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ABSTRACT

Here we update our progress on building a mitochondrial DNA (mtDNA) database and developing a useful panel of single nucleotide polymorphisms (SNPs) for bowhead whales (*Balaena mysticetus*). We utilize the SNP panel to investigate several aspects of population genetics for the BCB and Okhotsk populations, including Fst and heterozygosity. Results of these analyses are comparable to what has previously been found with mtDNA and microsatellites. Additionally, the SNP panel provides sufficient resolution to fingerprint individual whales. Currently, the mtDNA database contains sequences from 3 loci: cytochrome-b (474 individuals), ND1 (465 individuals) and the HVR1 portion of the control region (695 individuals). 368 individuals have complete data for all three loci.

INTRODUCTION

Genetic data have played a key role in conservation studies, including those related to bowhead whales. Our research group has been involved in long-term acquisition of mitochondrial DNA (mtDNA) to examine stock structure and effective population size estimates. Recent studies have incorporated three mtDNA loci: the control region, ND1, and cytb. The addition of two protein-coding genes to the highly variable control region data has increased the ability to resolve relationships and allowed more sophisticated analyses to be conducted (e.g. Phillips et al. 2012; Bickham et al. 2012).

Single Nucleotide Polymorphisms (SNPs) have successfully been utilized in evolutionary and population genetic studies across a wide variety of organisms, including non-model organisms (Helyar et al. 2011). They have been used to investigate population structure, phylogenetic relationships, historical demography, and many other population- and species-level characteristics. Specifically in whales, SNPs have been identified for bowheads (e.g. Morin et al. 2010; Baird et al. 2015), sperm whales (Morin et al. 2007), humpback whales (Schmitt 2013), and gray whales (DeWoody et al. 2016).

Because SNPs are analyzed by their sequence, they have a distinct advantage over the most commonly used bi-parentally inherited nuclear markers, microsatellites, which are analyzed by estimation of their fragment size. Using certain methods for SNP genotyping, they can be more easily reproduced among different labs and thus a public database can be established and built upon, study by study, as is presently done for mtDNA. Morin et al. (2012) compared the relative statistical power of SNPs and microsatellites. They concluded that a panel of 29 (42 linked and unlinked SNPs) bowhead SNP loci provided similar power as compared to a panel of 22 microsatellites in their ability to detect low levels of differentiation (Fst=0.005-0.03) among bowhead populations when sample sizes were at least N=20 per population. The microsatellite panel performed better when used for estimates of *Ne* and for assignment tests.

Baird et al. (2015) reported the results of their trials to identify an expanded panel of single nucleotide polymorphisms (SNPs) for bowhead whales (*Balaena mysticetus*) and to determine the most reliable and replicable method to genotype whales for the identified SNPs. They reported that a panel of 96 SNPs had been developed specific to bowheads, and that their preferred method of genotyping would be the Fluidigm SNPtype assay.

Here, we update progress on utilizing the Fluidigm platform to genotype bowheads from the BCB and Okhotsk stocks. We also update the progress of building a mitochondrial DNA (mtDNA) database for bowheads. We utilize the SNP data to update population genetic analyses of bowhead whales.

Obtaining robust databases of mtDNA and SNP data will improve our ability to continue to address issues relating to bowhead stock structure. Additionally, in future studies we plan to use these data to estimate genetic interchange between BCB and Canadian or Okhotsk populations and revisit estimates of historical demography. A complete understanding of these issues will serve a critical purpose for the IWC in its quota recommendations.

METHODS

mtDNA sequence data.—Bowhead whale DNA was extracted, amplified, and sequenced using the methods presented in LeDuc et al. (2008) and Phillips et al. (2011). Three mitochondrial loci were used: the hyper variable region-1 (HVR1) of the mtDNA control region, the complete cytochrome-b (cytb) gene and the complete ND1 gene. These resulted in sequence lengths of 397bp, 1140bp, and 957bp, respectively. Acquired sequences were compared to existing haplotypes from previous studies deposited in GenBank and a haplotype code was assigned to each individual.

Single nucleotide polymorphisms (SNPs).—Baird et al. (2015) outlined the procedures used to select a panel of 96 SNPs specific to bowheads. This SNP panel contained a combination of SNPs identified by Morin et al. (2010) and loci reported in Baird et al. (2014). Here, we used the Fluidigm SNPtype assay to genotype 285 whale samples for the 96-SNP panel reported in Baird et al. (2015). Of these, 252 samples were from the BCB stock and 33 were from the Okhotsk stock. Fluidigm's BioMark software was used initially to automatically call genotypes. Editing was then conducted by eye (Fig. 1). If no clear distinction among genotypes could be discerned for a particular locus, that locus was discarded for final data analysis.
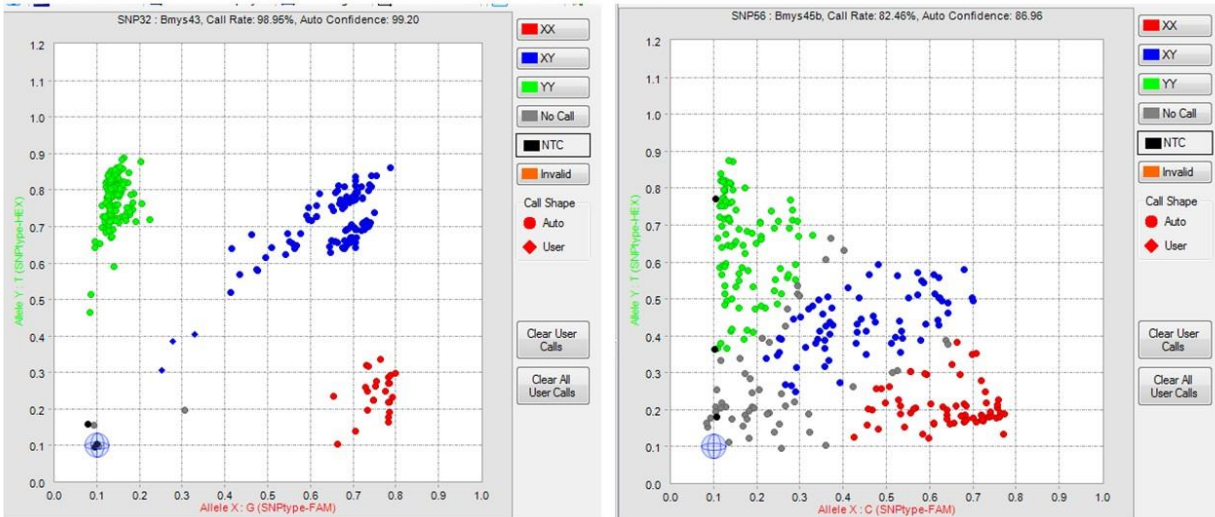

Figure 1: Examples of genotype calling for a clean locus (left) and a locus that was disregarded based on a lack of distinction among genotypes (right). Each dot represents fluorescence values for one individual.

We conducted several tests of quality control of the SNP data. First, we submitted duplicate samples on different plates to ensure that identical calls were obtained for each duplicate. We also submitted mother/fetus pairs to determine that fetuses had at least one allele that matched one of their mother's at each locus. Finally, we submitted samples used by Morin et al. (2010) to determine if they matched calls made by those authors for loci that we used from their study. Samples duplicated in Morin et al. (2010) and this study include 02G2, 02S2, 02S5, 01S3, 96B18, 05B29, 96B8, and 96B16.

Population genetic analyses of autosomal SNPs.---GenAlEx version 6.502 (Peakall and Smouse 2012) was used to compute genetic distances among all samples. We used Arlequin version 3.5 (Excoffier et al. 2005) to calculate Fst among the BCB and Okhotsk populations. GENEPOP version 4.4 (Raymond & Rousset 1995) was used to calculate observed and expected heterozygosities, and deviation from Hardy-Weinberg equilibrium.

RESULTS

Mitochondrial DNA results. —To date, the mtDNA database contains sequences from 3 loci: cytochrome-b (474 individuals), ND1 (465 individuals) and the HVR1 portion of the control region (695 individuals). 368 individuals have complete data for all three loci.

Overall SNP results. —The panel of SNP loci included 82 autosomal loci, 1 Y-chromosome locus, and 13 X-chromosome loci (= 96 total loci). Of these 96 total loci, 48 autosomal loci, 1 Y-chromosome locus, and 6 X-chromosome loci were of high enough quality to use for our final dataset. Results below include data only from the autosomal loci. Sex chromosome SNP data will be used in future studies.

Autosomal SNP results.—Below is a summary of various aspects of results from the autosomal SNPs.

Quality control. —Any samples that yielded <95% complete data were discarded from further analyses. Using that criterion, 42 of the 285 samples were discarded. This left four duplicated (for quality control) pairs of samples. Two of these pairs had identical genotypes across all loci. One pair had a single allele difference across all loci. The one remaining pair had several mismatches and was therefore disregarded from further analysis.

Of the loci that passed our quality standards (produced clear distinction among genotypes), the SNPs originally used in the Morin et al. (2010) study showed complete matches in the genotypes called by the methods used by Morin et al. (2010) and our Fluidigm SNPtype assays. One possible exception appears to be sample 96B16 for the BH395 locus. Morin et al. (2010) report this sample's genotype at that locus as "T?T" whereas our analysis showed CT. We interpret the question mark as uncertainty in the call by Morin et al. (2010) and do not consider this to be evidence of a mis-match among methods.

All mother/fetus pairs were determined to have at least one of the mothers' alleles present in her fetus for each locus.

Genetic Distances. —We examined genetic distance among all samples. With the exception of the purposefully duplicated samples, there was only one instance of two samples having identical genotypes. Those samples were 97B15 and 97B16. They were collected (harvested) sequentially in Barrow, Alaska during the same hunting season (1997) on the same day and in the same general location. They also have the same mtDNA haplotype. Thus, it is possible that they represent a mixup of samples or a single whale having been given more than one sample number. With the exception of this sample pair, all other samples have unique genetic fingerprints across the autosomal loci based on this analysis.

Heterozygosity.—Expected and observed heterozygosity was calculated for each population, BCB and Okhotsk, independently. The results are shown in Tables 1 and 2, respectively.

Table 1: Heterozygosity and Hardy-Weinberg Equilibrium Statistics for the BCB population (n=222). Significant values at p<0.05 are highlighted in bold.

Locus	Number of alleles	Observed Heterozygosity	Expected Heterozygosity	Departure from Hardy-Weinberg equilibrium (p-value)	Heterozygote excess (p-value)
BH108	2	0.27273	0.28644	0.3429	0.8305
BH42b	2	0.69369	0.45411	**0**	**0**
Bmys2	2	0.24775	0.2631	0.4394	0.8699
Bmys28	2	0.42723	0.47356	0.191	0.9425
Bmys34	2	0.18919	0.22093	0.058	0.9887

Bmys41	2	0.10909	0.11945	0.2089	0.955
C5	2	0.07207	0.06963	1	0.7557
BH43	2	0.3	0.30963	0.6631	0.7574
Bmys13	2	0.25225	0.24729	1	0.5081
Bmys21	2	0.00901	0.00899	1	0.9977
Bmys29	2	0.23423	0.21414	0.2143	0.1325
Bmys35	2	0.17526	0.19356	0.2486	0.9468
Bmys5	2	0.00905	0.00903	1	0.9977
PKM	2	0.43243	0.44842	0.6528	0.7537
BH34	2	0.15	0.17676	**0.0089**	0.9911
BH404	2	0	0.00899	**0.0023**	1
BH60	2	0.21622	0.20031	0.3246	0.2018
Bmys14b	2	0.43694	0.43767	1	0.5735
Bmys22	2	0.4955	0.48319	0.7807	0.4053
Bmys3	2	0.45045	0.4424	0.8792	0.4546
Bmys43	2	0.36036	0.40347	0.1327	0.9598
BH368	2	0.26364	0.25558	0.7936	0.436
BH410b	2	0.49774	0.5003	1	0.5837
BH92	2	0.34685	0.35226	0.8489	0.6689
Bmys23	2	0.48372	0.47921	1	0.5026
Bmys44	2	0.55204	0.48389	**0.0376**	**0.0245**
Bmys51	2	0.12162	0.11448	1	0.4312
CHY	2	0.51429	0.38295	**0**	**0**
Bmys31	2	0.12613	0.13406	0.3102	0.9105
Bmys24	2	0.13964	0.13791	1	0.6614
BH92-2	2	0.29412	0.4889	**0**	1
BH382	2	0.40826	0.35182	**0.0194**	**0.0109**
Bmys25	2	0.38739	0.34478	0.0781	**0.0444**
Bmys17	2	0.83256	0.49141	**0**	**0**
BH414	2	0.45946	0.45411	0.8832	0.4908
BH387	2	0.34234	0.33453	0.8413	0.4501
CSF2	2	0.51364	0.50088	0.7872	0.4037
Bmys7	2	0.24771	0.2244	0.2176	0.0986
Bmys39	2	0.67117	0.48867	**0**	**0**
BH42a	2	0.49541	0.49402	1	0.5384
BH387-2	2	0.28378	0.2631	0.3097	0.1796
Bmys4	2	0.42523	0.41055	0.7382	0.3635
Bmys33	2	0.58559	0.42592	**0**	**0**
Bmys19	2	0.49099	0.49258	1	0.5736
BH395	2	0.48649	0.50048	0.6887	0.7097
FES	2	0.32883	0.32663	0.8364	0.5517

Bmys9	2	0.57658	0.42239	**0**	**0**
Bmys40	2	0.50485	0.43999	**0.0389**	**0.0232**
AVERAGE		0.352365	0.328476458		

Table 2: Heterozygosity and Hardy-Weinberg Equilibrium Statistics for the Okhotsk Population (n=17). (N/A indicates one of two scenarios: only one allele was observed for that locus in this population, or only one individual had >1 allele in the population.) Significant values at p<0.05 are highlighted in bold.

Locus	Number of alleles	Observed Heterozygosity	Expected Heterozygosity	Departure from Hardy-Weinberg equilibrium (p-value)	Heterozygote excess (p-value)
BH108	2	0.35294	0.51337	0.328	0.9688
BH42b	2	0.64706	0.45098	0.1081	0.0886
Bmys2	2	0.05882	0.05882	N/A	N/A
Bmys28	2	0.35294	0.29947	1	0.5889
Bmys34	2	0.11765	0.11408	1	0.9697
Bmys41	1	N/A	N/A	N/A	N/A
C5	2	0.35294	0.29947	1	0.5889
BH43	2	0.29412	0.25847	1	0.7116
Bmys13	2	0.41176	0.3369	1	0.4627
Bmys21	1	N/A	N/A	N/A	N/A
Bmys29	2	0.05882	0.05882	N/A	N/A
Bmys35	2	0.09091	0.09091	N/A	N/A
Bmys5	1	N/A	N/A	N/A	N/A
PKM	2	0.23529	0.29947	0.4111	0.957
BH34	2	0.29412	0.25847	1	0.7116
BH404	1	N/A	N/A	N/A	N/A
BH60	2	0.35294	0.47059	0.3372	0.9473
Bmys14b	2	0.41176	0.3369	1	0.4627
Bmys22	2	0.52941	0.45098	0.6085	0.4366
Bmys3	2	0.35294	0.29947	1	0.5889
Bmys43	2	0.29412	0.25847	1	0.7116
BH368	2	0.17647	0.16578	1	0.9091
BH410b	2	0.47059	0.37077	0.5201	0.3428
BH92	2	0.05882	0.05882	N/A	N/A
Bmys23	2	0.52941	0.50802	1	0.6248
Bmys44	2	0.35294	0.47059	0.3372	0.9473
Bmys51	2	0.35294	0.37077	1	0.8226
CHY	2	0.17647	0.16578	1	0.9091
Bmys31	2	0.23529	0.2139	1	0.8211
Bmys24	2	0.11765	0.11408	1	0.9697

BH92-2	2	0.11765	0.47059	**0.003**	1
BH382	2	0.125	0.12097	1	0.9677
Bmys25	2	0.47059	0.37077	0.5201	0.3428
Bmys17	2	0.23529	0.47059	0.0989	0.997
BH414	2	0.29412	0.25847	1	0.7116
BH387	2	0.64706	0.50802	0.3428	0.2493
CSF2	2	0.52941	0.50802	1	0.6248
Bmys7	2	0.41176	0.45098	1	0.8282
Bmys39	2	0.375	0.31452	1	0.5656
BH42a	2	0.47059	0.49911	1	0.775
BH387-2	2	0.64706	0.45098	0.1081	0.0886
Bmys4	2	0.41176	0.3369	1	0.4627
Bmys33	2	0.17647	0.16578	1	0.9091
Bmys19	2	0.47059	0.37077	0.5201	0.3428
BH395	2	0.58824	0.49911	0.6244	0.3995
FES	2	0.23529	0.42781	0.0888	0.9943
Bmys9	2	0.17647	0.16578	1	0.9091
Bmys40	2	0	0.12874	**0.0345**	1
AVERAGE		0.319578864	0.313910455		

Population Fst.—We calculated pairwise Fst between the BCB and Okhotsk populations. This calculation was based on the final dataset that excluded one sample of each duplicated individual. Thus, the Fst calculation was based on 239 samples (17 from Okhotsk and 222 from BCB). The calculated Fst value was 0.05418 and was significant at $p < 0.05$.

Heterozygosity and Hardy-Weinberg.—For the BCB population, 12 loci significantly deviated from Hardy-Weinberg Equilibrium (Table 1). Of those, 9 loci deviated by having an excess of heterozygotes compared to their expected values. For the Okhotsk population, two loci deviated significantly from Hardy-Weinberg Equilibrium (Table 2). In those instances, the loci were observed to exhibit lower heterozygosity than expected. Average observed heterozygosity was slightly higher in the BCB population (~ 0.35) than the Okhotsk population (~ 0.32).

DISCUSSION

The SNP genotyping method we have chosen, Fluidigm SNPtype analysis, has proven to be a reliable and replicable method to genotype bowheads. Because loci in this study were derived from previously published genome and transcriptome data (Baird et al. 2015, Keane et al. 2015), the loci are not anonymous. An added benefit is that the loci/primers and genotyping method can easily be transferred across labs. Scored SNP genotypes will be directly comparable among collaborators' labs, an advantage not seen by using microsatellites (Morin et al. 2004) and certain other methods of SNP genotyping such as ddRAD. Additionally, we have shown that SNP data can be replicated even when using different methods of analysis, as demonstrated by the comparison of the Morin et al. (2010) data and our data. Morin et al. (2010) used Amplifluor genotyping chemistry (Millipore, Billerica, MA, USA), while we utilized Fluidigm SNPtype technology. Primers for our study were developed independently, and they may be different from those used by Morin et al. (2010).

As demonstrated here, the addition of SNP data to our existing mtDNA database has increased the power of our ability to examine the intricacies of population genetics of bowhead whales. Our ability to fingerprint individual whales will lead to future studies that can address issues such as the timing of migration of family groups and aid in the identification of previously tagged whales.

The Fst value between BCB and Okhotsk populations calculated here from the autosomal SNPs (0.05418) is similar to the value calculated using 3 mitochondrial loci in previous studies (0.097; Bickham et al. 2012) and both were significant. The Fst values calculated using this panel of SNPs was also comparable to the value calculated in Morin et al. (2012) using a panel of 42 SNPs (many of which were linked). The Fst value calculated based on the 42 SNPs was 0.037. Based on microsatellites, Fst was calculated at 0.035 (Morin et al. 2012). In every analysis, Fst values between BCB and Okhotsk populations were significant.

The analysis of heterozygosity in both the BCB and Okhotsk populations reveals several characteristics about those populations. In the BCB population, for the loci not at HWE, most had significant excesses of heterozygotes. The opposite is true for the Okhotsk population. While more analyses will be required to determine the exact cause of this phenomenon, we interpret these results to indicate that the BCB population is quite large and not experiencing high levels of inbreeding, which usually act to depress heterozygote frequencies. The Okhotsk population is much smaller and may be experiencing inbreeding. These conclusions support previous findings of a large BCB population (Givens et al. 2016) and small Okhotsk population (Vladimirov 1994; see also Brownell et al. 1997).

The sex chromosome SNPs not discussed here will be used in separate studies, in combination with the mtDNA haplotype data, to examine historical demography of bowheads.

Future studies will involve increasing the number of SNP loci to 96 usable loci. We plan to utilize genome sequence data (Keane et al. 2015) for Greenland whales to identify variable sites present in the Eastern Canadian Arctic population that may also be variable in the BCB population. Although our present panel of SNPs is sufficient for fingerprinting individuals, having a panel of 96 SNPs is the most cost-effective strategy for genotyping using the Fluidigm SNPtype platform.

LITERATURE CITED

Baird, A. B. , R. S. Suydam, J. C. George, and J. W. Bickham. 2014. Update on mtDNA and SNP database for bowhead whales. Paper SC/65b/BRG13 submitted to the International Whaling Commission Scientific Committee.

Baird, A. B., A. R. Rimmelin, C. Q. Tran, J. C. George, R. S. Suydam, and J. W. Bickham. 2015. Continued development of mtDNA and SNP databases for bowhead whales. Paper SC/66a/BRG/12 submitted to the International Whaling Commission Scientific Committee.

Bickham, J. W., R. M. Huebinger, C. D. Phillips, J. C. Patton, L. D. Postma, J. C. George, and R. S. Suydam. 2012. Assessing molecular substitution patterns in mitochondrial control region compared to protein coding genes in bowhead whales: update of SC/63/BRG13. Paper SC/64/AWMP9 submitted to the International Whaling Commission Scientific Committee.

Brownell, R. L., Jr., A. M. Burdin, S. A. Blokhin, and A. A. Berzin. 1997. Observations on bowhead whales (Balaena mysticetus) in the Shantar Archipelago, western Okhotsk Sea. International Marine Biological Research Institute Reports 7: 1-7.

DeWoody, J. A., N. B. Fernandez, A. Brüniche-Olsen, J. D. Antonides, J. M. Doyle, P. San Miguel, R. Westerman, C. Godard-Codding, and J. W. Bickham. 2016. Novel single nucleotide polymorphisms from functional genes in the gray whale (*Eschrichtius robustus*) genome provide a powerful genotyping platform. Paper SC/66b/SDx presented to the IWC Scientific Committee (unpublished). 68 pp. [Available at http://www.iwcoffice.org]

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1: 47-50.

Givens, G.H., S.L. Edmondson, J.C. George, R. Suydam, R.A. Charif, A. Rahaman, D.L. Hawthorne, B. Tudor, R.A. Delong, and C.W. Clark. 2016. Horvitz-Thompson whale abundance estimation adjusting for uncertain recapture, temporal availability variation and intermittent effort. *Environmetrics*, 27: 134-146.

Helyar, S. J., J. Hemmer-Hansen, D. Bekkevold, M. I. Taylor, R. Ogden, M. T. limborg, A. Cariani, G. E. Maes, E. Diopere, G. R. Carvalho, and E. E. Nielsen. 2011. Molecular Ecology Resources 11 (suppl. 1): 1-14.

LeDuc RG, Martien KK, Morin PA, Hedrick N, Robertson KM, Taylor BL, Mugue NS, Borodin RG, Zelenina DA, Litovka D, George JC. 2008. Mitochondrial genetic variation in bowhead whales in the western Arctic. Journal of Cetacean Research and Management 10: 93–97

Keane M, Semeiks J, Webb AE, Li YI, Quesada V, Craig T, Madsen LB, van Dam S, Brawand D, Marques PI, Michalak P, Kang L, Bhak J, Yim HS, Grishin NV, Nielsen NH, Heide-Jorgensen MP, Oziolor EM, Matson CW, Church GM, Stuart GW, Patton JC, George JC, Suydam R, Larsen K, Lopez-Otin C, O'Connell MJ, Bickham JW, Thomsen B, de Magalhaes JP. 2015. Insights into the evolution of longevity from the bowhead whale genome. Cell Reports. 10, 112-120.

Morin, P. A., G. Luikart, R. K. Wayne, and the SNP workshop group. 2004. SNPs in ecology, evolution, and conservation. TRENDS in Ecology and Evolution 19: 208-216.

Morin, P. A., N. C. Aitken, N. Rubio-Cisneros, A. E. Dizon, and S. Mesnick. 2007. Characterization of 18 SNP markers for sperm whale (*Physeter microcephalus*). Molecular Ecology Resources 7: 626-630.

Morin, P. A., V. L. Pease, B. L. Hancock, K. M. Robertson, C. W. Antolik. 2010. Characterization of 42 single nucleotide polymorphism (SNP) markers for the bowhead whale (*Balaena mysticetus*) for use in discriminating populations. Marine Mammal Science 26: 716-732.

Morin, P. A., F. I. Archer. V. L. Pease, B. L. Hancock-Hanser, K. M. Robertson, R. M. Huebinger, K. K. Martien, J. W. Bickham, J. C. George, L. D. Postma, B. L. Taylor. 2012. Empirical comparison of single nucleotide polymorphisms and microsatellites for population demographic analyses of bowhead whales. Endangered Species Research 19: 129-147.

Peakall, R. and P. E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28: 2537-2539.

Phillips, C. D., T. S. Gelatt, J. C. Patton, and J. W. Bickham. 2011. Phylogeography of Steller sea lions: relationships among climate change, effective population size, and genetic diversity. Journal of Mammalogy 92: 1091-1104.

Phillips, C.D., J. I. Hoffman, J. C. George, R. S. Suydam, R. M. Huebinger, J. C. Patton , and J. W. Bickham. 2012. Molecular insights into the historic demography of bowhead whales: understanding the evolutionary basis of contemporary management practices. Ecology and Evolution 3: 18-37.

Raymond, M. and F. Rousset. 1995. GENEPOP version 1.2: population genetics software for exact tests and ecumenicism. Journal of Heredity 86: 248-249.

Schmitt, N. T. 2013. Patterns of population genetic structure among Australian and South Pacific humpback whales (*Megaptera novaeangliae*). Ph.D. Dissertation, Australian National University, Canberra.

Vladmimirov, V. L. 1994. Recent distribution and abundance levels of whales in Russian far-eastern seas. Russian Journal of Marine Biology 20: 1-9.