, Hudson *et al.* 1992). We also used the nearest neighbour statistic, S_{nn} (Hudson 2000), which measures how often a sequence and its nearest neighbors (i.e., the sequences presenting the lowest number of differences in the dataset) belong to the same studied group. This estimator enabled the testing of data either with low or high haplotype diversity. For 2 highly differentiated groups, S_{nn} is near 1 whereas for 2 undifferentiated groups, S_{nn} is near 0.5 (Hudson 2000). Finally, we used the software Arlequin, V3.5.1.2 (Excoffier and Lischer 2010), in order to calculate fixation index estimators, F_{ST} and Φ_{ST}, analogs for haploid genomes to the F_{ST} calculated from nuclear diploid data (Weir and Cockerham 1984). All the genetic differentiation estimator calculations were performed using a permutation test of 10 000 replicates.

Results

Samples

Table 1 lists all of the 132 skin biopsy samples taken in the Karaginsky Gulf (n_{Kar}=33) and the Commander Islands (n_{Com}=99) from 2008 to 2016. DNA extraction and sex determination were achieved for all of the samples (Table 1). Eight microsatellite loci were amplified and analyzed successfully from almost all samples; genotypes were determined from all but 2 different sample/locus combinations. Identical microsatellite genotypes (all P_{ID} were less than 1.9×10^{-9}) showed that 4 whales were sampled twice in the same year and same locale (in Karaginsky Gulf in 2008, in the Commander Islands in 2009, 2013, and 2016) and that 4 whales were sampled 2 or 3 times around the Commander Islands (see Table 1 for details). No genotypes differing by only 1 or 2 loci were detected among the samples, supporting our conclusion that no other whales sampled several times were undetected because of genotyping errors. The duplicates of the whales from the same field season were removed from all analyses, and duplicates from different field seasons were included if they belonged to different compared groups, i.e., only in chronological analysis using inter-annual comparisons. Duplicates were removed from all other analyses.

Microsatellite Genotyping Analysis and Nuclear Differentiation

The 8 microsatellite loci studied were all polymorphic, with 7 to 15 alleles in the whole samples (Supplementary Table S1). Analysis with FreeNA did not detect evidence of significant frequencies of null alleles (r < 0.05 for all loci) and there was no evidence of linkage disequilibrium in any pairwise comparison of loci (P > 0.05 after Bonferroni correction). The expected heterozygosity calculated as an average for all loci was estimated at 0.76, with a range of 0.49–0.88 for the various loci considered independently (Supplementary Table S1). Overall observed heterozygosity was also determined to be 0.76, with a range between loci from 0.51 to 0.89 (Supplementary Table S1). F_{IS} values of all loci except 2 (GT575, F_{IS} = 0.091, P = 0.024 and GT211, F_{IS} = 0.080, P = 0.037) were not significantly different from zero (Supplementary Table S1). The overall F_{IS} was nonsignificant (F_{IS} = 0.003, P = 0.25, Supplementary Table S1).

We looked for genetic divergence between a priori groups, arranged on the basis of the sex of the animals, their sampling locale, and the years of their sampling, by calculating F_{ST} and R_{ST}. This comparative analysis of allelic frequencies did not reveal significant differences between whales from the Commanders and Karaginsky (F_{ST} = 0.004, R_{ST} = 0.005, both nonsignificant although the F_{ST} P value is <0.1, Table 2). However, a low but significant difference was detected between males and females from the 2 regions (F_{ST} = 0.0034, P = 0.027), although a nonsignificant R_{ST} = 0.006 (P = 0.08) did not confirm this difference (Table 2).

For the interannual comparison, some groups presented a low number of samples (Table 1), and some results must therefore be taken with caution. We compared humpback whales sampled in the Commanders in 2011, in 2013, and from 2014 to 2016, and those sampled in Karaginsky in 2008, 2009, and 2015. Interannual comparisons of humpback whales sampled in both sites revealed no strong temporal variation of allele frequencies. Only the Commanders 2011 and 2013 samples presented a significant F_{ST} value, not confirmed by R_{ST} calculation (F_{ST} = 0.011, P < 0.05; R_{ST} = 0.010, P > 0.05).

Table 1. Summary of samples from Karaginsky and the Commander Islands used in this study

	Year	Samples	Individuals	Males	Females	Microsatellites	MCR sequences
Karaginsky	2004–2006	36[a]	36	16	19	—	36
	2008	9	8[b]	5	3	8	8
	2009	9	9	1	8	9	9
	2015	15	15	11	4	15	15
n_{Kar}		**69**	**68**	**33**	**34**	**32**	**68**
Commanders	2004–2006	17	17	4	13	—	17
	2008	2	2[c]	1	1	2	2
	2009	10	9[d,e]	5	4	9	9
	2010	8	8	3	5	8	8
	2011	17	17[e,f,h]	6	11	17	17
	2012	4	4	1	3	4	4
	2013	36	35[c,e,f,g]	16	17	33	31
	2014	1	1	1	—	1	1
	2015	8	8	6	2	8	7
	2016	13	12[h,i]	3	9	12	11
n_{Com}		**116**	**113(108)**	**46**	**65**	**94**	**107 (102)**
All		**185**	**181(176)**	**79**	**99**	**126 (121)**	**175 (170)**

Humpback whale skin samples were collected near the Commander Islands (55°08'N, 166°10'E) and in Karaginsky Gulf (58°55'N, 164°20'E) from 2008 to 2016. SPLASH samples were collected in 2004, 2005, and 2006. Shown in the table are available samples, numbers of individuals, males and females for each locale and year, and numbers of microsatellite genotypes and MCR sequences determined. n_{Kar} and n_{Com} show the total number of samples for each locale, and numbers in brackets are numbers after removal of the same whales sampled in different years.

a: including one whale of undetermined sex. *b*: one whale was sampled twice in 2008. *c*: one whale was sampled twice in 2008 and 2013. *d*: one whale was sampled twice in 2009. *e*: one whale was sampled 3 times—in 2009, 2011 and 2013. *f*: one whale was sampled in 2011 and 2013, *g*: one whale was sampled twice in 2013, *h*: one whale was sampled in 2011 and 2016, *i*: one whale was sampled twice in 2016

Table 2. Microsatellite differentiation for all groups of samples defined *a priori.*

	Locale	Sex
	Commanders vs. Karaginsky	Male vs Female
Number of samples (N)	N_{Com} = 89 N_{Kar} = 32	$N_{Females}$ = 63 N_{Males} = 58
F_{ST}	0.004 (ns[(1)])	**0.0034***
R_{ST}	0.005 (ns)	0.006 (ns[(1)])

Genetic differentiation of microsatellite loci between *a priori* determined groups of humpback whales was estimated for all pairs of groups by calculating 2 indices, the F_{ST} and R_{ST} using Arlequin (Excoffier and Lischer 2010). Significant values ($P < 0.05$) are in bold, with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. "ns" $P \geq 0.05$. [(1)]$P < 0.1$.

No differences were detected on the basis of microsatellite polymorphisms between the Commanders and Karaginsky samples. The F_{ST} calculation (but not the R_{ST}) detected a low genetic differentiation between males and females.

The possible mixed origins of humpback whales in each locale prompted us to look for the existence of possible unpredicted genetic groups in the Karaginsky and Commanders samples using the Bayesian clustering approach implemented in the program Structure. There was no evidence of cryptic structure for the Commanders samples, for the Karaginsky samples or for both pooled: no specific and reproducible groupings of samples were obtained with or without admixture, assuming correlated alleles or not, for K = 1 to K = 5 (data not shown). The Supplementary Figure S1 shows the posterior probabilities determined from K = 1 to K = 5, highest for K = 1 except under the unrealistic conditions assuming a strong evolutionary separation and no gene flow between the 2 groups (no admixture and independent alleles).

mtDNA Control Region Diversity and Differentiation

We sequenced 653 bp, covering approximately 70% of the mtDNA control region from 117 different individuals (Table 1). We observed 22 variable sites, which defined 16 different haplotypes (15 haplotypes in the Commanders sample and 7 in the Karaginsky sample, Supplementary Table S2, Genbank accession numbers: KU893091–KU893108). We found no deletions or insertions; all of the variable sites were transitions and/or transversions. Among the global sample, we calculated a haplotype diversity of $Hd = 0.80$ and a nucleotide diversity of $\pi = 0.003$.

When the samples were subdivided into different strata (on the basis of locale, sex and the years the animals were sampled in each locale), each group presented haplotype diversity less than $1 - \frac{1}{\min(n1;n2)}$ (Table 3), so we used a χ^2 test in addition to F_{ST}, Φ_{ST} and S_{nn} calculations in order to assess the genetic differentiation between groups (Hudson *et al.* 1992). All 4 indices revealed a highly significant haplotype differentiation between individuals from the Commanders and Karaginsky (Table 3). Nine haplotypes among the 16 were unique to Commanders individuals and one haplotype was unique to Karaginsky individuals (Supplementary Table S2). Conversely, none of the differentiation indices was significant for

Table 3. Summary of the mtDNA diversity within and genetic differentiation between groups defined a priori in the 2008–2016 samples

	Locale	Sex
	Commanders vs. Karaginsky	Male vs. Female
Number of samples (N)	N_{Com} = 85 N_{Kar} = 32	$N_{Females}$ = 60 N_{Males} = 56
Haplotype diversity (Hd)	$H_{d\ Com}$ = 0.74 $H_{d\ Kar}$ = 0.70	$H_{d\ Females}$ = 0.81 $H_{d\ Males}$ = 0.77
Nucleotide diversity (π)	π_{Com} = 0.004 π_{Kar} = 0.002	$\pi_{Females}$ = 0.004 π_{Males} = 0.003
$1-\frac{1}{\min(n1;n2)}$	0.97	0.98
χ^2	**48.4*****	20.46 (ns)
S_{nn}	**0.72*****	0.53 (ns)
F_{ST}	**0.18*****	0.01 (ns)
Φ_{ST}	**0.14*****	0.00 (ns)

Mitochondrial DNA haplotype (Hd) and nucleotide (π) diversities were calculated for each group of humpback whales using the program DnaSP, V.5.10.01 (Librado and Rozas 2009). Genetic differentiation between groups was estimated for all pairs of groups by calculating 4 indices, the χ^2 and S_{nn} (calculated using DnaSP, V.5.10.01, Librado and Rozas 2009), the F_{ST} and Φ_{ST} (calculated using Arlequin, Excoffier and Lischer 2010). Significant values ($P < 0.05$) are in bold, with $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. "ns" $P \geq 0.05$.

All 4 indices indicate a strong genetic differentiation between Karaginsky and Commanders samples.

sex (Table 3) and between years of sampling (data not shown), and therefore there was no genetic differentiation linked to these parameters.

The mtDNA haplotypes identified during this study and those from the SPLASH program (Baker *et al.* 2013) overlapped at 472 bp. The truncated alignment of 472 bp involved the loss of only 1 SPLASH haplotype (haplotypes A+ and A– were no longer distinguished, Supplementary Table S2). Adding the SPLASH samples increased our sample size to n = 68 for Karaginsky Gulf and n = 102 for the Commander Islands (Table 1). All of the haplotypes determined in this study had a 100% identity with one SPLASH haplotype, although some were not previously sampled on the Russian feeding ground (Supplementary Table S2). Haplotype MnBer9_3, which was identical to the haplotypes A+/A– of Baker *et al.* (2013) for the truncated alignment, was the most represented in the Commanders samples (it was found in 46% of the samples). That was followed by haplotype MnBer9_2, identical to E1 (18% of the samples). The same MnBer9_2/E1 haplotype accounted for 50% of the Karaginsky samples, followed by haplotype MnBer8_2, identical to E6 (24% of the Karaginsky samples, Supplementary Table S2). This extended dataset provided results consistent with the ones obtained with the 2008–2016 only samples and confirmed the genetic differentiation observed between Karaginsky and the Commanders (Supplementary Table S3).

For the combined datasets, all 4 indices calculated were highly significant ($P < 0.001$) between the 2 locales, whereas all other calculations (between males and females, between years) were nonsignificant.

mtDNA Differentiation With Other Feeding Grounds

Pairwise comparisons of Commanders DNA haplotypes with those of all other Pacific feeding grounds, as represented in SPLASH (Table 4), provided highly significant differentiations with 5 out of 9 regions: Northern Gulf of Alaska (NGOA), Southeast Alaska (SEA), Northern British Columbia (NBC), Southern British Columbia–Washington (SBC-WA) and California-Oregon (CA/OR) (all P values < 0.001 for S_{nn} and χ^2 tests, and <0.05 for F_{ST} and Φ_{ST}, Table 4). No differences between the Commanders and Western Gulf of Alaska (WGOA) and Western Aleutians (WAL) were detected (all indices nonsignificant), and only Φ_{ST} detected a low but significant difference between the Commanders and the Eastern Aleutians (EAL). Bering Sea (BS) and the Commanders comparison provided significant values of differentiation for S_{nn}, Φ_{ST}, and χ^2 tests (Table 4).

Karaginsky samples were highly differentiated from all other feeding grounds (Table 4); all 4 indices of differentiation were significant for all pairwise comparisons, except for the WAL (F_{ST} and S_{nn} nonsignificant). The highest differentiations were observed between Karaginsky and NGOA, SEA, NBC (Table 4).

It is important to note that for all pairwise comparisons with the North Pacific feeding grounds, all the differentiation indices had higher values for Karaginsky Gulf than for the Commander Islands.

mtDNA Differentiation With Breeding Grounds

Commanders samples were differentiated from most North Pacific breeding grounds except Mexican (P values of all F_{ST} < 0.001, Table 5). The 3 Mexican breeding grounds were a notable exception, with Mexico-Revillagigedo and the Mexico mainland grounds not differing for F_{ST} (Table 5). The Karaginsky sample showed significant differentiation from all breeding grounds except the Philippines (PHI) (which has only a small sample size, Table 5). Indices of differentiation were highly significant (all F_{ST} and Φ_{ST} P values < 0.001), except for Okinawa (OK), Japan, which was weak but significant (Table 5).

Discussion

Our study is one of the first exploring humpback whales in their Russian feeding grounds from a genetic point of view. During the SPLASH study, the areas surrounding the Commander Islands and Karaginsky Gulf were thought to be hosting different humpback whales, because of the lack of photo-ID recapture between the 2 sites (Calambokidis *et al.* 2008). But the samples from multiple Russian sites were pooled due to the low size and revealed a significant divergence between the Russian and most of other North

Table 4. Pairwise differentiation indices (F_{ST}, Φ_{ST}, χ^2, and S_{nn}) calculated among the 2004–2016 Commanders and Karaginsky samples and all North Pacific feeding grounds

	Western Aleutians (WAL)	Bering Sea (BER)	Eastern Aleutians (EAL)	Western Gulf of Alaska (WGOA)	Northern Gulf of Alaska (NGOA)	Southeast Alaska (SEA)	Northern British Columbia (NBC)	Southern British Columbia - Washington (SBC-WA)	California-Oregon (CA/OR)
Number of samples (*N*)	N_{WAL} = 8	N_{BER} = 114	N_{EAL}= 36	N_{WGOA} = 96	N_{NGOA} = 233	N_{SEA} = 183	N_{NBC} = 104	N_{SBC-WA} = 51	$N_{CA/OR}$ = 123
					versus Commander (N_{Com} = 102)				
χ^2	7.83 (ns)	**34.89****	16.52 (ns)	22.16 (ns)	**74.18*****	**118.18*****	**73.06*****	**43.12*****	**95.89*****
S_{nn}	0.85 (ns)	**0.55*****	0.63 (ns)	0.52 (ns)	**0.65*****	**0.73*****	**0.67*****	**0.65*****	**0.69*****
F_{ST}	0 (ns)	0.01 (ns)	0.01 (ns)	0 (ns)	**0.04 *** **	**0.31 *** **	**0.22 *** **	**0.02 ***	**0.12 *** **
Φ_{ST}	0 (ns)	**0.04****	**0.05****	0 (ns)	**0.03****	**0.15*****	**0.09*****	**0.02***	**0.26*****
					versus Karaginsky (N_{Kar} = 68)				
χ^2	**30.82*****	**108.29*****	**68.93*****	**77.82*****	**194.28*****	**231.64*****	**152.11*****	**76.94*****	**96.5*****
S_{nn}	0.81 (ns)	**0.79*****	**0.81*****	**0.72*****	**0.86*****	**0.96*****	**0.93*****	**0.78*****	**0.75*****
F_{ST}	0.09 (ns)	**0.22*****	**0.22*****	**0.16*****	**0.33*****	**0.69*****	**0.59*****	**0.23*****	**0.15*****
Φ_{ST}	**0.18***	**0.22*****	**0.27*****	**0.15*****	**0.21*****	**0.60*****	**0.48*****	**0.16*****	**0.34*****

Genetic differentiation of mitochondrial DNA between regional feeding groups and, respectively, Commanders and Karaginsky samples was estimated for all pairs of groups of humpback whales by calculating 4 indices, the F_{ST} and Φ_{ST} (calculated using Arlequin, Excoffier and Lischer 2010) and the χ^2 and S_{nn} (calculated using DnaSP, V.5.10.01, Librado and Rozas 2009). Significant values ($P < 0.05$) are in bold, with $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. "ns" $P \geq 0.05$.

n: number of individuals in each sample.

Commanders samples presented no differentiations with WAL and WGOA, a low differentiation with BER and EAL, and higher differentiations with all other feeding regions (all indices highly significant). Karaginsky samples were highly differentiated from all other feeding area excepted WAL (Φ_{ST} significant).

Table 5. Pairwise differentiation indices (F_{ST}, Φ_{ST}, χ^2, and S_{nn}) calculated among the 2004–2016 Commanders and Karaginsky samples and all North Pacific breeding grounds

	Philippines (PHI)	Okinawa (OK)	Ogasawara (OG)	Hawaii (HI)	Mexico-Revillagigedo (MX-AR)	Mexico-Baja California (MX-BC)	Mexico-Mainland (MX-ML)	Central America (CENAM)
Number of samples (*N*)	N_{PHI} = 13	N_{OK} = 72	N_{OG} = 159	N_{HI} = 227	N_{MX-AR} = 106	N_{MX-BC} = 110	N_{MX-ML} = 62	N_{CENAM} = 36
					versus Commander (N_{Com} = 102)			
χ^2	**32.97****	**54.89*****	**48.79*****	**83.14*****	**28.13***	**38.50****	29.47 (ns)	**66.57*****
S_{nn}	**0.84****	**0.63*****	**0.59*****	**0.66*****	**0.54****	**0.56*****	**0.57***	**0.78*****
F_{ST}	**0.16*****	**0.15*****	**0.04*****	**0.09*****	0.01 (ns)	**0.01***	0.01 (ns)	**0.17*****
Φ_{ST}	0.02 (ns)	**0.11*****	**0.03****	**0.04****	0 (ns)	**0.02***	**0.06*****	**0.32*****
					versus Karaginsky (N_{Kar}=68)			
χ^2	15.22 (ns)	**23.02***	**59.85*****	**228.27*****	**82.86*****	**81.27*****	**63.79*****	**56.08*****
S_{nn}	0.73 (ns)	**0.55****	**0.67*****	**0.90*****	**0.73*****	**0.72*****	**0.71*****	**0.76*****
F_{ST}	0.02 (ns)	**0.02***	**0.08*****	**0.43*****	**0.14*****	**0.13*****	**0.14*****	**0.12*****
Φ_{ST}	0.04 (ns)	**0.03****	**0.06*****	**0.25*****	**0.14*****	**0.13*****	**0.18*****	**0.43*****

Genetic differentiation of mitochondrial DNA between regional breeding groups and, respectively, Commanders and Karaginsky humpback whales was estimated for all pairs of groups by calculating 4 indices, the F_{ST} and Φ_{ST} (calculated using Arlequin, Excoffier and Lischer 2010) and the χ^2 and S_{nn} (calculated using DnaSP, V.5.10.01, Librado and Rozas 2009). Significant values ($P < 0.05$) are in bold, with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. "ns" $P \geq 0.05$.

n: number of individuals in each sample.

Commanders samples presented the lower differentiations with the Mexican breeding grounds, particularly MX-AR and MX-ML, while Karaginsky presented no differentiation with PHI and low differentiation with OK.

Pacific feeding and breeding regions (Baker *et al.* 2013). The only regions which had no mitochondrial genetic differences from the pooled Russian sample set were WAL (Western Aleutians) among feeding regions and PHI (the Philippines) among breeding regions (Baker *et al.* 2013). Here, we provide analysis on a finer scale with a larger sample size, allowing us to distinguish between 2 feeding sites within the Russian feeding ground spaced about 500-km apart. We observed a significant divergence in maternal lineage between the 2 feeding sites. Thus, we offer new pieces of evidence for the already demonstrated fidelity of humpback whales to their feeding sites in the North Pacific, as revealed by genetic differences between groups in agreement with the observation of Baker *et al.* (2013). A key finding of our study is that the scale of feeding ground fidelity identified here is smaller than what has been documented in the North Pacific

(Baker *et al.* 2013). This finer geographical scale suggests a strong conservation impact of our study following the identification of the unique local stock in Karaginsky Gulf.

A Fine-Scale Fidelity to Feeding Sites, Possibly Culturally Transmitted

The analysis of microsatellite polymorphism did not detect strong genetic differences between humpback whales feeding in the 2 study sites (F_{ST} = 0.004, P < 0.1). When samples from the 2 feeding sites were combined, microsatellite polymorphisms met the expectation of the Hardy–Weinberg equilibrium. A moderate differentiation exists between males and females (F_{ST} = 0.0034, P < 0.05, R_{ST} = 0.007, P < 0.1), regardless of locale and years considered. Analysis of mtDNA haplotypes did not reveal any differentiation between males and females.

In contrast, a highly significant mtDNA difference was indicated by all 4 calculations (F_{ST}, Φ_{ST}, χ^2, and S_{nn}) between humpback whales sampled at the 2 feeding sites. Such mitochondrial divergences have often been explained, at larger geographic scales, by the fidelity of humpback whales to feeding grounds (i.e., Baker et al. 1990, 1993, 2013, Palsbøll *et al.* 1995; Larsen *et al.* 1996), and most likely represent a case of gene-culture coevolution in whales (Baker *et al.* 2013; Whitehead 2017).

Humpback whales show different feeding strategies in the 2 studied feeding sites. In the coastal waters around Karaginsky Island, they forage most probably on schooling fishes, while they eat more euphausiids off the Commander Islands (Filatova *et al.* 2013). Yet humpback whales are known to be opportunistic predators, consuming available prey (Filatova *et al.* 2013). Thus, the fidelity for each site highlighted in our study may be linked to a particular migratory destination rather than to foraging preference. Here, we suggest that the significant mtDNA difference between Karaginsky and the Commanders may be representative of a maternal lineage differentiation between 2 feeding sites on what has been considered the same feeding ground.

The average length of time of lactation for humpback whales was first estimated at 10.5 months by Chittleborough (Chittleborough 1958) with some observations suggesting that the separation between calf and mother happens after the return to the breeding ground (Baker *et al.* 1987). Even though Baraff and Weinrich (1993) observed that the separation can occur already on the feeding ground, calves may learn the migration route from their mother on their first trip from the calving ground to the feeding ground. The mtDNA differentiation between Karaginsky and the Commanders could therefore be explained by a cultural transmission of the migration route leading to a fidelity over generations to a specific feeding site within a particular feeding ground. Other studies suggest that similar divisions of humpback whale groups can also occur in other regions of the North Pacific (Wright *et al.* 2015). Fidelity to specific feeding sites at small geographic scales, sometimes linked to specific culturally transmitted feeding behaviors, may be more common than previously assumed for humpback whales.

The absence of differentiation of microsatellite allele distribution between Karaginsky Gulf and the Commander Islands highly contrasts with mtDNA haplotype differences between the 2 locations. Baker *et al.* (2013) already observed a lower level of nuDNA differentiation when compared to mtDNA between North Pacific breeding grounds, which could be at least partially explained by a male-mediated gene flow. In addition, the mixed origins of Karaginsky and Commanders humpback whales are demonstrated by the comparison of mtDNA haplotype distributions with North Pacific breeding grounds (see later). These mixed origins, including most likely common Asian breeding grounds as suggested also by photo-ID studies (Titova *et al.* 2018), when added to the low nuDNA differentiation among North Pacific breeding grounds (Baker *et al.* 2013), could explain the absence of nuDNA differentiation between Karaginsky Gulf and the Commander Islands.

No significant temporal variations were observed, although the samples have been taken over a 13-year period (2004–2016) including the data from the SPLASH program. We observed a low and slightly significant nuclear difference between individuals sampled in the Commanders in 2011 and in 2013 (only for F_{ST}, R_{ST} was nonsignificant). This suggests that a temporal effect might exist. As whales from different breeding grounds utilize this feeding ground, it is possible that the difference is driven by variation in the proportion of sampled animals from different breeding grounds in these 2 years. More research could clear up this uncertainty.

Comparison Between Karaginsky Gulf, the Commander Islands and the Other North Pacific Feeding and Breeding Grounds

The availability of mtDNA haplotypes derived for the entire North Pacific during the SPLASH program allowed the comparison of mtDNA haplotype distributions at a large geographic scale. This large-scale comparison has reinforced the differences between the Commander Islands and Karaginsky Gulf.

The Commander samples appeared closely related to the Western Aleutians (WAL), Eastern Aleutians (EAL), and Western Gulf Of Alaska (WGOA) feeding grounds. No significant differentiation indices were calculated with these 3 feeding grounds. Higher levels of differentiation were determined for all northeast Pacific feeding grounds from Northern Gulf Of Alaska (NGOA) to California-Oregon (CA/OR).

Commander samples were similar to WAL also in terms of the feeding behavior revealed through the stable isotope analysis. WAL samples had the lowest $\delta^{13}C$ and $\delta^{15}N$ values of all North Pacific feeding grounds (Witteveen *et al.* 2009). The samples from the Commander Islands, when analyzed separately from other Russian feeding grounds, also showed similarly low values, indicative of pelagic low trophic level prey (most likely euphausiids, Filatova *et al.* 2013). This indicates that the whales from the Western Aleutians and the Commander Islands have similar feeding and habitat preferences, which are likely to be culturally transmitted. These preferences may reduce the number of exchanges between coastal and offshore feeding areas (e.g., Karaginsky Gulf and the Commander Islands), providing partial separation between regions that are geographically close but ecologically distinct. On the other hand, they might stimulate exchanges between the Commander Islands and the Western Aleutian Islands, which are situated at the same distance from each other as the Commanders and Karaginsky Gulf, but have similar habitat. In combination with the genetic similarity, this suggests that the Commander Islands and the Western Aleutian Islands likely comprise a single feeding ground.

The Commanders were significantly different (P < 0.01) from most North Pacific breeding grounds, but weaker or nonsignificant levels of differentiation were calculated for comparisons with the Mexican grounds, particularly with Mexico-Revillagigedo (MX-AR) and Mexico-Mainland (MX-ML). The higher values of differentiation indices were determined with Okinawa (OK), Hawaii (HI) and Central America (CENAM). As already demonstrated by Baker *et al.*

(2013), these observations confirm that, in the North Pacific, there is not always a direct correlation between the geographical distance from a feeding to a breeding ground and humpback whale genetic similarity in the 2 grounds (i.e., Mexican mainland breeding grounds are more distant to the Commander Islands than HI and OK).

Karaginsky samples appeared highly differentiated from almost all other North Pacific feeding grounds, with differentiation indices having higher values and lower *P* values in all pairwise comparisons than those performed with the Commanders samples. Comparisons with WAL presented the lower indices values, but the low number of WAL samples (n = 8) precludes drawing a clear conclusion. Karaginsky samples are highly differentiated from all North Pacific breeding grounds except 2. The higher differences occurred with HI, CENAM, and the 3 Mexican mainland grounds (contrasting greatly with Commanders samples). Indices of low values, less significant, were determined with the OK breeding ground, not with Ogasawara (OG). No significant differentiation was detected with PHI (all indices nonsignificant), but caution must be taken because of the low number of samples (n = 13), although χ^2 and S_{nn} have been shown to be powerful indicators for low number samples (Hudson *et al.* 1992; Hudson 2000).

It can be noted that the 4 differentiation indices calculated between Karaginsky and the Commanders have values similar to the ones determined for more distant feeding grounds. For instance, the F_{ST} calculated between Karaginsky and the Commanders is in the same range as between each of the 2 areas and CA/OR ($F_{ST\ \text{KAR-COM}}$ = 0.18; $F_{ST\ \text{COM-CA/OR}}$ = 0.12; $F_{ST\ \text{KAR-CA/OR}}$ = 0.15, all 3 $P < 0.001$). The mtDNA difference that we determined between the 2 sites is therefore linked to a genetic distinction between 2 groups of humpback whales that can be compared to differences on a much larger geographic scale between the major grounds of the North Pacific.

With the exception of PHI and Karaginsky, all pairwise comparisons between our study sites and all the North Pacific breeding grounds have at least 2 differentiation indices with significant values (among the 4 calculated). Karaginsky Gulf and the Commander Islands appear therefore to represent mixed feeding sites, where humpback whales from different breeding grounds mix. If the low number of PHI samples (n = 13) does not preclude the detection of an actual genetic differentiation, the absence of differentiation could nevertheless indicate a direct link between Karaginsky and PHI, as it is the case in the northeast Pacific between CENAM and CA-OR (Baker *et al.* 2013). But the relatively low values of differentiation indices between Karaginsky and OK also suggest the existence of some migratory connections between the 2 sites, which is in good agreement with recent photo-ID matches (Titova *et al.* 2018). The Commanders samples appear to be more closely linked to the Mexican grounds, particularly MX-AR and MX-ML. In contrast to mtDNA, nuDNA polymorphisms revealed very low genetic differentiation between Karaginsky and the Commanders, significant only at $P < 0.1$. It can therefore be hypothesized that humpback whales from different breeding grounds could share fidelity, regularly coming to both of the feeding sites. PHI and OK could represent good candidates for these breeding grounds.

Implications for Management

Baker *et al.* (2013) discussed the importance of considering feeding regions when defining units to conserve, in particular because of the maternally inherited traditions of migration, illuminated by the divergence of mtDNA, leading to distinctive cultural groups. Filatova *et al.* (2013) also proposed, on the basis of different ecological characteristics, the existence of 2 different feeding sites for humpback whales in the areas surrounding the Commander Islands and Karaginsky Gulf. We have reinforced this proposal by adding a genetic basis to the ecological and behavioral differences. We have identified fidelity to specific feeding sites within what has been considered the same feeding ground, reflected in mtDNA polymorphisms, and therefore hypothetically due to a cultural transmission. We have revealed that the maternal lineage divergence between the 2 Russian sites was of similar magnitude as the mtDNA divergences observed by Baker *et al.* (2013) between feeding regions (i.e., feeding grounds). Conservation policies should take this diversity into account, to preserve feeding grounds to the same extent as breeding grounds, since the loss of a feeding ground, even partial, would also correspond to the loss of genetic heritage, of a particular culturally transmitted behavior, and maybe the loss of optimal habitat (Baker *et al.* 2013).

In the Bering Sea, some humpback whale feeding grounds (including Karaginsky Gulf) overlap with potentially detrimental human activities including shipping routes and fishing activities. Humpback whales will likely be exposed to increasing anthropogenic disturbances in the future, elevating conservation concerns (Van Waerebeek *et al.* 2007; Alter *et al.* 2010; Smith and Stephenson 2013; Christiansen *et al.* 2014). The Commander Islands have been largely protected since 1993 within the Commander Islands *zapovednik* (IUCN Category Ia) in order to conserve the birds and marine mammals in these waters. This marine protected area was then classified as the Commander Islands State Biosphere Reserve in 2002 (Hoyt 2011). In contrast, Karaginsky Gulf is not a marine protected area; there is considerable fishing activity and potential hydrocarbon development, as well as shipping activity. Nevertheless, our study demonstrated that Karaginsky humpback whales are distinct from whales in all other North Pacific feeding areas and may represent a fairly high proportion of the Philippines/Ogasawara DPS. They should therefore deserve specific attention, and we highlight the potential value of extending habitat protection to Karaginsky Gulf. Being the second largest feeding area in the Russian Pacific and exclusively hosting the whales from the small Asian population, this region is a good candidate for a new marine protected area.

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Data Accessibility

We have deposited the primary data underlying these analyses as follows: Mitochondrial DNA sequences: Genbank accessions KU893091–KU893108.

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