GENETIC RELATIONSHIPS AND EVOLUTIONARY HISTORY OF EXTANT BOWHEAD WHALE POPULATIONS, *Balaena mysticetus*

A Dissertation

by

RYAN MICHAEL HUEBINGER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2007

Major Subject: Genetics

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ABSTRACT

Genetic Relationships and Evolutionary History of Extant Bowhead Whale Populations, *Balaena mysticetus.* (August 2007)

Ryan Michael Huebinger, B.S.; B.S., Texas A&M University;

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Bowhead whales, *Balaena mysticetus*, are large baleen whales that are characterized by an Arctic distribution. In order to provide proper management recommendations to the International Whaling Commission, a panel of twenty-five microsatellite markers was developed. This panel consisted of pure cytosine-adenine (CA) repeats and was selected for their ability to consistently amplify and for their consistency for allele designations. This panel was utilized to investigate stock structure within the Bering-Chukchi-Beaufort population of bowheads. Over thirty reports and manuscripts utilized these data for providing management recommendations to the International Whaling Commision.

From this work it was concluded that there is presently one stock of bowhead whales within the Bering-Chukchi-Beaufort seas. This data was also utilized to examine the genetic relationships of the remaining extant bowhead populations. The microsatellite data, in combination with sequences from the mitochondrial control region and the NADH dehydrogenase subunit 1 (ND1) region, were used to examine population structure. The Okhotsk Sea was identified as the most divergent population of bowheads. Relationships among the extant populations demonstrated high levels of effective gene

flow between populations. Gene flow appeared to be female-biased in relation to the Okhotsk Sea, whereas gene flow between Canada and the Bering-Chukchi-Beaufort populations appears to be equal between the bowhead whale sexes.

DEDICATION

To my family and friends

ACKNOWLEDGMENTS

First I would like to thank Dr. John Bickham for allowing me to come back to Texas A&M to undertake my Ph.D. studies with his guidance. It has been a great experience. I would also like to thank my Ph.D. committee members, Dr. Jim Cathey, Dr. Rodney Honeycutt, and Dr. Spencer Johnston. Thank you for your guidance and expertise throughout my graduate career.

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To all the members of the United States Scientific Delegation to the International Whaling Commission and the scientists of member countries that analyzed my dataset for the implementation review of the bowhead quota. It has been an experience that I will never forget, and was ecstatic to be involved in the entire process.

Dr. Ed Louis Jr. and the genetics lab of the Henry Doorly Zoo in Omaha, Nebraska, thank you for allowing me to come to the lab to develop the microsatellite panel that was utilized for my dissertation and for the implementation review of the bowhead quota.

Thank you to the National Oceanic and Atmospheric Administration, Alaska Eskimo Whaling Commission, North Slope Borough, and Senator Ted Stevens for funding the research undertaken for my dissertation.

Dr. Robert Trujillo and all the members of the Bickham lab for their assistance over my tenure in the lab.

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Finally, to my family and friends. Thank you for your patience over the years and

trying to understand how someone can stay in school so long. I'm done!

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CHAPTER I

INTRODUCTION

Bowhead whales have been harvested by Inupiat Eskimos for over 2000 years. The bowhead whale has become part of their culture and a species upon which they depend for food. This subsistence harvest has been regulated by the International Whaling Commission (IWC) to ensure the levels of harvest will not impact the survival and recovery of the species. The harvest is now regulated because commercial whaling in the late 1800s and early 1900s reduced the Bering-Chukchi-Beaufort Seas (BCB) bowheads from a population of over 15,000 whales to under a 1,000 whales. Cessation of commercial whaling occurred in 1914. Since that time the bowhead whales have recovered to an estimated 12,000 individuals and continuing to grow at a rate of 3.4% per year. As part of the management of the species the IWC, safe hunting quotas are established based upon statistical population modeling and assessment methods, with priority given to recovery of the whale population as well as the nutritional and cultural needs of the aboriginal people. The management of the species has been based upon the assumption that the BCB bowhead population constituted a single stock from both a biological and management perspective. Evidence supporting the single stock hypothesis is summarized by Rugh et al. (2003). During the 2004 IWC implementation review of aboriginal subsistence harvest quotas of bowhead whales, some analyses of early genetic data found indications of genetic heterogeneity. Of these findings, the most notable was

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This dissertation follows the style of *Molecular Ecology*.

a temporal pattern identified in the fall migration of bowheads past Barrow, commonly referred to as the 'Oslo Bump.' This pattern indicated that individuals migrating past Barrow in the fall of each year were less genetically similar than would be expected if the individuals were about 5-11 days apart in their migration. These lines of evidence pointed to multiple stocks within the current BCB region. If there were in fact two stocks in the BCB, this would be a cause for concern relative to the management of the whale stock and the aboriginal harvest quota set by the IWC. In addition to the multi-stock scenarios presented at the 2004 IWC Scientific Committee meeting, the reliability of the microsatellite dataset was called into question. After conclusion of the 2004 IWC commission meeting, the aboriginal subsistence harvest quota for bowhead whales was put into an instant implementation review of the quota to be completed at the 2007 IWC meeting in Anchorage, AK. In order to properly address the questions put forth by the IWC Scientific Committee, we were charged with developing a new panel of microsatellites specific for bowhead whales and determining if the initial indications of genetic heterogeneity were biologically real. Additionally, increases in the number of whales analyzed for these markers were recommended. The following chapters detail the effort and culmination of genetic research undertaken as part of the implementation review of the aboriginal subsistence harvest quota for BCB bowhead whales.

CHAPTER II

CHARACTERIZATION OF TWENTY-FIVE MICROSATELLITE LOCI IN BOWHEAD WHALES, *Balaena mysticetus*

The bowhead whale (*Balaena mysticetus*) is named for its large bow shaped head that can comprise nearly 1/3 the total length of the animal. This species is characterized by an arctic distribution occupying waters adjacent to the sea ice edge, as well as "polynynas" or persistent areas of open water within the pack ice. It is the only species of baleen whale that has evolved the ability to live in the cold Arctic throughout the year (Montague 1993).

Commercial harvests of bowheads began in the early 16th century around Labrador. This was followed by exploitation of the Spitsbergen stock in the early 17th century and the mid-19th century saw the exploitation of the Okhotsk and Bering-Chukchi-Beaufort (BCB) stocks. The question of how many bowheads existed before the introduction of commercial whaling is still a point of contention, but was surmised to be a minimum of 50,000 whales for all stocks combined (Montague, 1993). At the end of commercial whaling in 1914, the total number of bowhead whales was estimated to be less than 2000 individuals for all stocks combined. Since its rapid decline, the BCB stock has rebounded to a current population size of at least 10,000 individuals (Zeh and Punt, 2004). Currently the only population that is annually whaled is the BCB stock. The International Whaling Commission (IWC) monitors and manages the aboriginal subsistence harvest by Inuits in Alaska where the majority of harvest occurs.

Genomic DNA was isolated from a female bowhead whale skin tissue (individual 02B10) using a standard protocol (Sambrook et al., 1989). A procedure for creation of a genomic library enriched for microsatellites was carried out as described in An et al. (2004). A brief overview of the enrichment protocol follows. Genomic DNA was digested with *Sau*3AI and then size selected utilizing Chroma Spin columns (Clontech Laboratories, Chroma Spin + TE-400). Fragments were then ligated to linker molecules described in An et al. (2004). Enrichment for CA repeats was accomplished utilizing biotynlated oligoprobe and Vectrex Avidin D (Vector Laboratories). Enriched fragments were then ligated into pCR 2.1 TOPO vector (Invitrogen, San Diego, CA). Ligated DNA was transformed into *E. coli*. Colonies that contained inserts were hybridized to a $(CA)_{10}$ oligoprobe. Screening of 1000 clones yielded 90 positives, which were sequenced on an ABI377 (Applied Biosystems; Foster City, CA). Primers for polymerase chain reaction (PCR) amplification were designed from the unique sequence regions flanking the microsatellites found in 61 of the positives. Loci were chosen for development based on a high repeat number and primers were designed using Oligo v.5.0 (National Biosciences, Inc., Plymouth, MN). Loci were determined to be polymorphic based on PCR fragment size when screened across 9 bowhead whales. Loci were screened for polymorphism by electrophoresing on 3% agarose gels.

Genomic DNA was isolated from tissue samples and PCR amplification was performed in a 25 µl reaction volume using an ABI2700 thermocycler (Perkin-Elmer; Foster City, CA) with approximately 50 ng of genomic DNA as template. Final amplification conditions consisted of 12.5 pmol unlabelled reverse primer, 12.5 pmol

fluorescently labeled forward primer, 1.5 mM $MgCl_2$, 200 μM each dNTP, and 0.5 units of *Taq* DNA polymerase (Promega; Madison, WI). The PCR amplification profile was 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, a primer-specific annealing temperature for 30 sec (Table 2.1), 72°C for 30 sec, ending with a single extension of 72°C for 10 min. For two loci, Bmy7 and Bmy8, a touchdown procedure was used for the amplification profile (Rooney et al., 1999).

Allele sizes were determined by fragment separation on an ABI3100 DNA Analyzer (Applied Biosystems, Inc; Foster City, CA). Fragment lengths were assigned by the GeneMapper software program (Applied Biosystems, Inc.) using GeneScan-400 [ROX] size standard. Heterozygosity values for each locus were calculated and tests for Hardy-Weinberg equilibrium of the genotypic frequencies were carried out using GENEPOP v3.1 software package (Raymond and Rousset, 1995). Additionally, loci were tested for linkage disequilibrium in GENEPOP v3.1 and no significant linkages were detected among these loci. Primer sequences, annealing temperature, repeat motif, GenBank accession number for each locus, number and size of alleles, and the heterozygosity values for each of the markers are presented in Table 1. Four loci were significantly out of Hardy-Weinberg equilibrium ($p<0.05$). Upon further analysis it was identified that one locus, Bmy47, was sex-linked locus. Disequilibria in the remaining three loci, Bmy18, Bmy38, and Bmy44, could be caused by null alleles present at the locus or due to random sampling error of genotypes. In a test for linkage disequilibrium, two sets of significant linkage were detected after utilizing a bonferonni correction (Bmy41 & Bmy54; Bmy7 & Bmy10). These loci could be physically linked, however

the linkage disequilibria also could result from the demographic bottleneck this species recently encountered. The number of alleles ranged from 4 to 22 alleles per locus.

This panel of loci will be utilized to examine a more extensive collection of bowhead whales throughout their range. From this more extensive collection questions regarding stock structure of the present populations will be examined in an effort to provide sound management advice for the continued aboriginal subsistence harvest of bowhead whales.

The authors would like to acknowledge the North Slope Borough, National Oceanic & Atmospheric Administration, Henry Doorly Zoo, and the Alaska Eskimo Whaling Commission provided funding for this study. We would like to thank the whaling captains, their crew, and the Eskimo villages that provided invaluable samples for this study.

Table 2.1. Microsatellite primers for bowhead whales. Primer sequences, repeat sequences, annealing temperatures, GenBank accession numbers, size range of amplification products, number of alleles (k), observed (H_O) and expected (H_E) heterozygosities, and Hardy-Weinberg equilibrium probabilities, for 25 *Balaena mysticetus* microsatellite loci.

Locus	Primer sequence (5' to 3')	Repeat motif	Annealing temp. (°C)	GenBank accession No.	Fragment Size (bp)	k	H_O	H_E	Prob HWE
Bmy1	F-6-FAM-AAA TCA ACC AGA ACA GGA GTC AAA CTT R-CGA TTT TTA TTT CAT CTT ATT TCC CTT	$(CA)_{15}$	50	EF538944	241-261	9	0.891	0.848	0.846
Bmy2	F-GAT GGG AGC CTG AAA CGA AGG TG R-HEX-TTG CCA ATC CTT TTC TAT TGA TGG AT	$(CA)_{17}$	53	EF538945	180-194	6	0.826	0.763	0.272
Bmy7	F-GAA ATT TGA AAC TAA TTT ATC CAT CGG R-6-FAM-GCT TCA GCA AAG TTT TCA GTA GGA T	$(CA)_{23}$	64-56[#]	EF538946	149-181	11	0.744	0.814	0.180
Bmy8	F-GAT GCT CAG AGA ATC CCA GGT C R-HEX-TTT ACC ACC CAT TTT TAC CCT G	$(CA)_{25}$	64-56[#]	EF538947	151-181	15	0.773	0.815	0.721
Bmy10	F-GCG CAG GAA GCT GTT CTC TTA CCC R-HEX-GCC CCA AGA GGA TTT CTC TGC A	$(CA)_{35}$	50	EF538948	212-256	21	0.956	0.934	0.585
Bmy11	F-6-FAM-AAG GAA ACA ATC AGA ATA AGG TGT CC R-GCT GCC CTT CAT TCT CTC AAA AGC	$(CA)_{33}$	50	EF538949	214-240	10	0.833	0.876	0.846
Bmy12	F-6-FAM-TTT GTC TCC CTG GGT AAC TTC TTG A R-GGT TAA TCC AAG GTG ACA TGA GCA	$(CA)_{38}$	53	EF538950	115-175	20	0.979	0.902	0.1631
Bmy14	F-GCT GGG TGT TTT GTT GTT GTT AAT GC R-6-FAM-ATG GTG KGG GAT TAG ATG AGT GTG C	$(GT)_{16}$	50	EF538951	171-179	4	0.524	0.530	0.612
Bmy16	F-HEX-ACT TGC AGA TGG TGT TTG AGT CTC T R-GAA GGC ACG GTC TCA ACT TGC T	$(GT)_{18}$	50	EF538952	207-219	7	0.714	0.761	0.301
Bmy18	F-GTT CCT GTT CTG CAA CAA GAT GGG R-6-FAM-GGA CGC TGG AAG AGT TAT GCT GA	$(GT)_{20}$	53	EF538953	107-139	16	0.854	0.902	0.025*
Bmy19	F-HEX-TGC CGC TGC CTC TGT ATT GG R-AAA GCA AGG TTA CAG AAA AGT C	$(GT)_{24}$	60	EF538954	104-134	15	0.936	0.875	0.281

Table 2.1. continued

Locus	Primer sequence (5' to 3')	Repeat motif	Annealing temp. (°C)	GenBank accession No.	Fragment Size (bp)	k	H_O	H_E	Prob HWE
Bmy26	F-6-FAM-CCC CAA GAG GAT TTC TTT GCA A R-GTG GCC TGG AAA TCA CAC CTC A	$(GT)_{30}$	56	EF538955	140-184	21	0.938	0.937	0.538
Bmy33	F-HEX-AAG GAA ATA AAT ATA ATT CTG TCT TCA GG R-GGG ACA GGA CTC ATT TTA TAC TGG A	$(CA)_{18}$	56	EF538956	133-157	11	0.857	0.801	0.493
Bmy36	F-6-FAM-AAG CCT AAT GAG GGT TAA TTG AAC C R-GCA GGG TTT TTC AAG AGC TGT CTA	$(GT)_{37}$	56	EF538957	154-200	22	0.98	0.939	0.863
Bmy38	F-6-FAM-AGT TCC CTC CTC TGA AAG TTC CTT G R-GAT GCC TGT TTC TGT GAG AGC CAC T	$(GC)_{10}(GT)_{19}$	56	EF538958	220-240	8	0.604	0.805	0.012*
Bmy41	F-6-FAM-TTG TGA GCG GTT AGT TTC AGA AGC R-GCC CAA ACA TGA GAT GTC TAA GGC A	$(CA)_{33}$	56	EF538959	188-232	17	0.88	0.851	0.173
Bmy42	F-GGT CCC AAT AAG AAT GCG TGT CA R-6-FAM-TTC TTG AGA TGG TAT AGG GAA CAC CTG	$(CA)_{21}$	63	EF538960	160-180	8	0.673	0.782	0.138
Bmy44	F-GAT TCC CCA TAA AGC ATT CTC TCC A R-HEX-CTG AGG CAG AGG TAA GTT TCC AAA	$(GT)_{33}$	63	EF538961	143-183	12	0.428	0.788	0.000*
Bmy47	F-6-FAM-TAG GCA ACA CTG AGG TCA CCA T R-GAT CCA TTC TCC AGT CCC CAA C	$(GT)_{26}$	60	EF538962	136-152	9	0.375	0.758	0.000*&
Bmy49	F-6-FAM-CAG CAG CAA AAT AAT TGA GAG GAC A R-GTA CAT GGC ATC TGA AGT CTG GTT T T	$(CA)_{25}$	60	EF538963	181-223	18	0.957	0.902	0.596
Bmy53	F-HEX-AGG AGC TGT CAA AGA ACA GAG GGA R-GCT AGT CTT CAG GTC ATT GTT TCC TTA	$(CA)_{27}$	63	EF538964	186-222	15	0.88	0.852	0.803
Bmy54	F-GTC GAT GCA ATT CAA GCT CTG G R-HEX-CCT CTG GCT TTA AAT ACC CCA ACT	$(CA)_{26}$	63	EF538965	155-169	7	0.714	0.717	0.631
Bmy55	F-GTG TTG AGC ATC TTT TCA TGT GCC R-6-FAM-CTC CCT GCT CTC CAT TTC TTG CT	$(GT)_{15}$	60	EF538966	211-221	6	0.756	0.745	0.371
Bmy57	F-GTA CTG CAG GCC TGA GAA TTG CAA R-HEX-TGG CAG TGG AAA AAT AAA CCC TTC	$(GT)_{21}$	60	EF538967	151-163	7	0.574	0.545	0.331
Bmy58	F-GAG GTG AAA TTT TAT TGA AAC TTT AGC AG R-6-FAM-TTG GCT TAC CAT TAG CTT ACT TTC AGT A	$(CA)_{36}$	60	EF538968	123-181	20	0.938	0.928	0.853

* denotes significant deviation from HWE $p<0.05$; # touchdown PCR as described in text;& sex-linked locus

CHAPTER III

MULTIDISCIPLINARY STUDIES OF THE BOWHEAD WHALE MICROSATELLITE DATA BY THE SCIENTIFIC COMMITTEE OF THE INTERNATIONAL WHALING COMMISSION

The International Whaling Commission (IWC) is the governing body that provides the quota for the aboriginal subsistence whale harvests of bowhead whales. In order to properly manage the whale stocks, it is imperative to have adequate data on a number of scientific issues, including stock structure. The IWC scientific committee meets annually to discuss the most current data available. In this process scientists present papers to the committee for discussion reporting the most up to date results of their research. The implementation review of the current bowhead harvest quota was examined at the 2004 IWC scientific committee meeting. Several issues were raised at the meeting causing concern that multiple genetic stocks may exist within the BCB population (Jorde et. al., 2004; Pastene et al., 2004). These papers pointed out that there may be multiple genetic stocks within the Bering-Chukchi-Beaufort Seas (BCB) and this would cause a change in the current procedures for management of the whale stock. In order to properly address questions of potential stock structure, an implementation review was initiated immediately after the 2004 meeting, with a scheduled completion at the May 2007 IWC meeting.

Certain parts of the data available in 2004 were called into question with regards to data quality, specifically the microsatellites utilized to generate the data. In an effort

to alleviate these perceived problems with the genetic data, a new dataset was proposed for providing management advice. Part of this new dataset was to include a new panel of microsatellites derived explicitly from bowhead whales designed to minimize the problems of high failure rate that characterized the data utilized in 2004 (Bickham et al., 2005 (Appendix A); Huebinger et al., 2006 (Appendix B)). Upon completion (see chapter II), this panel would be utilized to generate data from all whale samples available for the 2004 analysis as well as additional individuals that were harvested in subsequent years. Additionally, datasets were generated for the panel of 'new' microsatellites from two additional bowhead whale stocks (Sea of Okhotsk and eastern Canada).

Using the new panel of 25 microsatellite loci described in chapter I, I genotyped 367 individuals from the BCB and Okhotsk bowhead whale populations. An additional 47 individuals from Igloolik, Canada were genotyped by collaborators from the Department of Fisheries and Oceans, Canada (Postma and Lindsay). The final dataset included both the 11 'old' loci and the 25 'new' loci for 367 individual samples from the BCB and Okhotsk Sea stocks.

As part of the interdisciplinary, multi-institutional collaboration of the United States scientific committee delegation to the IWC, these data along with mitochondrial and single nucleotide polymorphism data produced by the Southwest Fisheries Sciences Center and modeled genetic data were utilized to make recommendations for the management of the BCB bowhead whale stock. My data were submitted as part of the Data Availability Agreement (DAA) of the IWC for analysis by member countries. As

part of the DAA, investigators from scientific delegations of Norway, Japan, and USA subjected these data to a multitude of analysis in order to provide proper management advice.

The lead statistician (Geof Givens, Colorado State University) of the U.S. scientific delegation utilized these data in a comprehensive analysis of genetic variability and possible population subdivision. Once these analyses were completed, a manuscript was prepared for presentation at the 2007 scientific committee meeting of the IWC (Givens et al., 2007 (Appendix C)). Upon completion of the scientific committee meeting, this manuscript will be modified for publication submission with Givens as the lead author. As part of the collaboration for the U.S. delegation, in addition to the actual generation of the microsatellite data, I assisted Givens with the analyses to independently corroborate statistical findings. Although Givens wrote the majority of the manuscript, as a collaborator I assisted in editing and finalization of the manuscript for the IWC meeting. Additional papers were submitted to the IWC scientific committee meeting in 2007 by member countries of the IWC (Jorde and Schweder, 2007; Kitikado et al., 2007). Additionally, the microsatellite data generated was utilized for error calculations (Morin et al., 2007 (Appendix D)) and modeling of data (Archer et al., 2007). As part of the scientific discussions during the meeting, scientists present written rebuttals as full papers (Givens, 2007; Martien et al., 2007) or as working papers (Huebinger et al., 2007 (Appendix E)). Working papers are only made available at the meeting. Upon completion of the meeting, the only record of 'working papers' were as notes in the final committee report. Table 3.1 lists 30 IWC papers and reports that

utilized the microsatellite datasets that I developed during the course of this study. Of course, few studies of population genetics of wildlife species receive such scrutiny, and perhaps this case is unparalleled in that regard. The purpose of this chapter is to help put into context the circumstances under which this study was conducted, and under which the data were utilized.

Upon discussion of the available data presented at the 2007 (including the microsatellite genetic data), it was concluded by the chairman that the evidence best supports a single genetic stock hypothesis for BCB bowhead whales. The scientific sub-committee (Bowhead, Right, and Gray whale; BRG) in which the research was discussed concurred with the chair's recommendation. Additionally, the standing working group for the Aboriginal Whaling Management Procedure (AWMP) utilized the stock structure conclusions reached within the BRG sub-committee to recommend that the bowhead Strike Limit Algorithm (SLA) is the best tool for giving management advice. The SLA is used to determine the risk associated with harvest levels and population sizes for a period 100 years from present. Based upon all available knowledge and the results from the SLA, present whale strike and whale catch limits for BCB bowheads are acceptable.

Table 3.1. Scientific papers presented to the IWC that utilized data developed by Ryan Huebinger for the BCB bowhead implementation review.

Bickham et al. 2005, SC/57/BRG19 **Appendix A**	Update on development of bowhead microsatellite panel
George, J.C. et al. 2005, SC/57/BRG21	Update on stock structure research of BCB bowhead whales
Givens, G.H. et al. 2006, SC/A06/AWMP3	Preliminary analysis of stock structure based on new microsatellites-AWMP intersessional meeting
SC/58/Rep2, 2006	Intersessional AWMP workshop on bowhead implementation review
Huebinger, R.M. et al. 2006, SC/58/BRG11 **Appendix B**	Progress on development of new microsatellite markers
Givens, G.H. et al. 2006, SC/58/BRG19	Preliminary analysis of stock structure base on new microsatellites
Givens, G.H. et al. 2007, SC/J07/AWMP2	Genetic differentiation in bowhead whales from the western Arctic
Kitakado, T. et al. 2007, SC/J07/AWMP3	Stock structure analyses for BCB bowheads using microsatellite data
Martien, K.K. et al. 2007, SC/J07/AWMP5	Genesis of simulated genetic data to emulate empirical data
Jorde, P.E. and Schweder, T. 2007, SC/J07/AWMP7	Brief analysis of BCB bowhead whales using microsatellite data
SC/59/Rep3, January 2007	Intersessional AWMP workshop on bowhead implementation review
SC/59/Rep4, March 2007	Intersessional AWMP workshop on bowhead implementation review
George, J.C. et al. 2007, SC/59/BRG3	Stock structure research summary of BCB
Morin, P.A. et al. 2007, SC/59/BRG8	Single Nucleotide Polymorphism (SNP) development and analysis
Givens, G.H. et al. 2007, SC/59/BRG11	Brief analysis of SNPs for BCB bowhead whales
Givens, G.H. et al. 2007, SC/59/BRG14 **Appendix C**	Microsatellite patterns of genetic differentiaion in bowhead whales
Morin, P.A. et al. 2007, SC/59/BRG15 **Appendix D**	Genotype error rates for bowhead microsatellite dataset
Archer, E. et al. 2007, SC/59/BRG17	Simulation of BCB bowhead whale populations and compared to empirical microsatellite data
Skaug, H.J. and Givens, G.H. 2007, SC/59/BRG20	Relatedness among individuals in BCB bowhead microsatellite data
Postma, L. et al. 2007, SC/59/BRG21	Update on bowhead whale research in Canada
Jorde, P.E. and Schweder, T. 2007, SC/59/BRG27	Analysis of stock structure for BCB bowhead whales with genetic data

Table 3.1. continued

Kitakado, T. et al. 2007, SC/59/BRG30	Update on stock structure analysis of BCB bowhead whales with microsatellite data
Givens, G.H. 2007, SC/59/BRG32	Note on Fis for bowhead whales sampled at Barrow
Martien, K.K. et al. 2007, SC/59/BRG34	Note on using STRUCTURE to infer number of populations in the BCB bowhead whale stock
Taylor, B.L. et al. 2007, SC/59/BRG35	Synthesis of lines of evidence for BCB bowhead population structure
Schweder, T. 2007, SC/59/BRGWP1	Estimating Size of two hypothesized stocks for AWMP trials
Kitakado, T. and Pastene, L.A. 2007, SC/59/BRGWP2	Response to SC/59/BRG34
Jorde, P.E. 2007, SC/59/BRGWP3	Note on genetic disequilibrium in BCB bowheads
Huebinger, R.M. et al. 2007, SC/59/BRGWP4 **Appendix E**	Reassessment of genotypes for 'problem individuals'
Jorde, P.E. 2007, SC/59/BRGWP7	Clarification of Hardy-Weinberg tests between BRG14 and BRG27

CHAPTER IV

PHYLOGEOGRAPHY OF EXTANT BOWHEAD WHALE (*Balaena mysticetus*) POPULATIONS BASED UPON MICROSATELLITE AND MITOCHONDRIAL DNA

The bowhead whale (*Balaena mysticetus*) lives in the high latitudes of the Northern hemisphere. It occupies the cold arctic waters adjacent to the sea ice edge, as well as "polynynas" or persistent areas of open water within the pack ice. It is the only species of baleen whale that has evolved the ability to live in the cold Arctic throughout the year. All other baleen whales migrate to tropical waters during winter months. In order to survive in these extreme climatic conditions, the bowhead has evolved highly specialized physiological and morphological characters. Adaptations by the bowhead include having extremely thick blubber (50cm) and an epidermis up to 25mm thick, which have allowed the bowhead to survive in a climate where the water temperature is commonly below 0ºC.

There are historically five described stocks of bowhead whales (Spitsbergen, Baffin Bay-Davis Strait, Hudson Bay-Foxe Basin, Bering-Chukchi Beaufort Seas (BCB), and Sea of Okhotsk) (Moore and Reeves, 1993). Definitions of stocks were based primarily on the commercial fishery history of bowhead exploitation. The evidence for the biological independence of these populations is weak (Woodby and Botkin, 1993) and has been questioned (Heide-Jorgensen et al., 2006; Borge et al., 2007). In fact, based on satellite tracking and genetic analysis, the HudsonBay-Foxe

Basin and Davis Strait-Baffin Bay stocks are considered to be one population by the Canadian government (Heide-Jorgenson et al., 2006; Lianne Postma, personal communication) With the exception of the Okhotsk Sea population, all populations are classified as high Arctic populations (Figure 4.1).

The distribution of these populations is currently geographically disjunct; however, the patterns of sea ice have changed dramatically over the last 10,500 years. Remains from fossil whales suggest that the high Arctic populations were possibly connected prior to the establishment of the M'Clintock Channel sea-ice plug, around 8,500 years ago (Dyke et al., 1996). Limited numbers of molecular studies have been completed. The primary focus of the molecular studies has been the BCB population (Rooney et al., 1999;2001; Hunter, 2005; LeDuc et al., 2007; Jorde et al., 2006). Nevertheless, a few studies have focused on the Okhotsk Sea (MacLean 2002; Leduc et al., 2005), Eastern Canadian Arctic (Postma et al., 2005), and historic samples of bowheads (Rastogi et al, 2004; Borge et al., 2007). Recent analysis by Borge et al. (2007) found a large number of shared mitochondrial haplotypes between the BCB and historic samples from the Spitsbergen area.

All of the bowhead whale stocks have been extensively harvested commercially. Over the time period of commercial whaling, all of the stocks were reduced to a small fraction of their pre-whaling abundances. In the case of the Spitsbergen stock, it was essentially harvested to extinction. After the cessation of commercial whaling in 1914, the BCB and Canada populations have rebounded (George et al., 2004; Cosens and

Richard, 2006), while the Okhotsk and Spitsbergen stocks continue to be imperiled (MacLean, 2002).

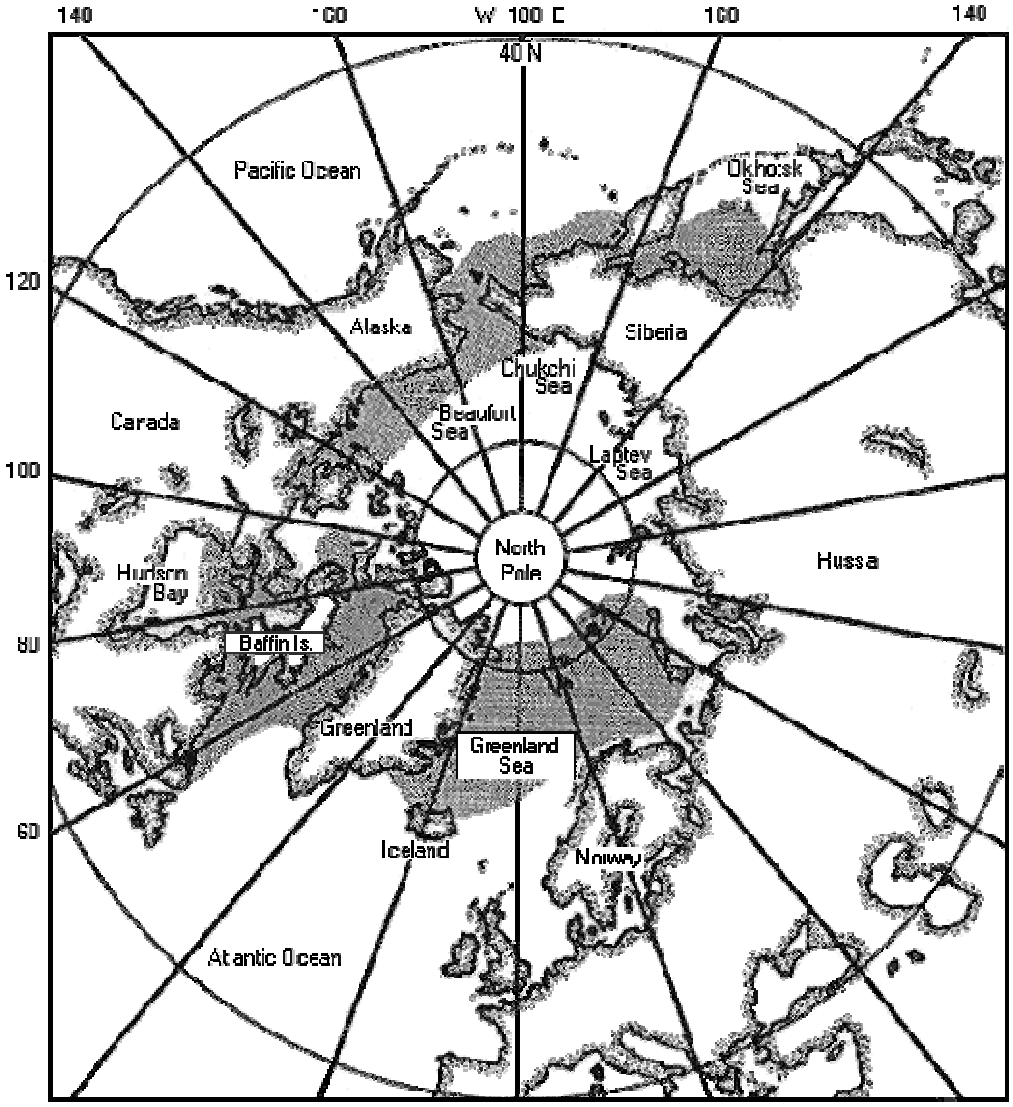

Figure 4.1. Historic distribution of bowhead whales. Shaded areas demonstrate areas that whales were found before the implementation of commercial whaling. Figure is taken from Shelden and Rugh (1995).

Molecular genetic techniques were utilized to delineate phylogeographic structuring of cetacean populations. In addition, these techniques have been utilized to

ascertain the level of differentiation between populations and the amount of genetic exchange that occurs between populations. This evidence can be used to determine the level of exchange and the degree of sex-biased dispersal. Cetacean populations can potentially have high levels of dispersal and mixing between populations. In light of this, molecular genetic markers have been utilized to determine the levels of gene flow and differentiation in cetacean populations (Brown Gladden et al., 1999; Lyrholm et al., 1999; Harlin et al., 2003; Cassens et al., 2003, 2005).

In the present study we utilized two mitochondrial gene sequences (ND1 and control region) to examine genetic maternal lineages. In addition to the mitochondrial sequences, we utilized data from 22 microsatellite nuclear markers (Huebinger et al, 2007). A portion of the microsatellite data was utilized to examine population structuring within the BCB population (Givens et al., 2007). A portion of the whales genotyped for microsatellites were sequenced for both mitochondrial genes. Through the combination of these two types of markers, we hope to delineate the present population genetic structure of extant bowhead whale populations and determine the current levels of genetic exchange among groups.

Materials and Methods

A total of 725 bowhead whale samples were collected. The individuals from the Okhotsk Sea and Eastern Canadian Arctic were from biopsies and sloughed skin. The majority of the BCB samples were obtained via necropsy sampling of whales that were

part of aboriginal subsistence harvests. A small number of BCB DNA samples were obtained from either remote biopsy darting of whales or from bone samples of previously harvested whales (for mtDNA analysis). Table 4.1 presents sampling locations and number of samples from each location.

Table 4.1. Summary of number of samples and sampling location for each population.

Sea of Okhotsk
- Ulbanskiy Bay and Konstantina Bay (n = 63)

Bering-Chukchi-Beaufort Seas
- Saint Lawrence Island (n = 56)
- Chukotka (n = 15)
- Diomede (n = 1)
- Point Hope (n = 6)
- Barrow (n = 231)
- Wainwright (n = 7)
- Kaktovik (n = 15)
- Mackenzie Delta (n = 13)

Canadian Arctic
- Pelly Bay (n = 8)
- Repulse Bay (n = 16)
- Igloolik (n = 176)
- Pangrintrung (n = 88)
- Hudson Bay (n = 3 control region only)
- West Greenland (n = 41 control region only)

Details of the sample collection and locations for the Sea of Okhotsk are reported by MacLean (2002). Sea of Okhotsk samples are from the Shantar Archipelago in the Sea of Okhotsk (Figure 4.2). Sampling locations for the BCB and eastern Canadian Arctic are shown in Figures 4.3 and 4.4, respectively.

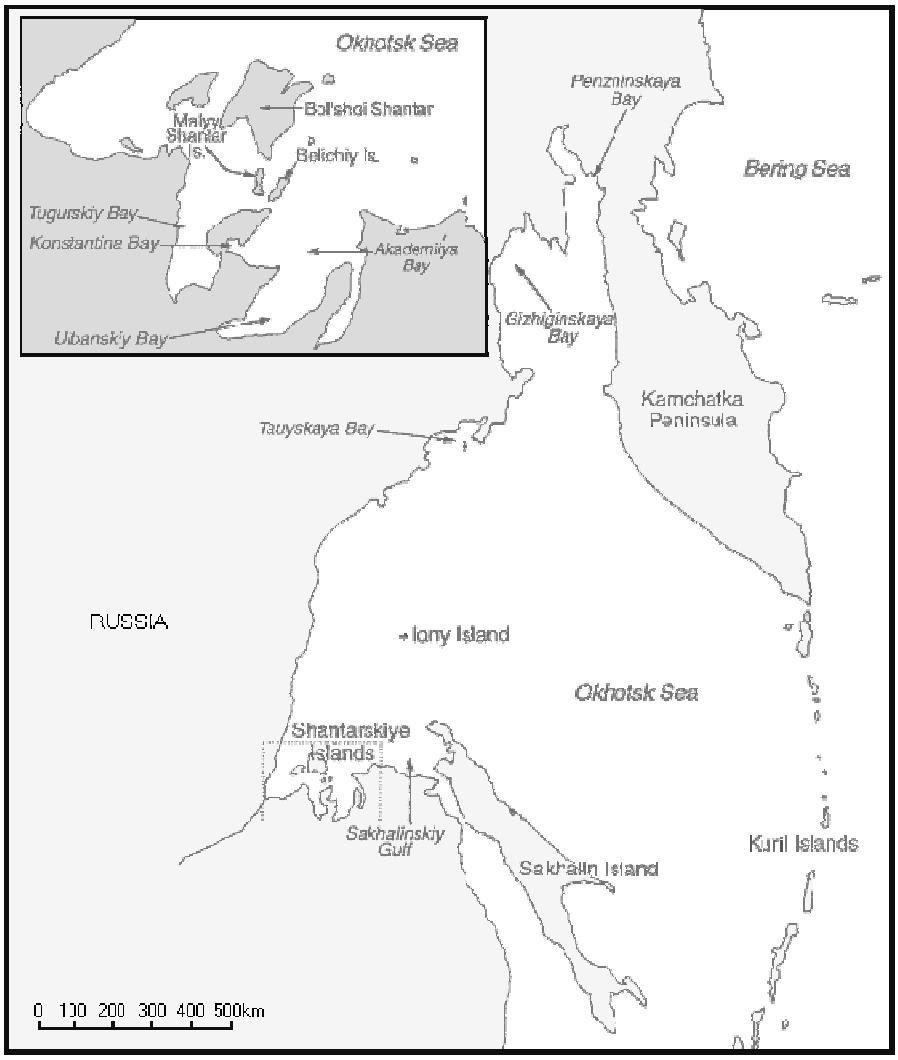

Figure 4.2. Sampling areas of bowhead whales in the Sea of Okhotsk described by Maclean (2002). Figure is taken from Shelden and Rugh (1995).

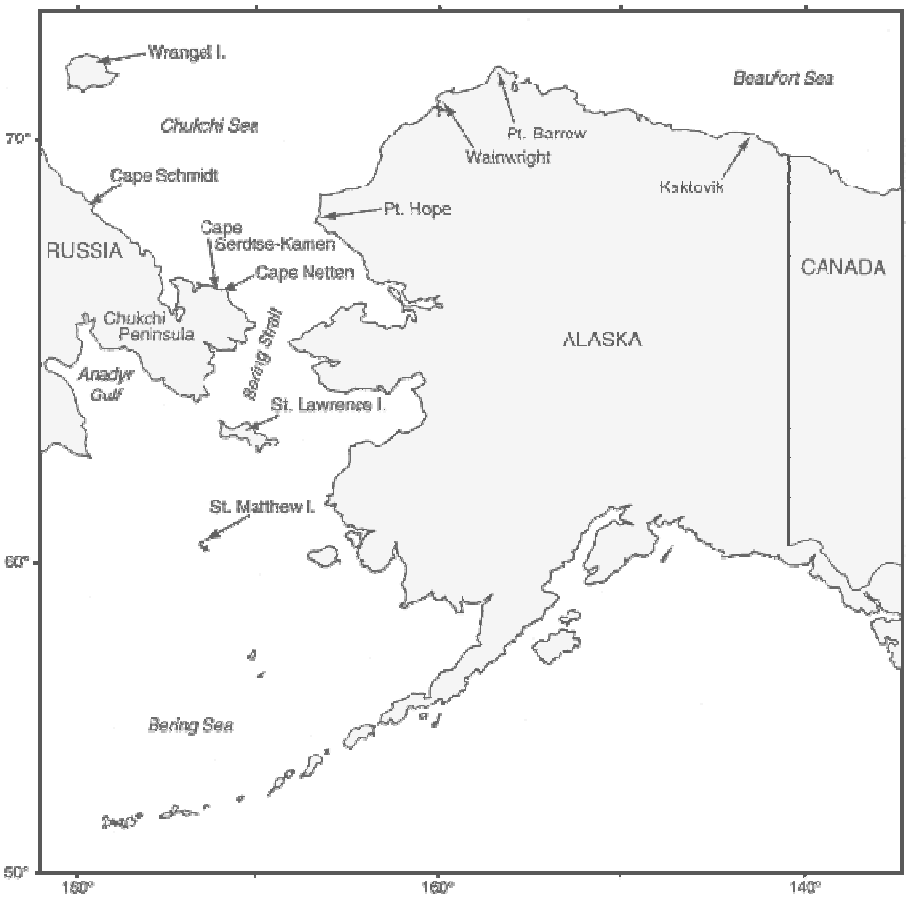

Figure 4.3. Sampling locations of bowhead whales in the Bering-Chukchi-Beaufort Seas. Figure is taken from Shelden and Rugh (1995).

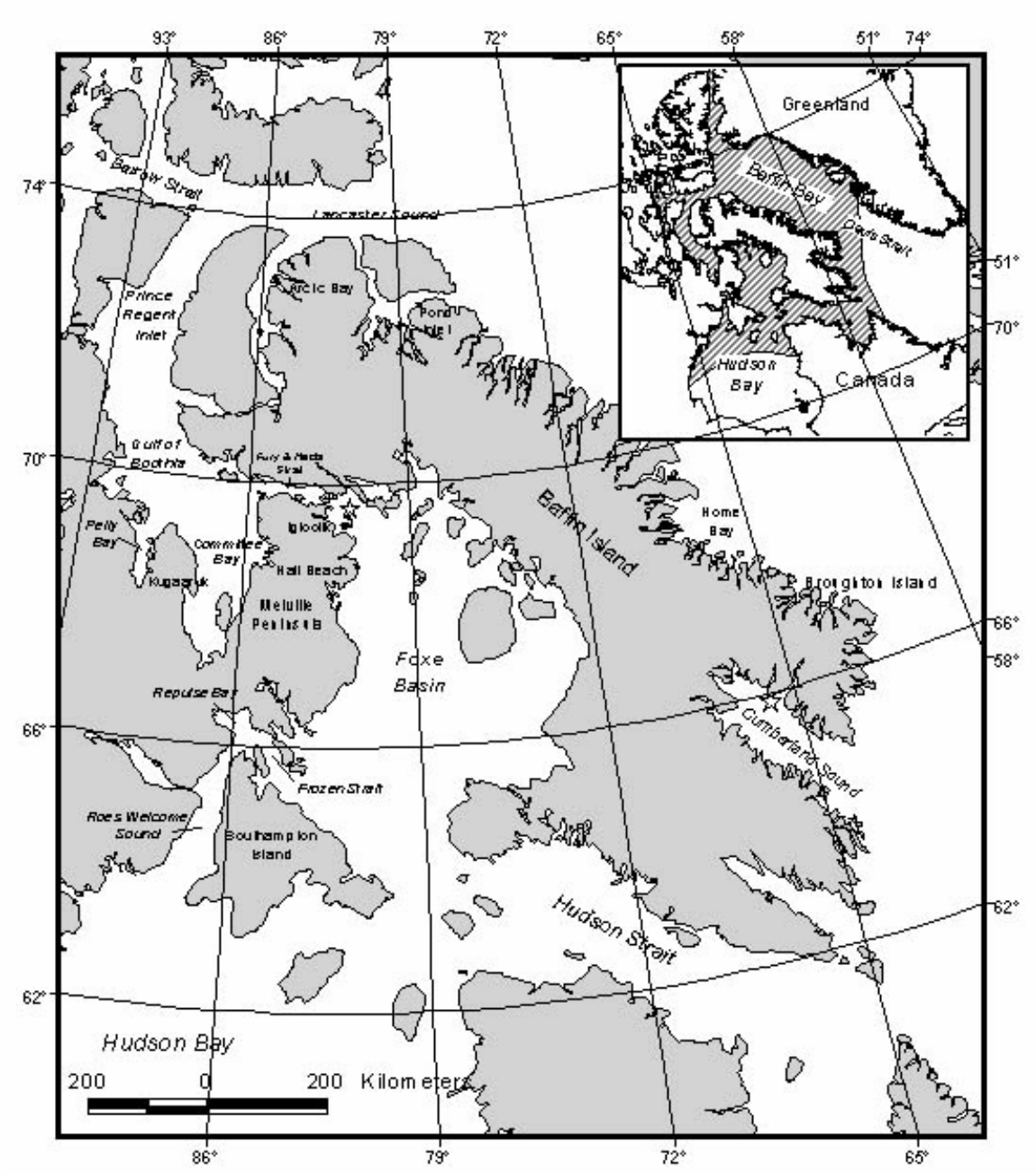

Figure 4.4. Map of Eastern Canadian Arctic depicting placenames where samples were collected. Figure is from Dueck et al. (2006).

A small portion of each sample was digested with proteinase k utilizing standard protocols. Genomic DNA was extracted utilizing Sigma's extraction kit (Sigma-Aldrich,

St. Louis, MO). Mitochondrial sequences and haplotypes for the control region were from previously published papers (MacLean, 2002; LeDuc, 2007; Postma, 2005). Amplification of the ND1 mitochondrial fragment was accomplished according to Cronin et al. (1996) with LGL 381 and LGL563. PCR amplicons were visualized through agarose gel electrophoresis. Successful amplicons were subsequently purified using Qia-quick PCR purification columns (Qiagen, Valencia, CA). Fragments were sequenced with internal primers (287 [5'CCT ACG TGA TCT GAG TTC AGA CC3'] and Whale_ND1_550 [5'GTC TAT TCC ATC CTA TGA TCC3']) utilizing ABI Big Dye v 3.1 (Applied Biosystems, Foster City, CA) according to the manufacturers standard protocols. Sequences were analyzed using an ABI3100 (Applied Biosystems, Foster City, CA) and subsequently aligned using Sequencher v4.2 (Gene Codes, Ann Arbor, MI) with final alignment and nucleotide calls confirmed by eye.

Nuclear microsatellite variation was determined utilizing primers described in Huebinger et al. (2007). Twenty-two of the twenty five loci were utilized in this analysis. Bmy 38, Bmy44, and Bmy47 were excluded from the analysis for reasons stated in Givens et al. (2007). Microsatellites were electrophoresed on an ABI3100 (Applied Biosystems, Foster City, CA) Allelic size calls were made in comparison to 400HD-Rox size standard and analyzed using Genemapper v4.0 (Applied Biosystems, Foster City, CA).

For each mtDNA dataset (ND1 and control region) we utilized Arlequin (Excoffier et al., 2005) to calculate the number of haplotypes (H) and nucleotide diversity (π) within populations. Estimates of pairwise Fst and number of migrants

between populations for the mitochondrial sequence data were also calculated with Arlequin. DNAsp (Rozas et al., 2003) was used to calculate Φst and Tajima's D statistic. Neighbor-Joining phylograms were constructed using MEGA v 3.1 (Kumar et al, 2004).

For the microsatellite dataset, tests for departure from Hardy-Weinberg equilibria were conducted in GENEPOP (Raymond and Rousset, 2004), utilizing exact tests based on Markov chain iterations (Guo and Thompson, 1992). Tests for genotypic linkage disequilibrium were tested between each pair of loci using the GENEPOP implementation of a MCMC exact test for independence. For the microsatellite data, estimates of Fst were computed for populations using the method of Weir and Cockerham (1984). Bounds for the 95% confidence intervals for Fst were obtained by jackknifing over loci as computed by the program FSTAT (Goudet, 2004). Utilizing Arlequin v 3.1, the effective number of migrants per generation was calculated between populations (Excoffier et al., 2005). Population structure was investigated using STRUCTURE v2.0 (Pritchard et al., 2000; Falush et al., 2003). For each STRUCTURE run a burn-in of 50,000 iterations were utilized and $1x10^6$ iterations for data collection, using the admixture with correlated allele frequencies model. Differing numbers of population sizes (K) were tested within STRUCTURE to determine the highest log-likelihood of the number of inferred populations. Tests for genetic bottlenecks were performed using BOTTELNECK (Cornuet and Luikart, 1996; Piry et al., 1999).

Results

Mitochondrial DNA control region sequences were analyzed for 725 bowhead whales from the three extant populations (BCB, Okhotsk, and Canada/Greenland). Of the 397 nucleotides scored there were 47 polymorphic sites, of which 39 were parsimony informative sites. These polymorphisms generate 78 different haplotypes within the control region. Neighbor-Joining phylogram of the bowhead control region haplotypes is shown in Figure 4.4. For the ND1 region 284 individuals were analyzed. A total of 957 nucleotides were examined for the ND1 region per individual. For the sequences analyzed 40 polymorphic sites were found, of which 24 sites were parsimony informative. This variation resulted in 37 different haplotypes for the ND1 region being identified. A summary of the mitochondrial statistics is listed in Table 4.2. Neighbor-Joining phylogram of ND1 haplotypes is shown in Figure 4.5. Estimates for Fst and Φst for the mitochondrial genes are listed in Table 4.3. Additionally, the effective number of migrants calculated for each mitochondrial gene is summarized in Table 4.4.

Microsatellite DNA A total of 621 individuals for the three extant populations of bowhead whales were analyzed for 22 nuclear microsatellites. A summary of the number of individuals, average number of alleles, and heterozygosity is described in Table 4.2. Values for the Fst comparisons between populations and the estimated number of migrants per generation are listed in Table 4.5. Graphical plots of the STRUCTURE outputs for the comparisons between all populations for differing values of k is shown in Figure 4.7. Comparisons utilizing STRUCTURE for only BCB and Canada is shown in Figure 4.8. Numbers on the X-axis in the STRUCTURE plots denote sampling locations within each population as given in the legend. Pairwise allelic differentiation between populations (BCB, Canada, and Okhotsk) were significant ($p < 0.00$). Tests for genetic bottlenecks with BOTTLENECK were non-significant for heterozygosity excess and no mode shift was detected for all populations tested.

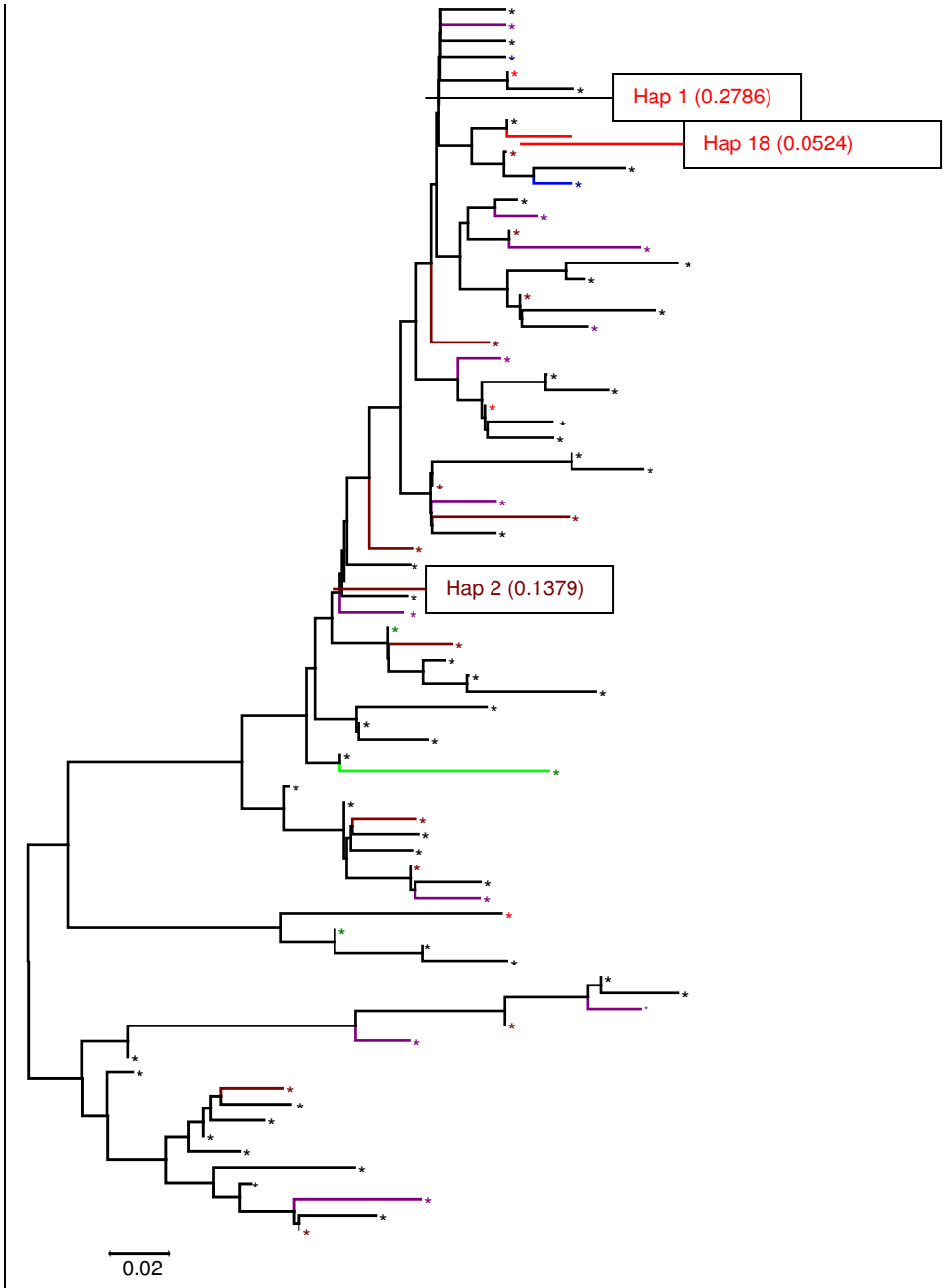

Figure 4.5 Neighbor-Joining phylogram of control region haplotypes. Frequency of each haplotype is listed next to each node in parentheses. Nodes designated with an asterisk have a frequency of less than 5%. Haplotypes are colored with the respective for which population they were identified within: Black--BCB only, Purple--Canada only, Blue--Okhotsk only, Red--BCB/Canada/Okhotsk, Maroon--BCB/Canada, Green--BCB/Okhotsk. Total number of control region sequences analyzed was 725.

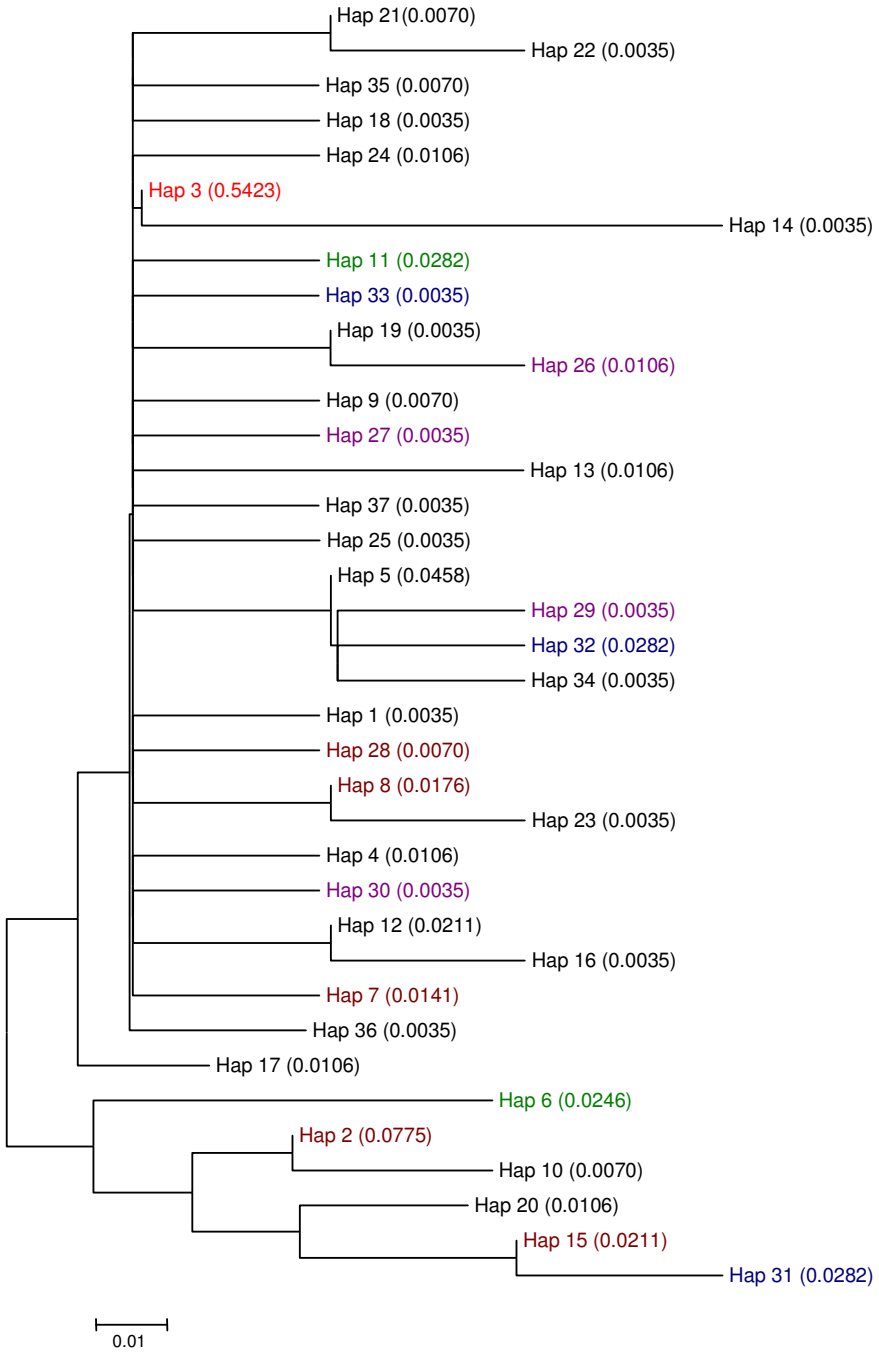

Figure 4.6 Neighbor-Joining phylogram of ND1 haplotypes. Frequency of each haplotype is listed next to each node in parentheses. Haplotypes are colored with the respective for which population they were identified within: Black--BCB only, Purple--Canada only, Blue--Okhotsk only, Red--BCB/Canada/Okhotsk, Maroon--BCB/Canada, Green--BCB/Okhotsk. The number of whales sequenced = 284.

Table 4.2. Summary of genetic variability statistics for (a) mitochondrial control region, (b) mitochondrial ND1 gene, and (c) nuclear microsatellite data. Number of unique haplotypes in each population is shown in parentheses next to the total number of haplotypes for each population.

Populations	All	BCB	Canada	Okhotsk
(a) mtDNA-control region				
N*	725	383	279	63
No. of haplotypes	77	65(45)	30(11)	6(1)
H	0.896±0.008	0.92±0.01	0.856±0.014	0.720±0.046
π	0.089±0.049	0.097±0.053	0.073±0.042	0.088±0.049
Tajima's D	-1.05736	-0.99297	-1.03562	1.4242
(b) mtDNA-ND1 gene				
N	284	182	51	51
No. of haplotypes	37	30(21)	10(4)	6(3)
H	0.695±0.03	0.718±0.036	0.500±0.085	0.736±0.046
π	0.038±0.025	0.035±0.024	0.025±0.019	0.057±0.035
Tajima's D	-2.15575*	-2.17153*	-1.96940*	0.08864
(c) microsatellite DNA				
N	621	302	256	63
K	16.9	15.73	14.524	10.045
Ho	0.8178	0.8143	0.8178	0.7778
He	0.8331	0.8229	0.8331	0.7849

Table 4.3. Genetic differentiation in terms of pairwise F-statistics. Above the diagonal, genetic distance-base Fst values are given for the mtDNA sequence data; Below the diagonal Φst values for the mtDNA sequence data. First line, control region; second line, ND1 gene. * denotes significance at $p < 0.05$.

	BCB	Canada	Okhotsk
BCB	X	0.02062* 0.02457*	0.04729* 0.05174*
Canada	0.02114 0.01067	X	0.07632* 0.09333*
Okhotsk	0.04919 0.05563	0.07125 0.07225	X

Table 4.4. Summary of effective number of migrants (Nm) for mitochondrial data. Rates of migration for the ND1 gene are above the diagonal, whereas rates for the control region are below the diagonal

	BCB	Canada	Okhotsk
BCB	X	19.852	9.164
Canada	23.746	X	4.85714
Okhotsk	10.073	6.0517	X

Table 4.5. Summary of Fst values and number of effective migrants (Nm) for the nuclear microsatellite data. Fst values with 95% confidence intervals are shown above the diagonal. Effective number of migrants are shown below the diagonal. * denotes significance at p <0.05.

	BCB	Canada	Okhotsk
BCB	X	0.005±0.002*	0.034±0.008*
Canada	55.44	X	0.037±0.009*
Okhotsk	6.91	6.36	X

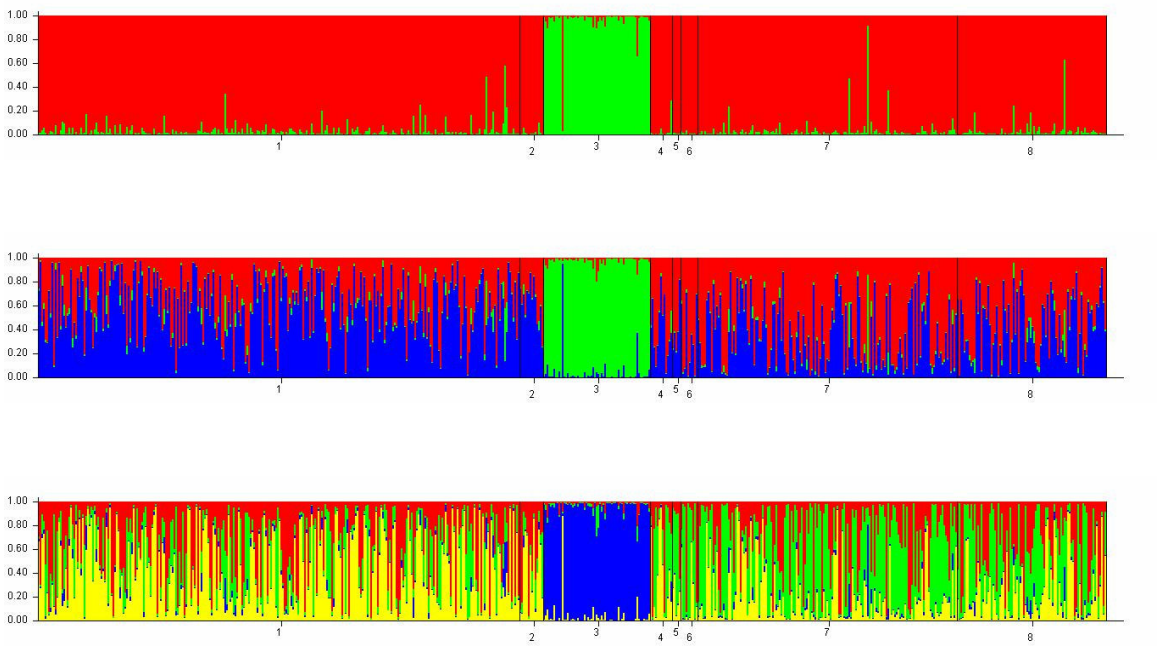

Figure 4.7. Graphical representation of individual probabilities of population assignment for k=2, k=3, and k=4. Comparisons used only individuals from the BCB and Canada population. Along the x-axis are the populations sampling locations: BCB (1), Chukotka (2), Okhotsk Sea (3), Mackenzie Delta, (4), Pelly Bay,CA (5), Repulse Bay, CA (6), Igloolik (7), Pangritrung, CA (8)

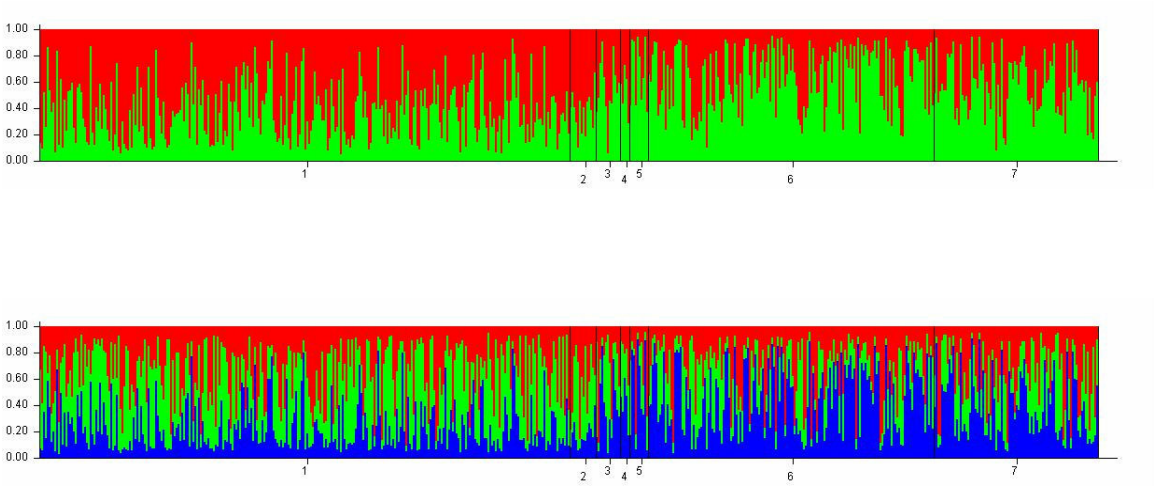

Figure 4.8. Graphical representation of individual probabilities of population assignment for k=2 and k=3. Comparisons used only individuals from the BCB and Canada population. Along the x-axis are the populations sampling locations: BCB (1), Chukotka (2), Mackenzie Delta (3), Pelly Bay,CA (4), Repulse Bay, CA (5), Igloolik (6), Pangritrung, CA (7)

Discussion

Throughout their recent history, bowhead whale populations they have experienced a marked decline due to commercial whaling. Some of the populations (BCB and Canada) have recovered to levels significantly above the levels to which they were reduced at the cessation of commercial whaling. Some whales born before the cessation of commercial whaling are still currently alive and reproducing. Although bowheads have a long gestation interval, the long-lived nature of bowheads has contributed to the recovery of the BCB and Canadian populations. The BCB and Canada populations are approaching pre-commercial whaling population levels. The Okhotsk population however continues to remain at levels similar to the size at the end of commercial whaling. In addition, the migratory and highly mobile nature of bowhead

whales has provided additional avenues for recovery and genetic exchange between populations.

Examination of mtDNA comparisons among populations revealed a high number of shared haplotypes. The Canadian and BCB populations exhibited a high number of haplotypes and of shared haplotypes for both mitochondrial genes compared to the Sea of Okhotsk, which possessed only 6 haplotypes for each mitochondrial gene examined. Haplotypes unique to the BCB and Canadian populations are considered to be rare as each unique haplotype appeared in a limited number of individuals. The reduced number of haplotypes exhibited in the Sea of Okhotsk population is probably caused by a multitude of effects. The Okhotsk population size before exploitation due to commercial whaling was presumably 5-fold smaller than either the BCB or Canadian population. Secondly, the population was reduced to only a few hundred individuals. The small population size, coupled with the populations lack of recovery could explain the smaller number of mitochondrial haplotypes found within the Okhotsk population.

Measures of the effective numbers of migrants (Nm) calculated for the both mitochondrial genes suggested lower levels of migration between the Okhotsk population and BCB and Canada, respectively, than was found between BCB and Canada. The reduced level of migration could contribute to the lower level of mitochondrial diversity seen in the Sea of Okhotsk bowheads. It was thought that the BCB and Sea of Okhotsk populations came into contact during the 'Little Ice Age.' If that in fact occurred, the mitochondrial data suggests there was very limited exchange between BCB and the Okhotsk during this time period.

Analysis of the microsatellite data yielded similar results to the mitochondrial comparisons of the Okhotsk population with the BCB and Canadian populations. The Fst for microsatellite data between the Okhotsk and remaining populations is six-fold higher than the comparisons between BCB and Canada. Additionally, allele frequencies between the Okhotsk and the other populations were significantly different. As shown in Figure 4.7, STRUCTURE was quite effective at detecting individuals from the Okhotsk population with a high probability. This finding is not unexpected however as STRUCTURE is normally effective at identifying individuals as members of populations when those populations have levels of differentiation above an Fst of 0.01. In instances were the Fst is lower, the program is normally unsuccessful at identifying plausible population divisions.

When comparing the BCB and Canada populations with mtDNA, it is also found that there are a large number of shared haplotypes for both control region and ND1. The instances of unique haplotypes in each population were found to be rare haplotypes that were found in single individuals (primarily male whales born before the end of commercial whaling). When comparing the Fst values between BCB and Canada, the values are low demonstrating little differentiation between these populations. As expected with low Fst values for the mitochondrial data, there is a high estimated number of migrants between these populations from these mitochondrial comparisons. These values support a high level of exchange between the BCB and Eastern Canadian bowheads. Additionally, the phylogram of haplotypes for each mitochondrial gene was unable to distinguish phylogenetic structure among the populations (Fig. 4.5 and 4.6).

Similar findings are found when the microsatellite data is used to compare the BCB and Eastern Canadian Arctic bowheads. Results from the microsatellite analysis also showed lower Fst and high numbers of migrants. When analyzing the STRUCTURE results for comparisons between BCB and Canada, the program was unable to identify individuals as two separate stocks. Although STRUCTURE was unable to identify individuals to a specific stock, individuals from the BCB were on average 68% of red ancestry, whereas the Canadian bowheads were 68% green ancestry on average when k=2 (Figure 4.8). The inability of STRUCTURE to identify two discreet stocks is not surprising due to the fact that the program has difficulties identifying groups when the level of differentiation is small.

The low level of differentiation and migration level between the BCB and Canadian bowheads is supported by several biological observations. Several cases of known migrants from Canada to the BCB have been identified through types of harpoons found embedded in whales harvested at Point Barrow. These unique whaling irons were only used for the commercial harvest of bowheads in the Eastern Canadian Arctic (IWC, 2001; Rugh et al., 2003). Additionally, satellite tracking of a single male bowhead whale from the BCB showed it was able to traverse extreme ice conditions to reach the North side of Banks Island. At that point, the bowhead would have found less ice conditions if it continued to migrate to Canada than it encountered on its return to the BCB migration route. Further, the distance to the Eastern Canadian Arctic was also a shorter geographic distance than the route back to the Canadian Beaufort Sea feeding grounds (Quakenbush, 2007).

Even though the difference found between the BCB and Canada is small, there is still a statistically significant difference for allelic frequencies between the two populations. This level of differentiation even with known migration between Canada and the BCB is still sufficient to recognize two different biological stocks. Each of the populations has its own migration route. These routes do not overlap and they have separate breeding and feeding areas.

Through comparing the different markers analyzed, one can determine if there are differential rates of migration relative to the sex of the whales. If there is equal migration between the sexes, the mitochondrial rate of migration should be four-fold lower than the microsatellite migration rate due to the haploid maternal inheritance of mitochondria. If there was more migration attributed to males, the Fst of the microsatellite data when compared to the mitochondrial Fst data would be lower than a four fold difference. In the case of bowhead whales in the Sea of Okhotsk, the Fst (and subsequently the rate of migration) calculated from the mitochondrial data is only two-fold lower than rate calculated from the microsatellite data. This suggests that the migration between Okhotsk and the BCB is female biased. In examination of the migration between BCB and Canada, the migration appears to be equal between sexes or slightly male-biased.

Bowhead whales are potentially highly mobile and can survive extreme climatic conditions. This level of mobility and its long life-span have contributed to the present populations mixing and give hope to the ultimate natural restoration of the other historical populations which have not yet rebounded after the whaling restrictions were

put in place for this species. Although there is overall differentiation between populations, there is still migration between the separate stocks of bowheads. This higher migration rates between Canada and BCB populations has lead to a low level of differentiation between High Arctic stocks and perhaps expedited the recovery of both populations. While a substantially lower migration rate has led to a moderate level of differentiation between the Okhotsk population and the High Arctic populations, there appears to be sufficient migration to lead to a natural recovery of the Sea of Okhotsk population.

CHAPTER V

CONCLUSION

Work for the implementation review of the aboriginal subsistence harvest quota of BCB bowheads was initiated in 2004. As part of the review, multiple meetings over multiple years were held to discuss and analyze the most current data as it related to stock structure of bowhead whales within the BCB. The culmination of the scientific effort and discussion was at the 2007 Scientific Committee meeting of the IWC in Anchorage, AK. At the meeting the chair of the Bowhead-Right-Gray (BRG) Whale committee and the chair of the standing working group on aboriginal whaling management procedure (AWMP) thanked the U.S. scientific delegation for their considerable effort in obtaining the data utilized in the implementation review. They noted that the examination of the available data was thorough and based upon this examination it was concluded that there is one stock of BCB bowheads. Based upon the recommendation that there is one stock, the present management scenario used for determining the aboriginal subsistence harvest quota for BCB bowheads is adequate to provide management advice. Utilizing the advice given by the scientific committee, the IWC commissioners passed by consensus of all members a quota of 280 strikes over the next five years. This maintained the present level of catch limits set forth by the IWC.

The additional analysis of bowheads from the extant stocks demonstrated differing levels of separation between stocks. With this differentiation, there was shown large levels of migration between some populations. This limited the level of differentiation between the BCB and Canada, but demonstrated the dynamic highly

migratory nature of bowhead whales. Further analysis of bowhead whale stocks will be undertaken utilizing nested clade analysis of the available data. Examination of the migration rates of male bowhead whales through sequencing of male specific DNA markers could provide useful insight into the dispersal rates of male bowheads particularly as it appears that female bowhead may be the dominant dispersers as discussed in Chapter IV.

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APPENDIX A

SC/57/BRG19 **PROGRESS REPORT ON THE DEVELOPMENT OF NEW MICROSATELLITE MARKERS FOR BOWHEAD WHALES**

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Abstract

This is a progress report for the bowhead whale genetics project being conducted at Texas A&M University. The objective of the first phase of the study is the development of a new and expanded panel of microsatellite loci from bowhead whales. This phase of the study was successful and considerable material now exists from which the new loci are being developed.

Introduction

Population genetic and conservation genetic studies based on microsatellites are a well-accepted means of addressing management conservation issues. An advantage of this approach is that there is virtually an unlimited number of loci that can potentially be studied. However, a serious problem with the method is that primers have been designed for a relatively small number of species at the present time. A perusal of the literature shows that most studies done on wildlife species are opportunistic in that loci that have already been described by other workers are used. Frequently these loci are from related but non-target species and typically the loci are a hodge-podge of different microsatellite repeat motifs. This raises the concern that scoring may not always be accurate or consistent and that different microsatellite motifs might evolve under different constraints. As with our initial study of bowhead whale microsatellites in which we found highly variable results from locus to locus, this does not represent an optimal approach to the problem (Bickham et al., 2004). Therefore, in this project we have taken a more systematic approach. Our initial focus is to develop a panel of approximately 25 loci from the target species (bowheads) that will likely include mostly, if not entirely, pure CA microsatellite repeats. The primers will be thoroughly tested for consistency and accuracy as well as variability within the species. The development of the new loci and their application should reduce the uncertainty about the quality and repeatability of the results.

Methods

From genomic DNA extracted from a bowhead whale skin sample three different enriched libraries were created. The libraries were enriched for microsatellites of the

following sequences: CA, GATA, and GTCT. We isolated 3000 microbial colonies from the CA library which showed evidence of a bowhead whale DNA insert. Hybridization using a CA probe of ~1000 colonies yielded 300 colonies (positives) with ostensible CA microsatellite repeats. The inserts to 90 of these positives have been sequenced. Sequence analysis shows that 70% of the sequenced clones have microsatellites that are potentially suitable for development. We therefore expect to have approximately 210 clones with microsatellites. We are presently analyzing the sequence data to determine how many of the approximately 210 loci will be developed. Some proportion of loci will be unsuitable due to any one of a number of reasons including having repeat motifs that are too large or too small, or the microsatellite might be located too near to one end of the insert, or the microsatellite might be a "degenerate repeat", or the flanking regions might be unsuitable for good primer design.

Results

Primers were designed and optimized for 23 loci. Another 10 loci have been sequenced but the primers not yet designed. And, there are another approximately 200 positives that remain to be sequenced if it becomes necessary to do so. We can expect that perhaps as many as 1/2 to ¾ of the loci could be usable but if not, we will repeat the procedure by hybridizing the second 1000 colonies, and even the third 1000 colonies if need be. In addition, we could hybridize the GATA and GTCT tetrameric microsatellite colonies as well. The tetramer libraries have had approximately 1500 colonies picked for each library. However, these have yet to be hybridized to check for positives.

Table A-1. Primer sequences for six microsatellite loci, optimization temperatures, fragment size, and results of tests for polymorphism. All loci were established from a bowhead whale DNA library enriched for CA repeats.

Locus	Temp	Size	Polymorphic	Primer Sequence
Bmy1	50^0 C	250	yes	F 5'-AAA TCA ACC AGA ACA GGA GTC AAA CTT R 5'-CGA TTT TTA TTT CAT CTT ATT TCC CTT
Bmy4	66^0 C	300	no	F 5'-TGA TAT GGG ACA GAC CAT GCA CT R 5'-GGC CTT TAT CCA CTG GAT GCT A
Bmy6	66^0 C	157	no	F 5'-TGA GGG TGG GGA AAC TGC CTT R 5'-TCA GGG TCA ATG TCA GGA ACG AG
Bmy10	50^0 C	250	yes	F 5'-CGC AGG AAG CTG TTC TCT TAC CC R 5'-GCC CCA AGA GGA TTT CTC TGC A
Bmy19	56^0 C	125	yes	F 5'-TGC CGC TGC CTC TGT ATT GG R 5'-GGC AAA GCA AGG TTA CAG AAA AGT C
Bmy20	60^0 C	234	yes	F 5'-AAG CTC CTC CCC TCT CAC TGG R 5'-GCG GGC AAG AGA GAA CGT CT

Table A.1 shows the primer sequences for six loci that have been tested for variability. All six loci gave clear and consistent bands of which we were able to score as variable or not variable on 1% agarose gels. Four of the six were variable and will be

employed in our ongoing studies of stock structure in bowhead whales. Additional new loci will be incorporated if they are tried and tested and found to be variable.

Discussion

We presently have enough material to make as many as 200 loci which is far more than are needed. From the initial battery of 33 possible loci we can expect that as many as 20 might be variable and suitable for use, extrapolating from our preliminary assessments of variability. The next step is to conduct an analysis on a small number of whales to determine which of the loci give us good PCR products and show variability in the population and can be consistently scored. Then, we will order fluorescence-labeled primers and begin the analyses of the large dataset. The objectives are to develop a battery of at least 25 loci that are variable, reliable and which can be consistently scored even in samples of not optimal quality. The likely timetable for finishing the development of the system is June 2005. By Spring 2006 we hope to finish the analyses of the whale samples and to be able to present the data at the 2006 IWC meeting.

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Bickham, J. W., D. D. Hunter, C. W. Matson, R. M. Huebinger, J. C. Patton, and J. C. George and R. Suydam. 2004. Genetic variability of nuclear microsatellite loci in Bering-Chukchi-Beaufort Seas bowhead whales (*Balaena mysticetus*): A test of the genetic bottleneck hypothesis. Paper SC/56/BRG18 presented to the Scientific Committee of the International Whaling Commission, June, 2004.

APPENDIX B

SC/58/BRG11 PROGRESS REPORT ON THE DEVELOPMENT OF NEW MICROSATELLITE MARKERS FOR BOWHEAD WHALES

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Abstract

To allow a more accurate assessment of genetic variation in bowhead whales a new panel of microsatellite loci has been developed at Texas A&M University. A small insert genomic library enriched for CA repeats was constructed and screened using standard protocols. A total of 196 identified clones were sequenced. A total of 61 loci were identified from among the sequences for further testing and appropriate primer pairs were designed for these loci. Of this group 34 loci yielded PCR products which exhibited clear polymorphisms on agarose gels. These 34 loci were labeled with fluorescent dyes and run on the ABI 3100 to determine their quality of amplification and their ease of analysis. A final set of 24 loci were selected from among the 34 tested for their consistent amplification and their ease in determining allele calls.

Introduction

Population genetic and conservation genetic studies based on microsatellites are a well-accepted means of addressing management conservation issues. An advantage of this approach is that there is virtually an unlimited number of loci that can potentially be studied. However, a serious problem with the method is that primers have been designed for a relatively small number of species at the present time. A perusal of the literature shows that most studies done on wildlife species are opportunistic in that loci that have already been described by other workers are used. Frequently these loci are from related, but non-target species and typically the loci are a hodge-podge of different microsatellite repeat motifs. This raises the concern that scoring may not always be accurate and consistent and that different microsatellite motifs might evolve under different constraints. As with our initial study of bowhead whale microsatellites in which we found highly variable results from locus to locus, this does not represent an optimal approach to the problem (Bickham et al., 2004). Therefore, in this project we have taken a more systematic approach. Our initial focus was to develop a panel of approximately 25 loci from the target species (bowheads) that will be exclusively derived from pure CA microsatellite repeats. The primers will be thoroughly tested for consistency and accuracy as well as variability within the species. The development of the new loci and their application should reduce the uncertainty about the quality and repeatability of the results.

Methods

From genomic DNA extracted from a bowhead whale skin sample an enriched library for CA microsatellite repeats was created. We isolated 3000 microbial colonies from the CA library which showed evidence of a bowhead whale DNA insert. Hybridization using a CA probe of ~1000 colonies yielded 300 colonies (positives) with ostensible CA microsatellite repeats. The inserts of 196 of these positives have been sequenced. Sequence analysis shows that 70% of the sequenced clones have microsatellite that were potentially suitable for development. A total of 61 loci were further selected from the sequenced clones and appropriate primer pairs were designed. These primer pairs were tested for successful PCR amplification. Those primer pairs that yielded quality PCR products were screened for polymorphism by electrophoresing on 3% agarose.

Results

A total of 34 loci demonstrated polymorphism on agarose and were subsequently labeled with fluorescent dyes. These 34 labeled loci were then amplified across a panel of individuals and run on an ABI 3100. The loci were analyzed for their quality of amplification and their ease of analysis. From this a total of 24 loci were selected based upon the results of the amplification of the test group of individuals. These 24 loci were then amplified across a larger group of bowhead whales.

Discussion

We presently have a panel of 24 loci that have been successfully run on 170 bowhead whales. Upon further analysis of the 24 individual loci, it was determined that one of the loci (Bmy47) has demonstrated linkage to the X chromosome. In addition, the loci selected for the panel have shown consistent amplification across the majority of samples including samples that were of questionable quality. These loci are all dinucleotide CA repeats derived specifically from bowhead whales. The timetable to finish the genotyping of all whales currently sampled will be the end of summer 2006.

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Bickham, J.W., D.D. Hunter, C.W. Matson, R.M. Huebinger, J.C. Patton, J.C George, and R. Suydam. 2004. Genetic variability of a nuclear microsatellite loci in Bering-Chukchi-Beaufort Seas bowhead whales (Balaena mysticetus): A test of the genetic bottleneck hypothesis. Paper SC/56/BRG18 presented to the Scientific Committee of the International Whaling Commission, June, 2004.

APPENDIX C

SC/59/BRG14 PATTERNS OF GENETIC DIFFERENTIATION IN BOWHEAD WHALES (*BALAENA MYSTICETUS)* FROM THE WESTERN ARCTIC

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Abstract

Analysis of 33 microsatellite loci for bowhead whales, including 22 new highly reliable markers, suggests present or historical departures from panmixia in Bering-Chukchi-Beaufort Seas bowhead whales. Although these bowheads are clearly genetically distinct from bowheads in the Sea of Okhotsk, we find significant patterns of genetic inhomogeneity among the Bering-Chukchi-Beaufort Seas samples. These samples exhibit strong and widespread departure from Hardy-Weinberg equilibrium, including significant evidence of a historical bottleneck consistent with gene drift after commercial exploitation or thousands of years earlier. There is also significant evidence that whales of detectably different ancestry intermingle during some spatio-temporal portions of the annual migration but partially segregate in other portions. The most notable such pattern is seen in migratory pulses passing Barrow in the fall. Estimates of F_{st} associated with our findings of genetic structure in Bering-Chukchi-Beaufort Seas bowheads are extremely small compared to values for comparisons with the known separate stock in the Sea of Okhotsk, and are also smaller than values obtained by separating suspected familial lineages within the Bering-Chukchi-Beaufort Seas samples. Furthermore, potential model misspecification provokes skepticism about some detected patterns, notably including the temporal ones. When analysis is limited to the most trusted markers and samples, sensitivity analyses show that most of our findings vanish and that the main sources of genetic signal in these data are scoring errors, familial relations, and birth year. We conclude that Bering-Chukchi-Beaufort Seas bowheads may comprise a complex spatio-temporal aggregation of animals with mixed and variable ancestry with an unknown degree of nonrandom mating, whose degree of genetic inhomogeneity is significantly less than what is seen between spatially isolated stocks. Despite these intriguing and complex

biological findings, we have found no convincing evidence that Bering-Chukchi-Beaufort Seas bowheads should be managed as more than one stock.

Introduction

Bowhead whales (*Balaena mysticetus*) have been hunted by aboriginal communities on the North Slope of Alaska, in the Bering Sea, and along the Chukotka Peninsula for centuries. These whales, known as the Bering-Chukchi-Beaufort Seas stock (hereafter BCB bowheads) migrate through this region and are hunted within the migration route at several villages on the Alaskan mainland coast, on islands including St. Lawrence Island, and on the Chukotka coastline in Russia.

BCB bowheads winter in the Bering Sea within the marginal sea ice edge and within "polynyas" or persistent areas of open water within the pack ice (Figure C-1). In spring, the whales migrate northward through leads and polynyas, past St. Lawrence Island, where the villages of Gambell and Savoonga are located. The Gambell hunt occurs directly offshore from the village. The spring Savoonga hunt occurs on the south side of the island, whereas the fall Savoonga hunt occurs on the north side roughly offshore from the village. The whales continue to migrate north through the Bering Strait and the Chukchi Sea, then most move east through the Beaufort Sea to summering areas. During migration bowheads pass other villages including Barrow, where the majority of the aboriginal hunt occurs. Estimates of population abundance and trends are also made near Barrow (George et al. 2004a). The extent to which spring migrants visit the Chukotka region is unclear although some 550-1200 whales have been estimated to pass by the Cape Dezhnev region heading northward in 2000 and 2001 (Melnikov and Zeh, 2006). Some whales may remain in the Chukotka region during summer (Melnikov et al. 2004), but bowheads mainly summer in the eastern Beaufort Sea. They migrate west and south again in the autumn. The autumn migration may be more geographically dispersed than spring, with many whales passing northern Chukotka (Moore et al., 1995). Fall migrants passing Saint Lawrence Island are hunted as late as January. A more thorough description of the migration is given by Rugh et al. (2003) and Moore and Reeves (1993).

The aboriginal subsistence hunt of these whales is managed by the International Whaling Commission (IWC). Safe annual hunting quotas are estimated using statistical population dynamics modeling and assessment methods, with priority given to whale population recovery as well as the nutritional and cultural needs of the aboriginal people. This management is predicated on the IWC's conventional wisdom that BCB bowheads constitute a single stock, from both a biological and management perspective. Evidence supporting this viewpoint is summarized by Rugh et al. (2003). Such evidence includes records of the spatio-temporal evolution of the historical commercial hunt, traditional knowledge from aboriginal hunters, persistent patterns of age-segregation in the annual migration, some previous analyses of mtDNA and microsatellite data, recovery in BCB bowheads of tags and harpoons from diverse regions, the highly labile nature of bowhead migration depending on factors related to ice, food, and anthropogenic disturbances, and apparent population growth rates in Chukotka beyond what could be attributed to a separate small stock (Rugh et al., 2003; IWC, 2001).

Despite the evidence supporting a single-stock hypothesis, there are several motivations for further stock structure research. First, whale stock / sub-stock structure is hypothesized or known to exist on quite modest spatio-temporal scales for some species, such as beluga whales (*Delphinapterus leucas*; O'Corry-Crowe et al. 1997). Second, several adjacent bowhead stocks—in the Sea of Okhotsk and in Canadian waters—may have been more closely related to

BCB bowheads long ago, and there is harpoon and tag recovery evidence of exchanges between stocks believed to be distinct (IWC, 2001; Rugh et al. 2003). Third, some analyses of early genetic data have found indications of genetic inhomogeneity among BCB bowheads (Jorde et al. 2007; Givens et al. 2004; Pastene et al., 2004). Of these, the most notable may be a temporal correlation feature referred to as the 'Oslo Bump' (as termed by IWC, 2006, p. 111), indicating that bowheads migrating past Barrow in the fall of each year are less genetically similar if they are about 5-11 days apart than at other temporal separations (Jorde et al., 2007). Finally, the historical period of commercial whaling of BCB bowheads (1848-1914) was distinguished by a very strong spatio-temporal pattern of exploitation, some age-selective hunting, and a severe depletion of the resource (Bockstoce and Botkin 1983; Bockstoce and Burns, 1993). The effects of commercial whaling could leave a persisting genetic imprint today, considering the long lifespan and long generation time of these whales. In particular, 5 of 84 landed bowheads aged using aspartic acid racemization exceeded 100 years old, with the oldest estimated to be 178 years old (Rosa et al., 2004).

This hypothesis of a potential historical genetic imprint of commercial hunting deserves further explanation. During the period of commercial whaling, BCB bowheads were severely depleted. Indeed, the cessation of commercial hunting was driven in part by whale depletion reaching levels that rendered whaling economically unviable (Bockstoce and Botkin, 1983; Burns et al., 1993). Since that time, BCB bowhead abundance has increased steadily (George et al. 2004a). Yet, at the point of maximum depletion, there may have been only a few hundred or fewer sexually mature females.

Recent biological data, most notably corpora counts (George et al., 2004b), suggest that long-lived, highly active female matriarchs may produce a large proportion of bowhead offspring. It is unclear whether males exhibit highly variable breeding behavior, although biologists' field notes of testes sizes in harvested animals show highly variable development of the male sexual organs (O'Hara et al. 2002). In the decades surrounding the end of the commercial harvest, the new calves produced may have originated from a small number of mothers and fathers. In recent decades, BCB bowheads have grown to be quite numerous and presumably genetically diverse.

Questions about bowhead stock structure are important for effective resource management and conservation. If we adopt the term 'management units' to describe groups of individuals among which the degree of connectivity is sufficiently low so that each group should be monitored and managed separately (Taylor and Dizon, 1999), then IWC debate about BCB bowhead stock structure is driven by uncertainty and disagreement about how much sub-structuring exists among BCB bowheads and whether patterns of disaggregation are of sufficient magnitude to warrant division of BCB bowheads into multiple management units. It is also important to look beyond genetics to determine what biological and other data can say about stock structure. Taylor (2004) discusses how biological, demographic, and management-related information can require quite different approaches to stock structure inference for different whale populations.

Palsbøll et al. (2006) argue that the identification of management units from genetic data should be based on the amount of genetic divergence at which populations become demographically independent, rather than on statistically significant rejection of the null hypothesis of panmixia. We agree with this viewpoint, but we begin our paper with several analyses that test the panmixia hypothesis against alternatives with varying degrees of spatio-temporal or other specificity. We emphasize consideration of the magnitude of genetic divergence in the discussion section, where we consider what levels of population substructure are consistent with our findings and what the corresponding management implications might be.

Color versions of the figures in this paper can be obtained from the IWC Secretariat.

Data

Samples

Our dataset is based on samples from 457 bowheads. The vast majority of these are tissue samples of varying quality obtained from harvested animals. Some samples were obtained via non-lethal biopsy (6 Barrow, at least 13 Chukotka, 64 Sea of Okhotsk, and 48 Igoolik, Canada). As described below, the two main datasets used for analysis comprise 414 and 281 of these samples, based on various data screening criteria.

Laboratory analysis

In 2004, preliminary results were reported from statistical analysis of 12 microsatellite loci (Givens et al., 2004). These markers were chosen opportunistically from the available literature. Methods describing DNA extractions, PCR, and genotyping procedures for these markers are detailed by Bickham et al. (2004) and generally followed the methods of Rooney et al. (1999a). Briefly, DNA was extracted from tissue (skin and underlying tissues) and used to amplify 12 microsatellite loci that had previously been shown to be variable in bowhead whale populations (LeDuc et al., 1998; MacLean, 2002; Rooney et al., 1999b). Fluorescence labeled primers were used, and the PCR products were scored using an ABI 377 automated sequencer following the methods of McLean (2002). This differs from the methods used by Rooney et al. (1999a) in that those investigators used radioactively labeled PCR products scored visually from autoradiographs. The 12 microsatellite loci included GATA028 (Palsbøll et al., 1997), EV1 and EV104 (Valsecchi and Amos, 1996), TV7 (Rooney et al., 1999a), and TV11, TV13, TV14, TV16, TV17, TV18, TV19, TV20 (Rooney et al., 1999b). The latter set of loci, TV11 through TV20, were derived from bowhead whales whereas the former three sets were derived from sperm whale (EV1), humpback whale (EV104 and GATA28) and bottlenose dolphin (TV7).

After finding tentative indications of genetic structure using these markers, a new set of microsatellite loci was developed to increase statistical power and to overcome some concerns about the quality and reliability of the original markers. A total of 34 new microsatellite loci was developed from a genomic library enriched for CA_n repeats (An et al., 2004). From the initial 34 loci, 25 markers were selected for ease of PCR amplification and consistency in being able to reliably score the locus across all individuals. The development of these markers was described by Huebinger et al. (2006).

Allele designations for all loci are based upon estimated sizes, in number of base pairs, of the amplified product. Differences in allele sizes are the result of additions or deletions of base pairs. Microsatellite loci typically evolve by the addition or deletion of whole repeats. As a result, dimeric repeat microsatellites usually have alleles that differ in size by multiples of two or tetrameric repeats by multiples of four. Except for GATA28, which is a tetrameric repeat, all other loci are dimeric repeats or complex modifications thereof.

Data quality screening

Genetic markers: Of the 12 original loci, one (TV18) exhibits short allele dominance (Jorde et al., 2004). It has been eliminated from further analysis. For the remaining old loci and the new 25 loci, we examined the data for possible scoring problems such as null alleles, short allele dominance, and stutter bands, using the Microchecker program (van Oosterhout et al. 2004), using all available data for Barrow whales. Perhaps from a laboratory point of view pre-

specification of rigid rules for excluding loci from analysis is a good idea, but from a statistical point of view decisions to exclude outlier data are most sensibly made by assessing the severity of outlying, the likely inferential impact of including or excluding the outliers, and the existence of plausible extraneous causes for the outlying. Two loci (BMY38 and BMY44) exhibited statistically significant homozygosity excesses that were far more extreme than other loci and which would be likely to unduly affect analysis results if this homozygosity reflected scoring problems rather than true genotypes. These loci also showed estimates of null allele frequencies (using the methods of van Oosterhout et al. (2004) and Chakraborty et al. (1992)) that were many times larger than for other loci. These null allele frequency estimates are shown in Figure C-2 (which also raises questions about TV7 and TV11). In the case of BMY38, the severe excess of homozygotes coincided with a significant and severe deficiency of genotypes of one repeat unit difference and a moderate deficiency of two repeat unit differences. In our view, the most likely explanations for these findings are extraneous factors not related to population structure, namely that BMY44 has a null allele(s) and BMY38 suffers from stuttering and/or null allele(s).

A third locus (BMY47) demonstrated linkage to the X chromosome. No other biochemical or scoring problems were identified in the remaining 22 new loci.

All of the remaining analyses reported here exclude TV18, BMY38, BMY44, and BMY47. Thus our analyses are based on 33 loci: 11 original and 22 new.

The 33 loci chosen for analysis are not comparable. One measure of data quality is the number of individuals for which scoring failed for each locus. Across loci, the median scoring failure rate for the 11 original loci was triple the rate for the 22 new loci. Figure C-3 shows the rates for each locus. Clearly it was much more difficult to score the original loci than the new loci (which were specifically designed for reliable scoring). Locus BMY2 was a special case in that all Igoolik samples (which were processed in a different lab than most other samples) failed to amplify. The hypothesized reason for this failure is an error within the commercially synthesized primers. Adjusted for these 47 cases, the failure rate for BMY2 was only 0.059.

Heterozygosity and genetic diversity are much higher in the 22 new loci than in the 11 original loci. For example, using the Barrow data the new loci have average heterozygosity of 0.815 compared to 0.693 for the original loci. Similarly, F_{is} is estimated to be 0.008 for the new loci and 0.027 for the original loci, with respective 95% confidence intervals of (-0.002, 0.020) and (0.002, 0.053) obtained by bootstrapping over loci.

Such discrepancies between the two groups of loci are exactly what we would expect since some of the original loci were not developed specifically for bowheads. Imperfectly matching primers could result in dropped alleles and other technical problems that would be manifested as excess homozygosity in the data. For example, we are particularly concerned about the suitability of TV7, which was developed for the genetically distant *Tursiops* genus. IWC (2005) elaborates concerns about TV7, noting among other issues that TV7 had been one of several loci derived from *Tursiops*, most of which exhibited symptoms of ascertainment bias. The other markers had been rejected in the lab, but TV7 was retained for analysis specifically because it was found to be out of Hardy-Weinberg equilibrium.

The 22 new loci have some important advantages over past markers. All the new markers were specifically developed for bowheads and seem to present very few biochemical or scoring difficulties. This is in part because the sequence of the primers designed for bowheads should precisely match the samples being analyzed, thus reducing important technical variables

influencing data quality. These markers were also designed and selected based on their ability to amplify consistently and with relative strength. Data for the new loci were generated on an ABI 3100 capillary machine, which is more sensitive for detecting the amplified products and does not have problems with bleeding over into another lane, compared to the ABI 377 machine used for the old loci. Finally, there is greater statistical power available when using a larger number of loci.

Given these issues, we question the wisdom of relying solely on analysis of the 33 loci. Instead, we should confirm that important findings are found equally well in the 22 new loci as in the overall dataset. A similar strategy of sensitivity analysis was suggested by IWC (2005), although the approach was partially motivated then by a need to ensure a sufficient sample size of loci. With the 22 new loci now available, the balance between locus sample size and data quality may warrant adjustment in favor of greater quality

	Samples with microsatellite data	**Final counts used in 33-locus analyses (Spring+Fall=Total)**	**Final counts used in 22-locus analyses (Spring+Fall=Total)**
Barrow	260	98+115=213	108+123=231
Chukotka	16	3+12=15	3+12=15
Commander Isl.	4	0+0=0	0+0=0
Gambell	9	5+4=9	5+4=9
Kaktovik	16	0+12=12	0+15=15
Little Diomede	1	1+0=1	1+0=1
Nuiqsut	5	0+5=5	0+5=5
Point Hope	7	6+0=6	6+0=6
Savoonga	19	6+10=16	6+10=16
Wainwright	7	7+0=7	7+0=7
Unknown	1	0+0=0	0+0=0
Igoolik, Canada	48	0	47
Sea of Okhotsk	64	0	62
Totals	457	126+158=284	136+169+47+62=414

Table C-1: Counts of BCB bowhead samples for primary analyses. No seasonal data are given for Igoolik and Okhotsk samples.

Samples: There is also the question of which whales to analyze. Following the recommendation of the AWMP SWG, we limited primary consideration to whales successfully scored on at least 30 of 33 loci (IWC, 2007). Whales from Okhotsk and Igoolik, Canada, were scored only on the new loci. In analyses using these samples and only the 22 new loci, whales were limited to those who were scored successfully on at least 20 of 22 loci.

We also deleted from consideration one sample of unknown origin, and three fetuses from Barrow whose mothers were already in the dataset. (There were 4 additional fetuses already deleted for insufficient loci scored, and 1 fetus retained because its mother was not analyzed.)

Applying these quality control criteria reduced our 33-locus dataset to 284 samples, of which 213 are from Barrow. Table C-1 shows seasonal and village totals. For analyses that included the Canadian and Okhotsk samples, the 22-locus dataset comprised 414 individuals including 231 from Barrow, 47 from Igoolik, and 62 from Okhotsk.

For the main dataset of 284 whales, 1.3% (119/9372) of the data are missing. The minimum, quartiles, and maximum percentages of missing data by locus are (0%, 0%, 0.4%, 1.8%, 8.1%). Of the 284 whales, the number of individuals missing scores for 0, 1, 2, and 3 loci were 200, 60, 13, and 11, respectively.

Methods

We tested for Hardy-Weinberg disequilibrium using the GENEPOP software (Raymond and Rousset, 2004). The test was specifically for the alternative hypothesis of heterozygote deficiency (Rousset and Raymond, 1995). The test statistic is the score statistic, namely the derivative of the log likelihood under the null hypothesis. Monte Carlo Markov Chain (MCMC) implementation of these methods (Guo and Thompson, 1992) used chain length of 1 million, batch size of 1,000, and burn-in of 30,000.

Linkage disequilibrium was studied using GENEPOP. Only the Barrow whales were used for this analysis.

A bottleneck analysis was conducted using Bottleneck v1.2.02 (Cornuet and Luikart, 1996). The principle of this analysis is that during a population bottleneck, alleles are lost more quickly than is heterozygosity. We used the one-tailed Wilcoxon sign-rank test under the two-phase mutation model to detect bottlenecks, analyzing only the Barrow samples.

Comparisons of allele frequencies between various temporal, spatial, and age-related groups were made with GENEPOP, using MCMC to approximate exact analysis of contingency tables as described by Guo and Thompson (1992). The same MCMC parameters as above were used. This allelic frequency test calculates the p-value of a two-way allele frequency table as the total probability of all possible tables having the same or smaller probability (under the null hypothesis) as the data table, with the constraint that the marginal sums of such tables match those of the data table (Fisher, 1935).

Estimation of F_{is} and F_{st} was carried out with the FSTAT software (Goudet, 2004). These calculations follow the approach of Weir and Cockerham (1984). Confidence intervals for F_{st} were obtained by bootstrapping over loci.

The STRUCTURE program (Pritchard et al., 2000; Falush et al., 2003) was used to identify potential clustering in the data. The admixture model with correlated allele probabilities was fit using 50,000 burn-in iterations and 1,000,000 iterations for estimation. We also used the no-admixture model with uncorrelated allele probabilities and the same Monte Carlo simulation settings. Runs were initialized randomly.

We used the technique known as Fisher's method throughout this paper to pool p-values across loci when necessary. This approach is based on the simple fact that $-2\sum_{i=1}^{k}\log p_i$ has a chi-square distribution with $2k$ degrees of freedom, where p_i are the locus-specific p-values. The

locus-specific test statistics used here do not have continuous distributions, so Fisher's method is approximate.

Some of our analyses rely on estimates of whale ages. For 21 samples, whale age was estimated from aspartic acid racemization (George et al., 1999; Rosa et al., 2004). Ages for 12 others were estimated from corpora counts (George et al., 2004b). Another 11 ages were estimated from stable isotope cycle counting in baleen (Lubetkin et al., 2004). In 12 additional cases, estimates were available using two of these methods, and the weighted average estimate was taken. For the remaining whales, direct age estimates were unavailable.

Many of our analyses are stratified by season. Because of the spatio-temporal nature of the migration, assignment to spring and fall seasons can be done unambiguously by dividing the calendar year exactly in half. The exception is for Gambell and Savoonga on St. Lawrence Island where hunting during the southward migration period extends into January, at which time the distinction between migratory and residence behavior is ambiguous in some cases. For these two villages, winter hunts ended in January and spring hunts began in April for our dataset. Winter hunts were classified as "fall".

Results

Disequilibria

There is strong and widespread Hardy-Weinberg disequilibrium among the Barrow samples. Eight of the 33 loci (5/22 new loci and 3/11 original loci) exhibit heterozygote deficiency at the nominal 0.05 significance level, for an overall p-value of 1.9×10^{-8} using Fisher's method. No significant deficiency is found for any other spatial stratum, or when St. Lawrence Island villages are pooled. The disequilibrium at Barrow is stronger in the fall ($p = 4.8 \times 10^{-7}$) than in the spring ($p = 0.015$).

Tests for a historical bottleneck were also highly significant. The one-tailed Wilcoxon sign-rank test p-value is 0.0035 using the Barrow samples, all 33 loci and the default parameters of 70% single-step mutations with multi-step mutation variance of 30. However, if the percentage of single-step mutations is changed to 95% and the variance to 12 (c.f. Piry et al. 1999), the p-values become non-significant. This bottleneck test can give false positive results when applied to data from a mixed-stock assemblage and other circumstances when testing assumptions are violated.

Linkage disequilibrium is also present in these data. At the nominal 0.05 level, 40 of 528 pairwise locus comparisons (7.6%) among the 33 loci showed significant linkage, using the Barrow data. The diploid number of the bowhead is 2n=42 (Jarrell, 1979), so there are 20 possible pairs of autosomes (since none of the loci are X or Y linked). Thus some physical linkage would be expected. However, there is no reason to suspect such linkage to be strong, and the 7.6% occurrence rate is too high to be explained by physical linkage. Population stratification or factors related to recent demographic history (e.g., gene drift from a bottleneck) can produce spurious findings of linkage disequilibrium. Fitness interactions between genes and inbreeding or other types of non-random mating are other possible causes of apparent linkage disequilibrium.

Spatial strata

Okhotsk and Barrow samples exhibit significantly different allele frequencies ($p < 1 \times 10^{-10}$). The Canadian samples also differ significantly from Barrow ($p < 1 \times 10^{-10}$).

The St. Lawrence Island samples (Gambell and Savoonga, pooled) exhibit significantly different allele frequencies from Barrow (overall $p = 0.006$), with locus-specific differences stronger than the nominal 0.05 significance level for 1/22 new loci and 4/11 original loci. Separating the two island villages, Barrow differs significantly from Savoonga ($p = 0.034$) but not from Gambell ($p = 0.35$). The Savoonga difference may be seasonal: spring Savoonga animals do not differ from either season at Barrow, but fall Savoonga differed significantly from spring Barrow ($p = 0.011$) and from fall Barrow ($p = 0.048$). Recall that the fall Savoonga harvest includes whales that may be wintering nearby in January. Sample sizes for these comparisons are very small, especially for spring Savoonga.

We found no significant allele frequency differences between the St. Lawrence Island villages of Gambell and Savoonga, despite traditional knowledge and hunter observations of some migratory variations (Noogwook et al., 2007). In the spring, Savoonga hunters hunt from the southwest side of the island, at Southwest Cape. They report that the whales they hunt approach from the southeast. However, they recognize another group of whales, which pass Southwest Cape far offshore and are available to Gambell hunters at the northwest tip of the island. The Gambell hunters confirm these observations saying that the bowheads they hunt approach Gambell from the southwest, and then head northeast after passing Gambell. Migratory traffic on these two paths is said to be negatively correlated, in that if the whales are seen in numbers at Southwest Cape they are unlikely to be available at Gambell at the same time. The hunters do not know

K	**log(P[Data|*K*])**
1	1
2	735
3	836
4	880
5	857

Table C-2: Estimates of the log of P[Data|*K*] for *K*=1,...,5, using the correlated admixture model in STRUCTURE.

whether these two paths past St. Lawrence Island represent routes of two distinct groups of whales, or whether they represent alternate routes chosen at various times by various portions of the same population of whales. Considering that both putative groups commingle in the passage between St. Lawrence Island and Chukotka during the early spring migration in a region where aerial surveys have reported a high frequency of mating behavior (Koski et al., 2005), some degree of interbreeding seems more plausible than not.

We found no other statistically significant spatial comparisons of allele frequencies among various villages/locations in the Bering-Chukchi-Beaufort Seas region. This includes comparisons involving Chukotka samples.

Temporal structure

Allele frequencies do not differ significantly at Barrow in spring versus fall.

Some significant genetic patterns were detected using the Bayesian cluster analysis provided by the STRUCTURE program. The results of our STRUCTURE runs are based on the combined data from the BCB, the Sea of Okhotsk, and Canadian samples, using only the 22 new loci since the two outgroups were not scored on the original loci. These results suggest temporal structure in the Bering-Chukchi-Beaufort Seas region.

The developers of STRUCTURE describe their method for statistical inference for the number of clusters (denoted K) as "dubious at best" because it is based on a crude integral approximation (Pritchard et al., 2000, p. 949). If one overlooks this criticism, it is possible to examine estimates of P[Data|K] and therefore posterior probabilities for K under, say, a discrete uniform prior. Table C-2 shows the estimated log(P[Data|K]) for various K values[1], for the correlated admixture model.

Table C-2 clearly shows that separating whales from Okhotsk from the whales from other regions (i.e., K>1) is strongly preferred compared to K=1. Choosing K=2 offers a greatly improved fit, but there are diminishing returns for K larger than 2. It is somewhat surprising that the Canadian samples do not cluster separately from BCB samples since allele frequencies differed significantly.

Pritchard et al. (2000) recommend that the numbers in Table C-2 be used as only as a rough guide and they note that the model we used (with correlated allele frequencies) is likely to overestimate K. Nevertheless Table C-2 exhibits classic signs of a "knee", which is recommended as the best indicator for choosing K. Therefore we view K=2 as clearly the best choice, with K=3 being the only reasonable alternative. Our use of these STRUCTURE results is mainly to identify a putative scenario with two BCB clusters, not to test its plausibility against other scenarios. Therefore, we examine the K=3 results below because they are the most easily interpretable results that yield multiple BCB clusters. These results provide some interpretable patterns that are not improved with larger K. We must also emphasize that clusters found by STRUCTURE may correspond to detectable genetic patterns caused by any sort of divergence from panmixia, ranging from mild inbreeding or gene shift to non-interbreeding substocks. We discuss later that the putative BCB clusters provided by STRUCTURE do not exhibit a substantial F_{st}.

Figure C-4 shows the STRUCTURE clusters for K=2 (top), 3 (middle), and 4 (bottom). Each color represents an estimated cluster for the chosen K, but the colors are not consistent across plots[2]. Each whale is indicated by a vertical strip, with colored bars apportioned to match its estimated ancestry from each cluster. The whales are separated into 15 spatial/seasonal groups divided by black vertical lines and labeled with indices. These groups are: 1=spring Barrow; 2=fall Barrow; 3=spring Savoonga; 4=fall Savoonga; 5=spring Gambell; 6=fall Gambell; 7=spring Chukotka; 8=fall Chukotka; 9=(spring) Diomede; 10=(spring) Point Hope; 11=(spring) Wainwright; 12=(fall) Kaktovik; 13=(fall) Nuiqsut; 14=Igoolik, Canada; 15=Okhotsk. Within each of these 15 groups, whales are ordered sequentially by calendar day from left to right. The preference for $K \geq 2$ is obvious in Figure C-4: the Okhotsk (group 15) whales clearly cluster into a separate group.

If the putative clusters within the BCB samples (for K=3) are to be taken at face value, then one must agree that they fail to exhibit clear spatio-temporal separation of a magnitude similar to the

[1] The estimates are adjusted by an additive constant of 38791.2 for clarity.
[2] This is a necessary inconvenience because cluster membership varies across panels.

differentiation seen between the known stocks of BCB and Okhotsk. The BCB clusters are highly mixed in virtually all locations and times. Furthermore, the ancestries of BCB whales are far more likely to be mixed or uncertain than for Okhotsk whales, where the ancestries are nearly all predominantly from a single source. Thus, it is important to consider whether the BCB clusters might represent genetic structure of a sort that is less definitive than the classic scenario of non-interbreeding substocks, or whether they represent true genetic differentiation at all. See the discussion.

Fall Barrow: Focusing on the results for BCB bowheads when K=3, a temporal pattern can be detected. For this portion of Figure C-4, notice that the fall Barrow migration (group 2) appears to exhibit alternating pulses of whales of red and green ancestry. To investigate further, we computed the conditional red ancestry (i.e., red/(green+red)) for each fall Barrow whale, and plotted this against capture date[3]. Figure C-5 shows the results, with one circle for each fall Barrow whale. The area of each circle corresponds to the whale's estimated age. Most years are color coded. An unweighted variable-span smoother has been fit to these data, with span chosen by cross-validation. This smoother (supsmu in Splus (Insightful, 2007)) was chosen for its ability to handle the uneven temporal spacing of whales. Joint 95% null bands (calculated as per Jorde et al. (2006)) for this smooth are shown with dotted lines. This graph shows a statistically significant pulsing pattern, with a red ancestry pulse dominating between two pulses of green ancestry, and perhaps other red pulses at each end of the migration.

The pattern in Figure C-5 can explain the Oslo Bump. The apparent pulses of whales of differing ancestry are exactly the sort of temporal migratory structure that could generate a finding like that of Jorde et al. (2007). Furthermore, the temporal separation of the peaks and troughs in Figure C-5 is about 10 days, which is roughly consistent with the findings of those authors. It is worth noting that the Jorde et al. (2007) analysis used only the 11 original loci, whereas the analysis in Figure C-5 uses only the 22 new loci.

We have also confirmed existence of the Oslo Bump (using all 33 loci) using the method of Givens and Ozaksoy (2006) with the fall Barrow data. Figure C-6 shows the result of that analysis where pairwise allele matching probability (vertical axis) is modeled to depend on pairwise capture time difference and on whether the alleles in the pair originate from the same or different whales. The model fit is shown by the solid lines (with the flat upper line showing the estimated same-whale match probability). The dotted lines show joint 95% confidence bands for the curve fit for the effect of capture time difference. Panel (a) shows the analysis using all 33 loci, whereas panel (b) limits the analysis to the new 22 loci only. In the main analysis, a significant effect is found ($p < 0.002$ using the bands method but $p = 0.230$ using the deviance method), indicating that whales caught in the same year about two weeks apart are less similar than whales caught more or fewer days apart. The two-week interval we detect is somewhat longer than the result from Jorde et al. (2007), but also consistent with Figure C-5. We interpret the conflicting p-values from the bands and deviance testing methods as an indication that the effect is statistically significant but fails to explain a large portion of the variation in genetic similarity. A comparison of the results in panels (a) and (b) indicates that the Oslo Bump signal is essentially confined to the original 11 loci. In both analyses we find that after controlling for the effect of capture time difference, there is still significant evidence of additional non-specific

[3] Of the 123 fall Barrow whales analyzed here, 1 had greatest ancestry assigned to neither the red or green cluster. In such cases, the conditional red ancestry might be misleading since, for example, (red, green, blue) ancestry of (0.03, 0.01, 0.96) has conditional red ancestry of 0.75. Deleting this whale from the smoothing analysis did not qualitatively change the fitted curve.

genetic inhomogeneity in the data (p = 0.008 for panel (a)). This suggests that the Oslo Bump is not the sole source—or perhaps not even the primary source—for the widespread disequilibrium reported above.

An important criticism of the nonparametric smooth of STRUCTURE results shown in Figure C-5 is that it makes no distinction between years. The timing of the bowhead migration is known to vary interannually due to weather, ice, and other unknown reasons. This means, for example, that October 1st does not correspond to the same point in the migration each year. (However, the fall migration is less affected than spring by weather and ice factors since the southward migration path is nearly all open water.) In this respect, the analysis methods of Givens and Ozaksoy (2006, and Figure C-6 here) and Jorde et al. (2007) are superior because they control for this interannual variation in migration timing

A specific temporal pulsing hypothesis has been suggested as a biological explanation for the Oslo Bump. This 'Chukchi Circuit Hypothesis' (Schweder et al., 2005) proposes the existence of two distinct subpopulations with sufficiently little mixing to maintain genetic distinctiveness. According to this hypothesis, one subpopulation follows the conventional migration path, whereas the other subpopulation leaves the Bering Sea in late May and June and migrates northwest along the Chukotka coast. The summering range of this group might be in the Chukchi Sea, with some fraction of these whales migrating south along the Barrow canyon, passing Barrow on their return trip to the Bering Sea in autumn. Genetic patterns in the fall Barrow data are hypothesized to be the result of the whales completing this Chukchi circuit as they pulse past Barrow in the fall amidst the main fall migration returning from the Beaufort Sea. The Chukchi Circuit whales passing Barrow must be sufficiently few in number not to have been seen summering or migrating southward toward Barrow by acoustical (30.5 hours), ship-based (64 hours), and aerial (8.3 hours) search efforts northeast of Barrow and in the Chukchi Borderland region (Anonymous, 2006).

Although Figures C.5 and C.6 confirm the Oslo Bump, Figure C-4 clearly refutes this Chukchi Circuit Hypothesis. Whales of both green and red ancestry appear to pass Barrow in the spring, intermingled, in large numbers. Significant heterozygote deficiency is present in Barrow in both spring and fall, although it is stronger in the fall. Thus, if our STRUCTURE classifications of green and red ancestry correspond to a biological reality, neither of these two groups avoids passing Barrow in the spring. Both groups are counted during Barrow census efforts. Assuming no bias in harvest availability or selectivity, the groups are of similar abundance. Estimated F_{st} for the red and green ancestry groups (discussed later) is extremely small, suggesting that any population subdivision represented by our results is much more subtle than was implied by the Chukchi Circuit Hypothesis.

Spring Barrow: A temporal pattern is also observed in the spring Barrow samples; see Figure C-7. In spring, there is a statistically significant (p=0.023) increase in red ancestry as the migration progresses, with the oldest whales having highest red ancestry passing at the end of the migratory period. The pattern is linear on the scale of the logit of red ancestry; a simple linear regression model was fit to this scale. The result is back-transformed in Figure C-7, and remains fairly linear over the range of the data. This figure also illustrates that the spring migration of BCB bowheads is highly organized by age, with only one apparent pulse of mothers and calves at the end (Angliss et al., 1995).

Sensitivity Analysis: Although the green and red ancestry clusters provided by STRUCTURE offer some interesting and provocative interpretations, it is important to assess how much evidence there is that this statistical phenomenon reflects a biological one. Greater confidence in

our STRUCTURE results is warranted if it can be shown that the green and red ancestry groups likely correspond to a biological reality, rather than perhaps to some peculiar samples, mis-scored loci, or happenstance of the uneven temporal sampling. To investigate such possibilities, we conducted several further experiments.

First, we noted that the fall result seemed to depend upon the particular clustering of whales captured in 2005. To investigate, we omitted the 2005 samples from the smoothing analysis and recomputed the results. The bump vanished entirely. The bump also vanished under two other sensitivity tests described in the discussion section.

We also conducted a cross-validation second experiment, where we deleted a random 10% of the BCB samples (30 whales) from the dataset and reran the STRUCTURE analysis. In fact, we repeated this ten times without replacement, effectively partitioning the BCB samples into ten 90% cross-validation subsets. In each run, the entire set of Canadian and Okhotsk samples was used. If the green and red ancestry clusters in the original run correspond to a biological reality, then we would expect whales sharing the same cluster in the original run also to share the same cluster in these cross-validation runs. For each same-cluster and different-cluster whale pair from the original run, we counted the number of same-cluster outcomes among the 8 (or rarely 9) cross-validation runs in which both members participated. Note that this eliminates confusion about arbitrary color designations across runs. Figure C- 8 shows the numbers of times same-cluster membership was assigned for pairs that were originally same-cluster. This figure also shows the results for whale pairs whose members were originally in different clusters. Ideally, the same-cluster pairs (black bars) would fall only in bins 8 and 9, whereas the different-cluster pairs (red bars) would fall only in bin 0. Figure C- 8 shows that there is fairly strong persistence in cluster membership across runs, considering that some whales have ambiguous ancestry.

Immigration

If one accepts the results of our K=2 STRUCTURE analysis (and optionally K>2), it appears that at least a few samples appear genetically consistent with membership in another stock. Furthermore, these results did not identify a distinction between BCB and Canadian samples. There are also other indications of (at least historical) immigration between BCB and Canada. In particular, there have been two documented incidents where whaling irons used in the western north Atlantic fishery were later found in whales taken in the Chukchi Sea (Bockstoce and Burns, 1993). Also, there have been at least 4 reports of European-made harpoons recovered from bowheads killed in the Bering and Chukchi Seas (Tomlin, 1957).

Furthermore, satellite tracking of one bowhead this year (Alaska Dept. of Fish and Game, 2007) and satellite imagery of ice coverage (AOOS, 2007) shows that this tagged whale traveled along the north shore of Banks Island in early August, thereby essentially crossing the most difficult sea ice barrier, as seen in **Error! Reference source not found.**. Considering current trends in arctic climate, the track of this whale suggests that transit between the BCB and Canadian stocks may be possible. Physical movement between regions is by itself insufficient for gene flow, of course.

Birth Year

The STRUCTURE analysis also yielded an interesting result related to whale birth years. Figure C-9 plots the conditional red ancestry against the estimated birth year of the whale. The STRUCTURE analysis was run on the entire 22-locus dataset, but in this figure only whales with reliable birth year estimates (see above) are plotted. The vertical line in the figure indicates

1914, when commercial whaling ended. The curve is a cross-validated lowess smooth of the data (Insightful, 2007).

The results show that whales estimated to have been born prior to the end of commercial whaling all had predominantly red ancestries. Whales born in the years shortly after the end of commercial whaling had predominantly ancestries associated with the other BCB cluster. Ancestries become increasingly evenly mixed for younger cohorts, although there may be some pulsing evident.

This pattern is statistically significant ($p<0.0002$). Since a key component of the signal is at one edge of the plot, the permutation null band approach was not appropriate here. Instead, we calculated the sum of squared residuals for the actual smooth, and compared it to null distribution values obtained from smooths on data where birth years had been permuted.

If this data signal corresponds to a biological reality, one possible explanation could relate to competitive exclusion. Perhaps whales of one type of ancestry were disproportionately wiped out by commercial whaling, and whales of other ancestry grew to have proportionally greater abundance, perhaps even filling in newly available range. As time progressed, the first group might have recovered and the two groups may now be intermixing. An alternative explanation could be that the second ancestry group was not pre-existing but was actually created by the gene drift occurring near the end of commercial whaling when overall abundance was severely depleted. Of course, Figure C-9 alone does not constitute sufficient evidence to elevate such hypotheses above mere speculation.

Another reason to reserve skepticism regarding this figure is that whale birth years are estimated with considerable variability, especially for very old whales. Uncertainty in birth year estimation is not accounted for in the smooth fit or the significance testing.

Discussion

The analyses presented here show clear evidence that the BCB bowhead samples are not in Hardy-Weinberg equilibrium. Our analyses also present the strongest evidence to date for a historical bottleneck, although the evidence is not conclusive and any bottleneck may have occurred recently or thousands of years ago. We have also found patterns of temporal genetic structure in the migration and a suggestion that some genetic structure may be related to whale birth year.

Before assessing possible explanations for the detected genetic inhomogeneity, it is worthwhile discussing the sensitivity of our findings to some of our analysis choices. Table C-3 summarizes some sensitivity tests we ran. In these tests, we repeated some of the key analyses described here, using different data subsets or other reasonable choices.

STRUCTURE results were rerun using the model for independent populations with uncorrelated allele frequencies and no admixture. The cluster memberships under this model with K=4 had only modest correlation (0.47) with those generated under the admixture model. We view this alternate model as less appropriate than the admixture choice because it is designed for populations having much lower potential historical mixing than two putative substocks that might have coexisted in the Bering-Chukchi-Beaufort Seas region over recent millennia.

Two other main sensitivity strategies were used. First, we examined results using only the 22 new loci, which we show above to be more reliably scored than the other 11 loci. Second, we eliminated some whales from analysis, recognizing that equilibrium tests can be quite sensitive

to minor amounts of laboratory scoring and labeling errors. Morin et al. (2007) cite several whales having extremely high influence on Hardy-Weinberg disequilibrium results; we deleted their top six offenders, which were homozygous for rare alleles at some locus. These deleted whales were 02B16, 02B6, 05B7, 99B3, 83B1, and 96B11. Using analysis of SNPs data, Morin and Hancock (2007) identify one pair of samples in our dataset that appear as possible duplicates despite having different identifying labels. We deleted 01B12. Skaug and Givens (2007) report analyses searching for closely related individuals such as parent-offspring pairs. We deleted the minimal number of individuals from our dataset to ensure that only one member of each of pair remained[4]. Thus we deleted 00B5, 00B11, 04KK1, 04B18, 02B17, 02B14, 02KK2, 95B4, 95B9, 92B3, 05H3_5, 96B3, 96B5, 96B7, 96B6, 97B12, 03B2, ARIG2003-13, ARIG2003-19, ARIG2003-27, BMIG01-27, BMIG01-29, BWCH13, BWCH14, BWCH16, BWCH2, RUS-BW000911.29, RUS-BW990906.02, RUS-BW990906.03, RUS-BW990906.04, RUS96-7, and RUS-BW000829.S5. Since the purpose of these deletions was to examine sensitivity of certain main results, the whales in this last group chosen for deletion were selected to minimize the number of deletions from St. Lawrence Island and fall Barrow. Also, note that many of these deletions are irrelevant for the sensitivity analyses in Table C-3. Thus we list the total number of deleted whales for each test.

Table C-3 shows that all evidence for spatial genetic differences vanishes when only the 22 new loci are analyzed. The findings of generic Hardy-Weinberg disequilibrium and the bottleneck result persist in the 22 new loci. Regarding the temporal pulsing at Barrow, the finding vanished under each sensitivity test. See Figure C-10 for the STRUCTURE results obtained after excluding 12 fall Barrow and 25 other special cases. The spring Barrow temporal trend remained under alternative modeling assumptions.

These sensitivity results are consistent with the hypothesis that the main sources of genetic signal in these data are scoring errors, familial relations, and birth year. The persistence of the spring Barrow temporal trend is consistent with this because that pattern is essentially driven by birth year due to the age-structured spring migration.

Some of our results are based on the red/green clusters identified by STRUCTURE, yet these clusters may not correspond to groups that are demographically distinct or biologically meaningful. The likelihood function at the core of the model-based STRUCTURE clustering method rewards population groupings that—as far as possible—are not in disequilibrium. The red/green clusters may simply be one of many artificial sample stratifications that reduce apparent disequilibrium. For the 22 new loci used with STRUCTURE, Hardy-Weinberg disequilibrium was greatly reduced (but not eliminated) after clustering. However, substantial disequilibrium remained in the 11 original loci even after clustering. Since BCB bowheads have recently experienced a period of severe population depletion and recovery, one might not expect yet to find Hardy-Weinberg equilibrium in the present samples. Thus, the STRUCTURE model is misspecified for our data. Whether the degree of misspecification is sufficient to render our red/green clusters unreliable is unclear.

[4] At the time of writing, we had only a preliminary list of possible related pairs. Therefore, the individuals listed here may not exactly match the final list generated by Skaug and Givens (2007).

Major Finding	Remains when…	Vanishes when…
Heterozygote deficiency, Barrow overall ($p=2x10^{-8}$)	*only new loci used (p=0.0002).	* only new loci used with 21 special cases deleted (p=0.12).
Heterozygote deficiency, Spring Barrow (p=0.015)		* only new loci used (p=0.20). * only new loci used with 9 special cases deleted (p=0.64).
Heterozygote deficiency, Fall Barrow ($p=5x10^{-7}$)	*only new loci used (p=0.00005). * only new loci used with 12 special cases deleted (p=0.049).	
Bottleneck (p=0.019 spring; p=0.014 fall)	* only new loci used (p=0.0037). *only new loci used with 21 special cases deleted (p=0.0046).	* single-step mutations changed to 95% and variance changed to 12, with any choice of loci and whales (p>0.05)
Allele frequency difference, St. Lawrence Island vs. Barrow (p=0.006)		* only new loci used (p=0.40). * only new loci used with 21 special cases deleted (p=0.16).
Fall Barrow temporal pulses in STRUCTURE output (uses only new loci)		* used model for independent stocks with uncorrelated allele frequencies and no admixture. * omitting 2005 whales from smoothing analysis. * 12 fall Barrow and 27 other special cases deleted.
Fall Barrow Oslo Bump using Givens & Ozaksoy (2006) analysis		* only new loci used.
Spring Barrow temporal trend	* used model for independent stocks with uncorrelated allele frequencies and no admixture.	
Birth year effect in STRUCTURE output (p<0.0002)	* used model for independent stocks with uncorrelated allele frequencies and no admixture (p=0.0002).	

Table C-3: Summary of sensitivity test results for key findings.

It is particularly troubling that STRUCTURE was unable to identify the Canadian whales, which had highly significantly different allele frequencies compared to Barrow. If the red/green clusters are demographically and biologically meaningful, then one would expect that STRUCTURE's detection of them would be accompanied (if not preceded) by detection of Canadian whales as a separate cluster. The fact that STRUCTURE instead assigned red/green ancestries indiscriminately among both BCB and Canadian whales suggests that a high degree of skepticism is warranted when considering the red/green clusters.

Strata	F_{st}	95% Confidence Interval
Canada vs. Okhotsk	0.039	(0.028, 0.051)
Barrow vs. Okhotsk	0.034	(0.026, 0.043)
Barrow vs. Canada	0.006	(0.002, 0.009)
Barrow vs. White Ventrum	0.005	(-0.003, 0.014)
Barrow vs. St. Lawrence Island	0.002	(-0.001, 0.006)
Red vs. Green	0.000	(-0.001, 0.001)

Table C-4: F_{st} estimates for various comparisons. In each case, the largest number of loci possible was used to compute the estimate, so the top three rows rely on only the 22 new loci, whereas the next two estimates rely on all 33 loci. The Red vs. Green comparison relies on only the 11 original loci, for reasons explained in the text. White ventrum whales are also discussed later in the text.

Another reason to remain cautious about our findings is that the magnitude of genetic structure seen in the BCB samples is smaller than what is seen when comparing these samples to some other regions. Our STRUCTURE runs clearly show how much less distinct any BCB structure is compared to splitting off Okhotsk samples. Table C-4 lists some estimates of F_{st} for various comparisons. Note that the estimates of F_{st} for comparisons with the known separate stock in the Okhotsk Sea are larger than for the speculative subdivisions of the BCB samples investigated here. The F_{st} estimate for the stratification by conditional green/red ancestry is based on the 11 original loci only. Using the 22 new loci that were the basis for the STRUCTURE clustering would produce a biased estimate because those clusters were empirically estimated essentially to maximize between-cluster differences and minimize within-cluster differences.

The small F_{st} values corresponding to some of our key findings of possible structure in the BCB population must be reconciled with the significant p-values for allele frequency differences. One way to reconcile these results is to consider that F_{st} describes the magnitude of a heterozygosity reduction relative to Hardy-Weinberg expectations, whereas the tests for allele frequency differences merely attempt to detect the existence of any differences (which might cause a Wahlund effect and hence a significant F_{st}). With the large number of whales sampled at Barrow and the large number of loci available for analysis, it is possible that the statistical power to detect differences provides resolution beyond the level of genetic differences commonly ascribed to non-interbreeding substocks.

Indeed, we confirmed this hypothesis about statistical power using the POWSIM program (Ryman, 2007). Targeting F_{st}=0.002 with three choices for effective population size and number of generations of drift, we found the power to reject the null hypothesis was roughly 0.75 to 0.90 using the observed sample sizes for SLI and Barrow and the observed allele frequencies in BCB whales. Statistical power to detect very small differences via hypothesis testing is therefore very strong in our dataset.

As suggested by Palsbøll et al. (2006), it is important to consider whether the significant differences we have found correspond to a magnitude of population differentiation that warrants population subdivision for management. While our findings here raise a lot of very interesting questions about genetic structure at various levels, the corresponding F_{st} estimates are quite small.

To better understand the importance of different F_{st} levels, we estimated the F_{st} corresponding to a division of samples believed not to represent substocks. Bowheads exhibit some phenotypic variation (e.g., in unpigmented skin patches, girth, chin patch size, and rostrum and peduncle shape), and at least five phenotypic variants[5] are recognized by native hunters including ingutuk, ingutuvuk, kiraliq, kiralivuk, and kiralivoak (Braham et al., 1980; Rooney et al., 2002). Detailed biologist field observations on harvested whales are rare, especially outside of Barrow, but we managed to identify 7 Barrow whales (2 spring and 5 fall) with distinctive white ventral patches and contrasted these whales to the remaining Barrow whales.

Before proceeding, it is worth considering the evidence that these seven whales are not representatives of a distinct separate stock. A white ventrum is far more plausibly a variation indicative of a familial lineage than substock differentiation because: (i) white ventrums do not correlate with any statistically significant spatial genetic structure, (ii) white ventrums do not correlate with the Oslo Bump and there are far too few white ventrum whales for them to be the source of the Oslo Bump signal, (iii) white ventrum whales do not cluster disproportionately in the red or green ancestry clusters, (iv) white ventrum whales appear to be too rare to represent a viable independent stock, and (v) to propose white ventrum whales as a spatially distinct second stock that nevertheless mixes at Barrow in both seasons is an unnecessarily extravagant elaboration when parsimony is more plausible.

Perhaps the most convincing evidence along these lines comes from analysis of the mtDNA of 411 BCB bowheads. A neighbor-joining tree was constructed (Swofford 2001) for the 68 mtDNA haplotypes using Tamura-Nei distances with a gamma distribution for the variation in mutation rates. In the midpoint-rooted tree, 4 of the 7 white ventrum whales (individuals 96B1, 02B6, 04B17, and 05B20) clustered with the most common haplotype, the fifth (04B13) had a haplotype seen in only one other sample (89B2), and the remaining two (89B5 and 97B6) shared another haplotype unique to those two individuals. Both of these rare haplotypes were quite distant from the most common haplotypes and therefore from the other white ventrum whales. For the haplotypic frequencies observed in the mtDNA dataset of 411 individuals, the probability that seven random individuals include the only instances of any non-unique haplotype is approximately 0.0023, so the shared rare haplotype here is not likely a coincidence. Furthermore, the probability of observing two individuals out of seven that share a rare mtDNA haplotype is likely much greater if the white ventrum patches are tracking a familial group or groups than if the patches are tracking two distinct stocks, unless the second stock is an extremely small group of mostly close relatives. Yet we have found no signal indicative of a small and very distinct second group; rather we have found a signal of possible separation into two large groups with low levels of distinctiveness. Furthermore, the fact that the majority of the white ventrum whales share the distant, common haplotype is inconsistent with the possibility that white ventrum whales constitute a small second group of close relatives. Taken together, this evidence strongly suggests that the white ventrum patches are tracking some microsatellite indicator of familial groups.

[5] Some of these pertain more often or exclusively to a specific gender.

Continuing with our analysis, then, we compared the microsatellites for white ventrum group to those for the remaining Barrow whales. F_{st} was estimated to be 0.005 with 95% confidence interval (-0.003, 0.014). The magnitude of this F_{st} is comparable or larger than the F_{st} values corresponding to our other main spatio-temporal findings regarding the putative red/green ancestry or Oslo Bump signal and the allele frequency contrast between St. Lawrence Island and Barrow. When the magnitude of these F_{st} values is compared to the magnitude of F_{st} estimates for comparisons between BCB, Okhotsk, and Igoolik, it is apparent that the microsatellite dataset provides the statistical power to detect genetic structure of various orders of magnitude that may have quite different management implications.

The BCB bowheads—like nearly any real biological population—are clearly not in Hardy-Weinberg equilibrium, but the biological interpretation of the genetic differences found here is unclear. We may be detecting substock structure, patterns of inbreeding or other nonrandom mating, residual effects induced by past spatio-temporal harvest patterns and/or recent population expansion, or effects of other phenomena such as natural selection, immigration, and gene drift in a finite population. In the present case of large abundance and sparse non-selective hunting, the magnitude of detected genetic differences is small relative to what might trigger severe conservation concerns. We have found no evidence for a small genetically distinct BCB substock, and no convincing evidence that BCB bowheads should be managed as more than one stock. While not discounting the need for continued testing of hypotheses about genetic structure and corresponding management implications, we believe the greatest import of our findings is that they may initiate a new dialogue about subtle patterns of mixing and disaggregation in this species leading to an improved understanding of BCB bowhead biology.

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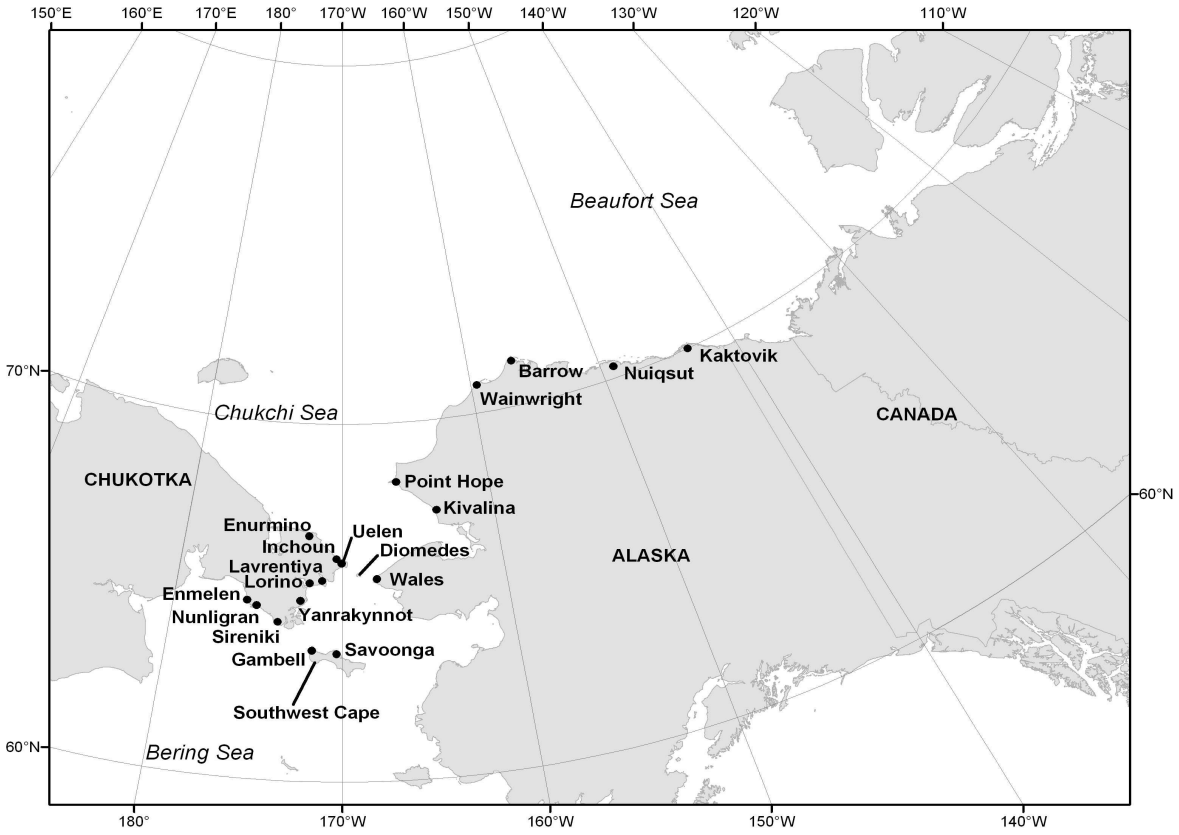

Figure C-1: Range of BCB bowheads and locations of aboriginal whaling villages.

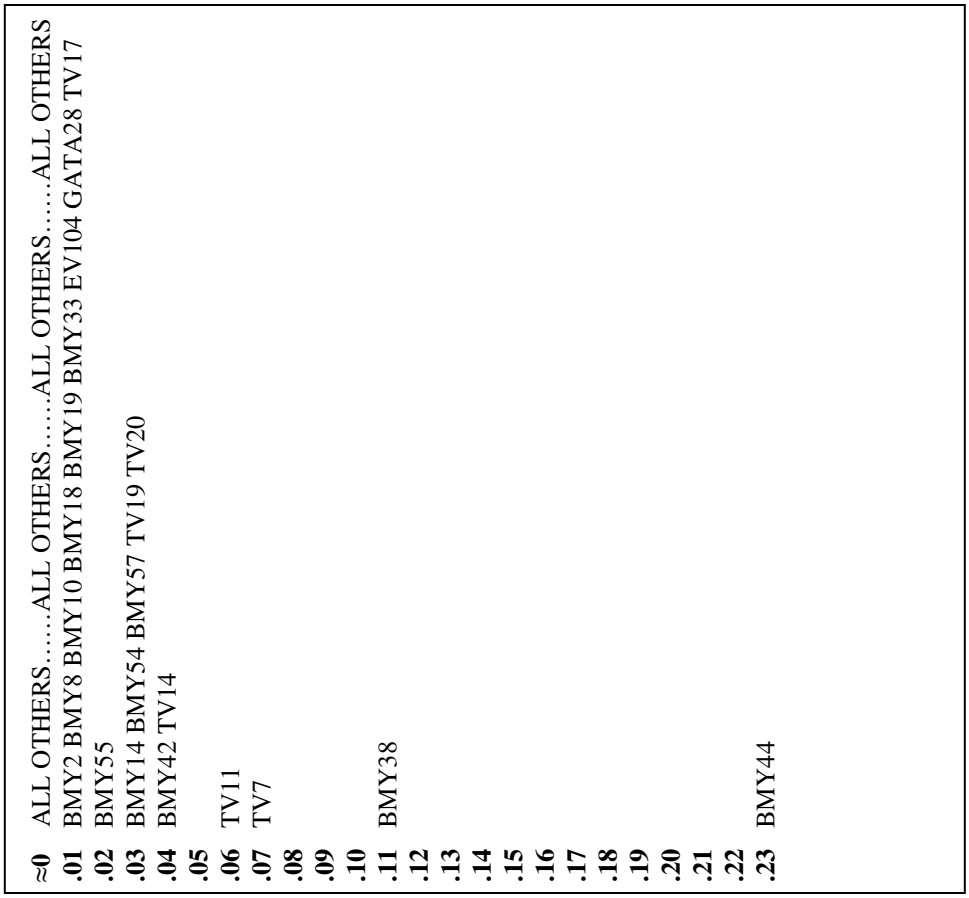

Figure C-2: Estimates of null allele frequency for each locus.

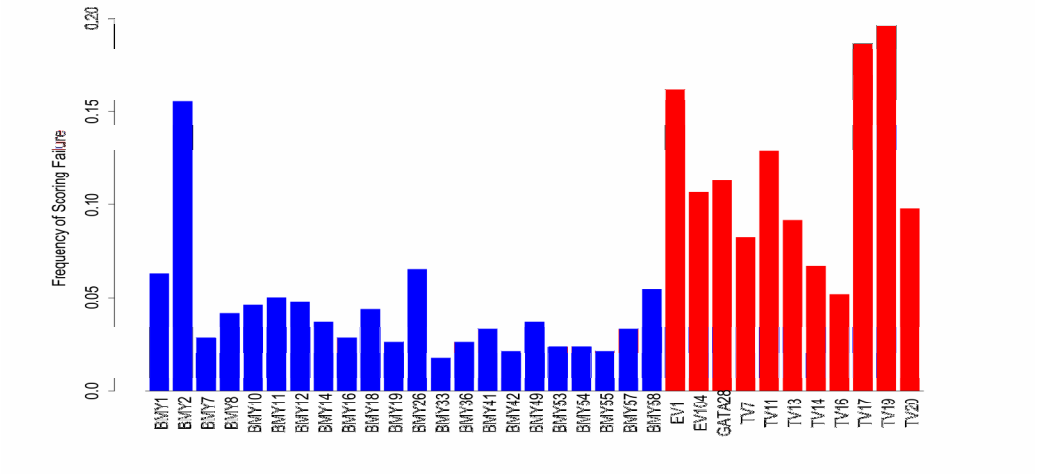

Figure C-3: Scoring failure rate for each locus. Adjusted for a systematic problem in the Canadian lab, the rate for BMY2 is 0.059.

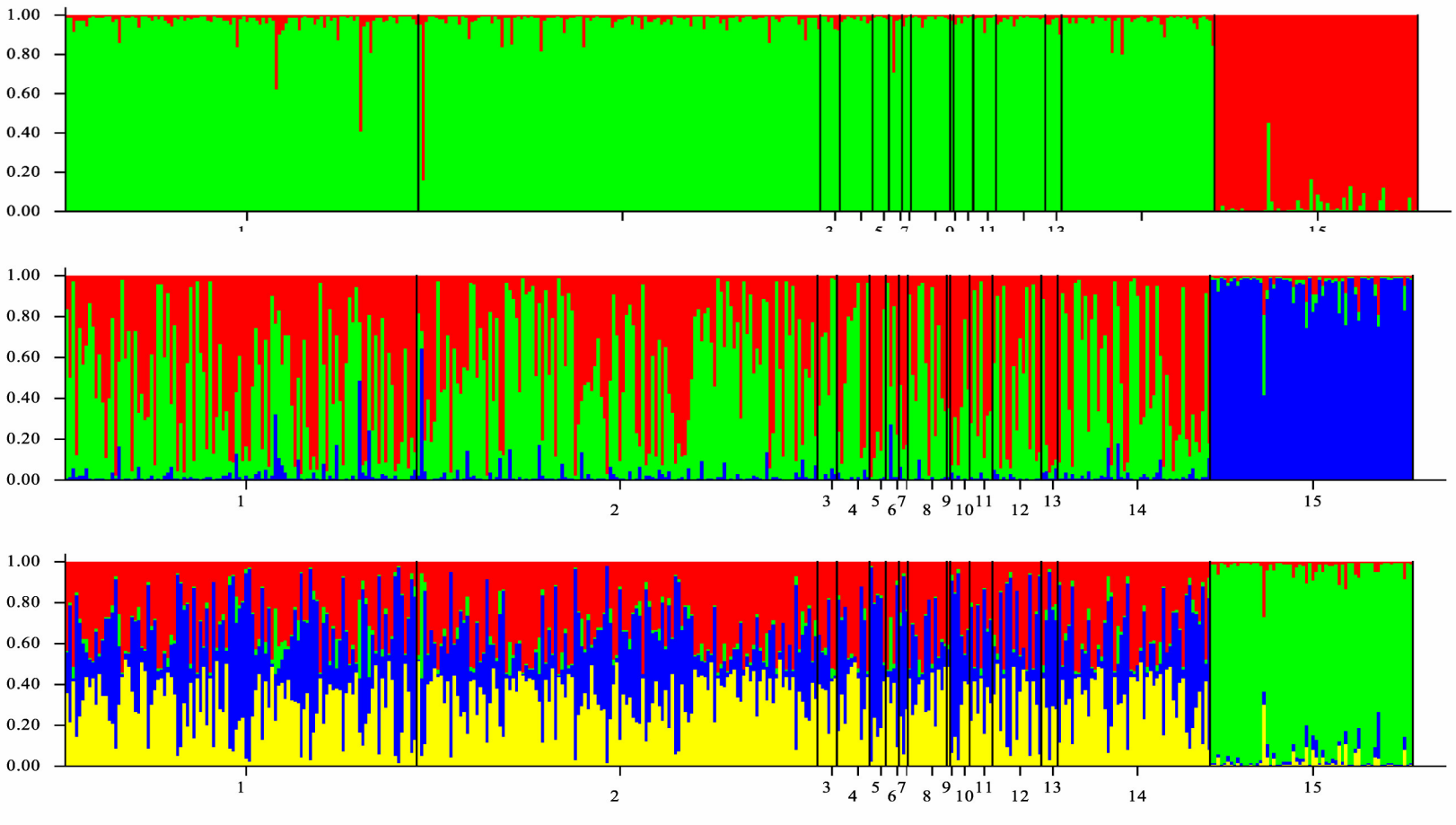

Figure C-4: STRUCTURE clustering results for K=3, 4, and 5, from top to bottom. Group labels are: 1=spring Barrow; 2=fall Barrow; 3=spring Savoonga; 4=fall Savoonga; 5=spring Gambell; 6=fall Gambell; 7=spring Chukotka; 8=fall Chukotka; 9=(spring) Diomede; 10=(spring) Point Hope; 11=spring Wainwright; 12=(fall) Kaktovik; 13=(fall) Nuiqsut; 14=Igoolik, Canada; 15=Okhotsk. Within each of these 15 groups, whales are ordered sequentially by calendar day from left to right.

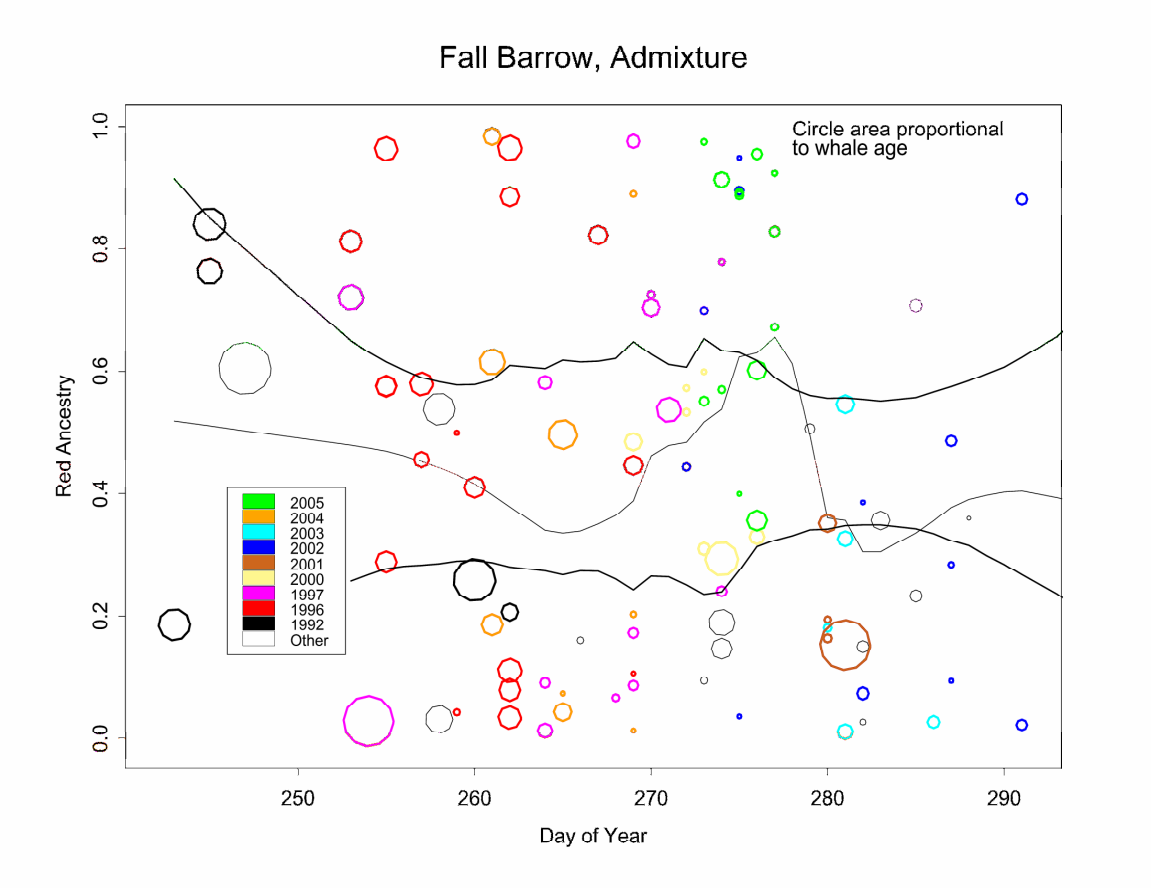

Figure C-5: Smooth fit and null 95% joint probability bands for estimated conditional red ancestries for fall Barrow whales, with capture year and whale age also indicated (with "Other" indicated as a lightweight grey circle rather than a heavy black one.)

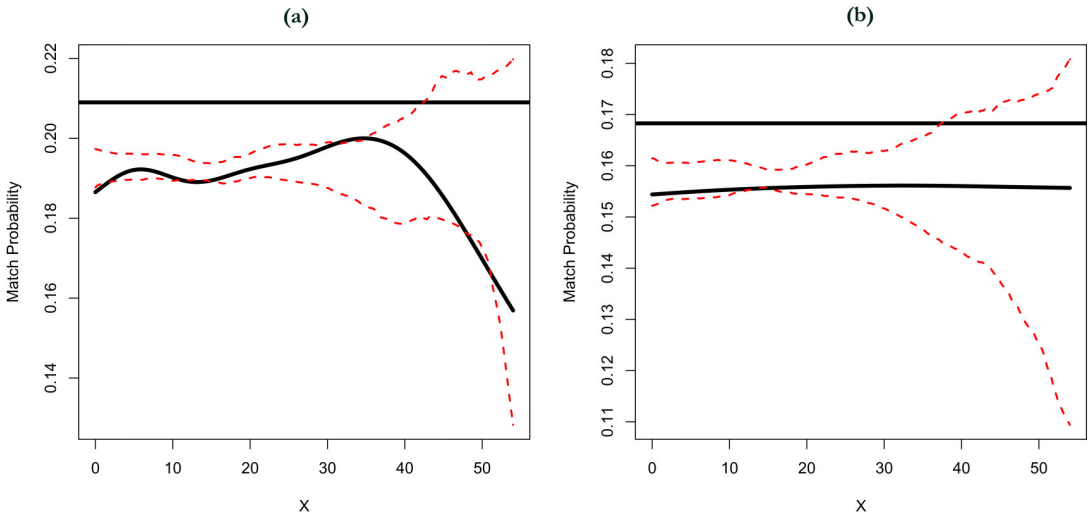

Figure C-6: Results of two analyses to detect the Oslo Bump using the method of Givens and Ozaksoy (2006). Panel (a) uses all 33 loci whereas panel (b) uses only the new 22 loci.

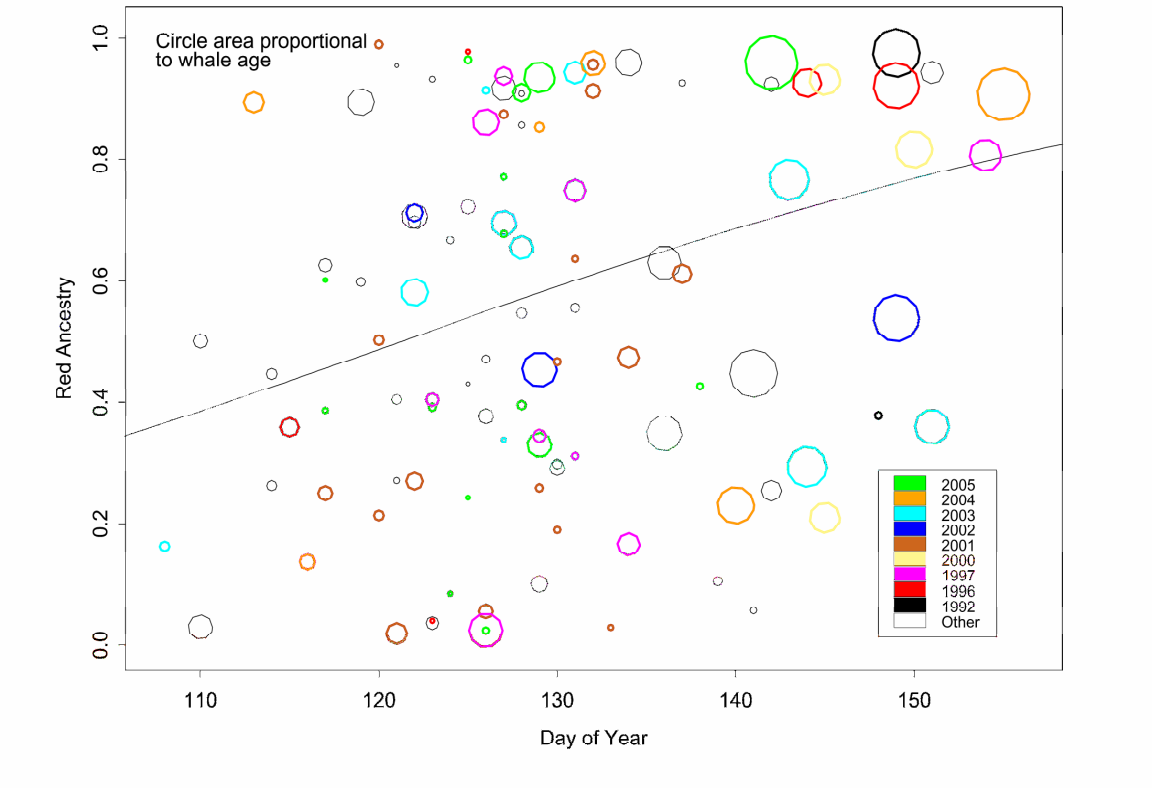

Figure C-7: Smooth fit and back-transformed linear regression fit for logit(red ancestry) versus capture date for spring Barrow samples. The slope of this regression was statistically significant (p=0.023).

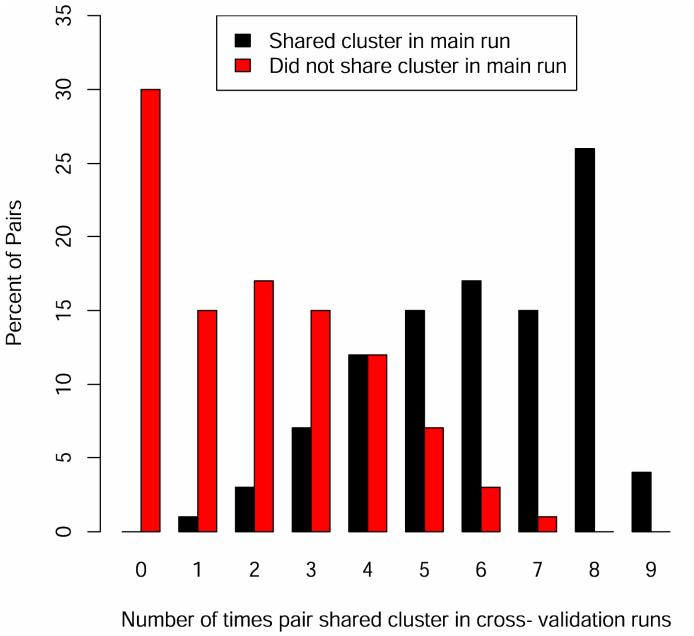

Figure C- 8: Outcomes from STRUCTURE cross-validation experiment.

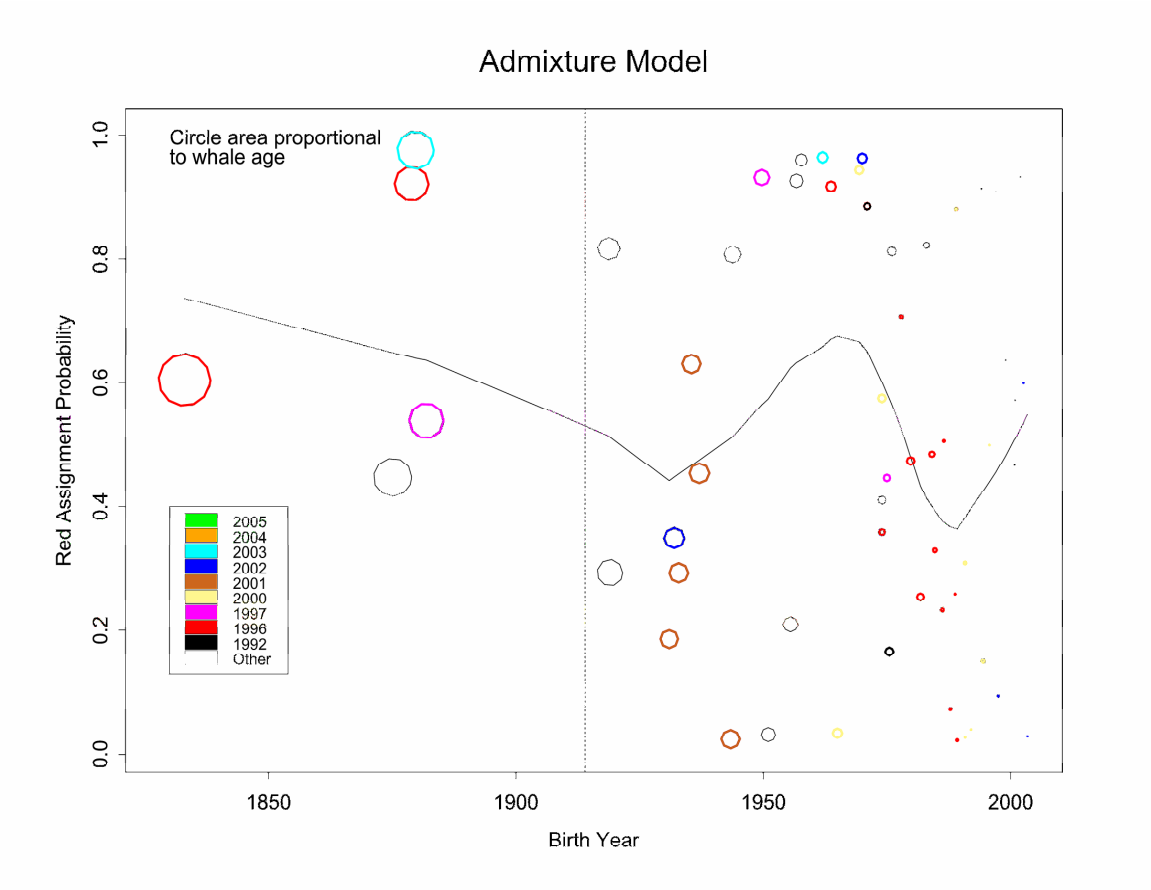

Figure C-9: Conditional red ancestry of whales plotted against estimated birth year.

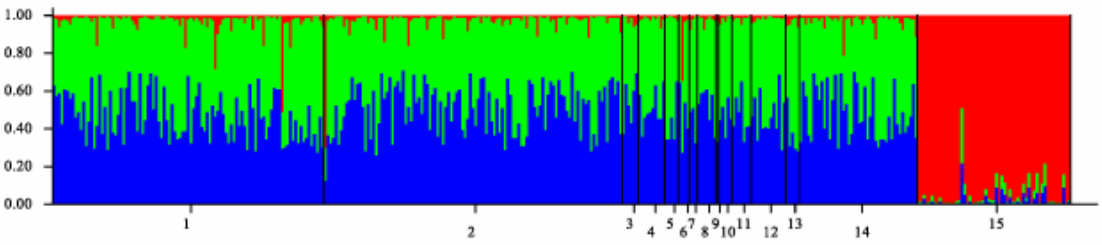

Figure C-10: STRUCTURE results for *K*=3 after omitting special cases.

APPENDIX D

SC/59/BRG15 ESTIMATED GENOTYPE ERROR RATES FROM BOWHEAD MICROSATELLITE DATA

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ABSTRACT

We calculate error rates using opportunistic replicate samples in the microsatellite data for bowhead whales. The estimated rate (1%/genotype) falls within normal ranges reviewed in this paper. The results of a jackknife analysis identified four individuals that were highly influential on estimates of Hardy-Weinberg equilibrium for four different markers. In each case, the influential individual was homozygous for a rare allele. Although these individuals have not been re-genotyped in the laboratory to determine whether the initial homozygous allele calls were correct, our result demonstrates that Hardy-Weinberg p-values are very sensitive to homozygosity in rare alleles for single individuals. This raises the possibility that even small, normal levels of laboratory errors can result in an overestimate of the degree to which markers are estimated to be out of Hardy-Weinberg equilibrium and hence overestimate the potential to infer stock structure. To avoid such bias, we recommend routine identification of influential individuals and multiple replication of those samples.

Introduction

Microsatellite genotypes are the most common type of marker used in studies of phylogeography, population structure, social structure, individual identification and paternity. Several characteristics of microsatellites make them nearly ideal markers for

many of these types of studies, including their ubiquitous presence in almost all organisms, high numbers of alleles and levels of heterozygosity, and rapid mutation rates. However, some of these characteristics also represent some of the most significant limitations. Microsatellite alleles can range substantially in size, resulting in the possibility of mis-scoring due to marker characteristics and sizing technology limitations. Replication slippage across repeats can result in 'allelic stutter' patterns that can then lead to incorrect genotype scoring. Reliance on electrophoresis to determine allele sizes introduces variation that can make it difficult to match allele sizes from samples genotyped in different laboratories, over time and technologies in the same laboratory, and even with fluctuating climatic conditions (Davison & Chiba 2003; LaHood et al. 2002).

The impact of genotyping error has been described for paternity (Gagneux et al. 1997; Hoffman & Amos 2005), individual identification (Bonin et al. 2004; Skaug & Øien 2004), and population size estimates based on genetic mark-recapture (McKelvey & Schwartz 2004). We are not aware of any publications to date that examine the potential effects of genotyping error on detection of population structure, though effects on associated parameters such as homozygosity (Gagneux et al. 1997; Taberlet et al. 1996), departure from Hardy-Weinberg equilibrium (HWE) , overestimates of inbreeding have been noted (reviewed in Bonin et al. 2004; Broquet & Petit 2004; McKelvey & Schwartz 2004).

In this paper, we review some of the types of errors that can commonly occur with microsatellites and what error rates are commonly reported in published studies. We then estimate the error rates for the current bowhead whale (*Balaena mysticetus*) microsatellite genotypes, and evaluate how they may affect population analyses. Additional effort to more accurately estimate error rates in these samples and genetic markers is ongoing, and will be presented as a working paper (Huebinger and Bickham).

Causes and types of microsatellite genotyping errors:

Genotyping errors can be broadly classified into three types:

1) Errors due to marker characteristics, such as allelic stutter, short allele dominance, and null alleles. The genotype patterns on gels or in capillary electrophoresis instruments can very substantially among markers, and some are inherently more difficult to genotype accurately.
2) Errors due to technological limitations. Allele sizing is based on migration of amplified PCR products through a matrix, propelled by an electric current, and visualized relative to a size standard to estimate or calculate allele sizes. Electrophoretic migration can be affected by both size and nucleotide composition of the alleles, plus the addition of fluorescent molecules for visualization, and is therefore not always perfectly correlated with the actual size of the amplified alleles, and allele sizes can differ by more or less than the size of the microsatellite repeat unit (e.g, a CA repeat can have alleles that differ on average by 1.8-2.2bp; Amos et al. 2007). In addition, electrophoresis is itself variable, and can cause allelic size differences of up to 7bp across time, technologies, and instruments (LaHood et al. 2002).

3) Errors introduced due to sample handling or data transcription. These are the errors that are most difficult to detect, and which can affect few or many samples, as in cases where samples are switched, or whole data sets get mixed by copy and paste or sort errors in spreadsheets.

We will review only error types 1 and 2, which are typical of all data sets and for which error rates have been estimated in other studies. Table D.1 summarizes error rates and the publications from which they were obtained. Error rates are typically calculated as the ratio of differing replicated genotypes to the total number of genotypes in the replication study (the per genotype error rate), though some studies calculate the error rate per allele, when multiple miss-calls at a locus can be detected. The latter is the most precise, and is how we have calculated genotype error rates. Because of this variation in the way error rates have been presented in the literature, it must be kept in mind that per genotype error rates will be roughly twice the per allele error rates in most cases. The data in Table D.1 represent the range of per genotype errors observed in microsatellite data sets, and include data obtained from non-invasive samples, which are expected to produce higher error rates than high quality samples (e.g. preserved skin tissue) because of the quantity and quality of extracted DNA. It is important to note that almost all studies that evaluated error rates reported a positive error rate, regardless of sample types used.

Table D-1

Published microsatellite per genotype error rates. The total error rate was calculated for those studies where error rates were reported separately for allelic dropout and false alleles. The composite genotype error is the theoretical frequency of incorrect composite genotypes assuming 10, 20 , or 30 loci with equal probability of error per locus (Bonin et al. 2004).

			Composite genotype error probability (%)		
Source	Error rate (%)	Sample source	10 loci	20 loci	30 loci
Paetkau 2003	4.9	hair	63	87	95
Paetkau 2003	6.2	hair	72	92	98
Paetkau 2003	4.1	hair	57	81	92
Paetkau 2003	4.6	hair	61	85	94
Paetkau 2003	2.8	hair	43	68	82
Paetkau 2003	3.9	hair	55	80	91
Paetkau 2003	1.9	hair	32	54	68
Paetkau 2003	16.7	hair	97	100	100
Paetkau 2003	4.3	hair	58	83	93
Paetkau 2003	5.2	hair	66	88	96
Paetkau 2003	2.1	hair	35	57	72
Paetkau 2003	4.8	hair	63	86	95
Paetkau 2003	5.9	hair	70	91	97
Paetkau 2003	1.2	hair	21	38	52
Paetkau 2003	1.7	hair	29	50	64
Paetkau 2003	0.0	hair	-	-	-
Paetkau 2003	7.8	hair	80	96	99
Bonin et al. 2004	0.8	**tissue**	15	27	38
Bonin et al. 2004	1.2-2.0	feces	21-22	38-55	52-70
Broquet & Petit 2004	8.0	feces	81	96	99
Broquet & Petit 2004	16.7	feces	97	100	100
Broquet & Petit 2004	2.1	feces	35	57	72
Broquet & Petit 2004	8.0	feces	81	96	99
Broquet & Petit 2004	2.0	feces	33	55	70
Broquet & Petit 2004	1.5	feces	26	45	60
Broquet & Petit 2004	35.0	feces	100	100	100
Broquet & Petit 2004	36.9	hair	100	100	100
Broquet & Petit 2004	0.4	hair	8	15	22
Broquet & Petit 2004	7.2	feces	78	95	99
Broquet & Petit 2004	15.2	feces	96	100	100
Broquet & Petit 2004	18.6	feces	98	100	100
Broquet & Petit 2004	18.3	feces	98	100	100
Broquet & Petit 2004	6.8	feces	76	94	99
Broquet & Petit 2004	18.3	feces	98	100	100
Broquet & Petit 2004	24.0	feces	100	100	100
Broquet & Petit 2004	1.0	feces	18	33	45
Broquet & Petit 2004	0.0	hair	-	-	-
Broquet & Petit 2004	0.0	hair	-	-	-
Broquet & Petit 2004	48.0	feces	100	100	100
Broquet & Petit 2004	0.0	feces	-	-	-
(Hoffman et al. 2006)†	0.8	**tissue**	15	27	38
Hoffman & Amos 2005	0.1-0.7	**tissue**	2-13	4-24	6-34
Hoffman & Amos 2005**	0.1-12.7	**tissue**	2-93	4-100	6-100

†20% of genotypes replicated in 2 labs could not be matched.

**based on review of human medical genetics studies

Error types and rates have been studied extensively, especially for non-invasive samples (Bonin et al. 2004; Broquet & Petit 2004; Hoffman & Amos 2005; Johnson & Haydon 2007; McKelvey & Schwartz 2004; Paetkau 2003). All find that genotyping errors are to be expected, but can be minimized with proper use of controls, replication, and marker selection. In particular, higher error rates are often associated with markers that have higher heterozygosity, more alleles, more stutter bands, and larger product sizes (Hoffman & Amos 2005). Error types are also not equally common. In the studies summarized in Table 1, many estimated error rates separately for allelic dropout separately from false alleles (scoring of a non-specific band) (Broquet & Petit 2004; Paetkau 2003).

Four of the most common types of genotyping errors create a bias towards increased homozygosity. These include allelic dropout, which was found to be much more common than false alleles in studies where the two were analyzed separately; null alleles; mis-interpretation of neighboring alleles as stutter; and short allele dominance, in which the larger alleles tend to have lower signal intensity, causing them to be missed in samples where the lowest signal allele falls below the detection threshold.

BCB bowhead microsatellite per allele error rates

Genotypes were generated for 409 bowhead whale samples from Alaskan and Russian waters (Givens et al. 2007). Not all samples were genotyped with every microsatellite marker; the number of loci for individual samples ranged from 22 to 33, though there were fewer completed genotypes for some samples. To examine error rates, we looked at pairs and groups of samples that represented duplicate samples of individual whales. In all cases, these samples were not known by the laboratory personnel to be duplicates prior to genotyping. Some represented multiple biopsies from free-swimming whales, and others were cases of inadvertent multiple samplings of harvested whales.

Some of the duplicates were identified as such after the samples yielded identical genotypes. In other cases, “near-matches” (samples whose genotypes differed by only one or a few loci) were revealed upon closer examination to actually be identical but to have suffered a genotyping error. The latter cases provided an estimation of the number of errors among the duplicates, which was then calculated as a fraction of the total number of allele calls among the duplicates (including both matches and near matches). The apparent causes of errors included allelic dropout, mistakes in allele sizing and transcribing errors.

The error rate given will be a slight underestimate since we did not account for a locus having missing data from one replicate but not the other; the number of scored markers in the final dataset was used for the total number of alleles called (i.e., two identical genotypes across 35 loci indicate 140 good calls). The bias of the estimate is likely to be minimal, since duplicate samples from a given individual tend to suffer from the same genotyping failures (i.e., missing data).

The matches that were detected by identical initial genotypes (no errors evident) included 7 pairs of replicates and one cluster of 8 replicates of the same individual. In aggregate, these indicated 1392 good allele calls.

There were 15 pairs of samples that initially were near matches that ended up being actual duplicates upon closer examination. These included 32 miscalled alleles over 1780 allele calls. Incorporating the correctly called replicates, this gives 32 mistakes over 3172 allele calls – very close to 1%. The mistakes did not seem to be biased toward any particular loci. The 32 mistakes were spread across 19 loci, with only one (Bmy19) including three mistakes, three including two mistakes, and 17 with only a single mistake. The total does not add up to 32 because it is simply a tally of occurrences of miscalls, not whether there was one or two miscalled alleles in a given genotype. Although 1% is not a high error rate, even this error rate led to only about half the replicates being detected (considering the group of 8 as separate replicates).

Twelve of the 32 miscalls were from homozygote/heterozygote differences, the likely candidates for allelic dropout. These were from 11 loci. The loci involved were TV7, TV14 (twice, same allele), TV20, Bmy14, Bmy16, Bmy19, Bmy26, Bmy42, Bmy44, Bmy53, and Bmy57. There did not seem to be any obvious pattern to these discrepancies with regard to allele sizes or frequencies; both common and rare alleles were involved and sizes could be either similar or very different. For 9 of the 11 loci, the allele that varied (present in one replicate but not the other) was the less common one. As a rough measure, this would seem to indicate an allelic dropout rate of 0.4% (12/3172).

Effects of errors on population genetic analyses

Of the 33 loci genotyped for 213 samples from Barrow, nine were found to be out of Hardy-Weinberg equilibrium (HWE), with a significant heterozygote deficiency. In order to determine if particular individuals were more influential than average on HWE, we conducted a jackknife analysis. In the jackknife, each individual was sequentially removed from the dataset and HWE was calculated again across all loci using the remaining 212 individuals.

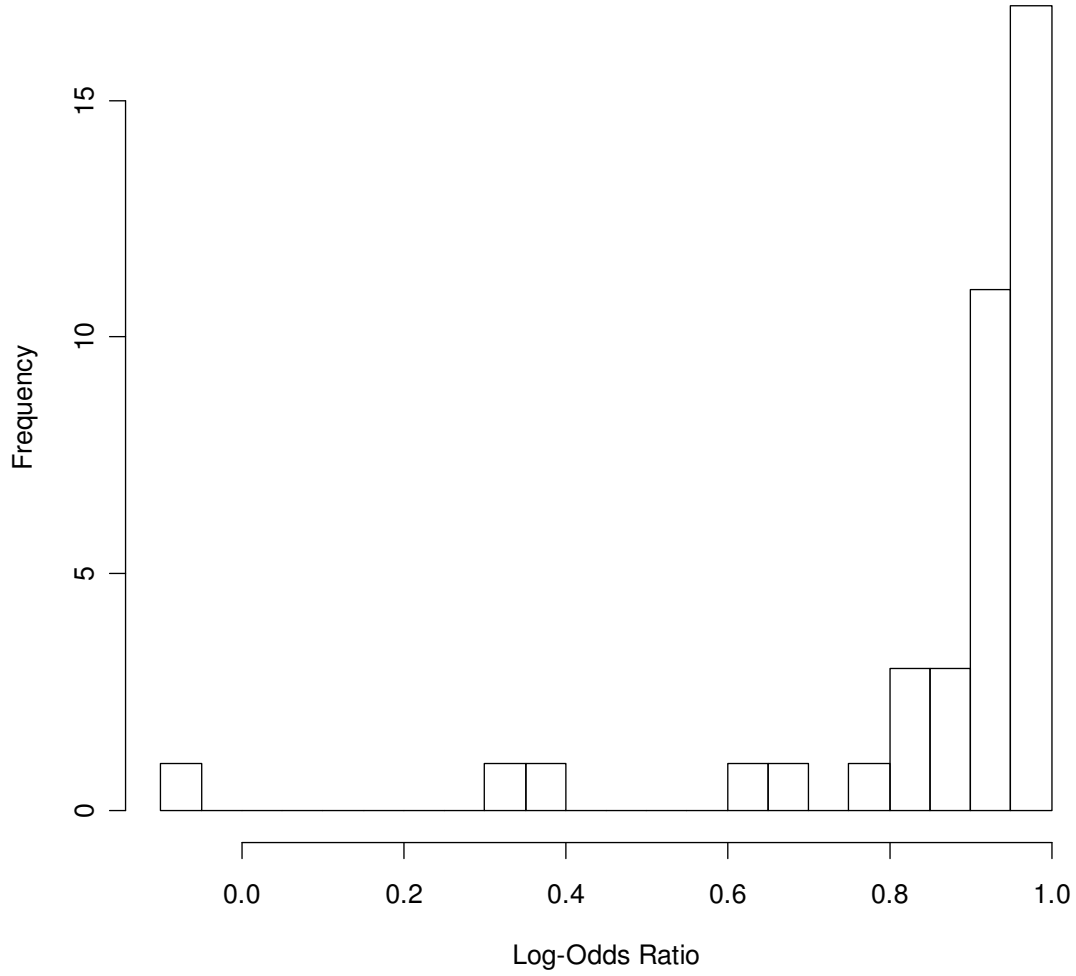
There were 40 cases where removal of a single sample changed a locus from being out of HWE to being in HWE ($p > 0.05$, Table D.2). Figure D.1 shows that there were five extreme jackknife replicates (log-odds ratio between jackknife and observed p-values < 0.7). In these cases, four different loci were involved (Bmy41, TV14, TV19, and Bmy18). Each of the samples in these replicates was homozygous for a rare allele (frequencies < 6%) at the locus under consideration. Additionally, the degree of the difference in the HWE p-value between the original and jackknife replicates was directly related to the frequency of this rare allele. This further suggests that these individuals are largely responsible for the findings of significant heterozygote deficiency.

The other 35 replicates where there was a change in HWE (log-odds ratios between 0.8 and 1) all occurred on loci Bmy18 and Bmy42. Of these replicates, 22 of the samples were homozygotes and 13 were heterozygotes at the locus. The distribution of log-odds ratios in this group is not significantly different from a normal, suggesting that the change in HWE in these cases was due to stochasticity either in sampling or the MCMC assessment of HWE.

Table D-2. Jacknife replicates where significant Hardy-Weinberg equilibrium was found to be non-significant ($p > 0.05$).

Locus	ID	Obs p-value	Jackknife p-value			Odds Ratio	Odds Diff	Genotype	Allele Freq
Bmy41_1	02B16	0.006	0.579			-0.062	5.433	233 / 233	0.005
TV14_1	02B6	0.025	0.228			0.334	2.440	93 / 93	0.017
TV19_1	05B7	0.004	0.114			0.376	3.412	184 / 184	0.013
Bmy18_1	99B3	0.049	0.130			0.641	1.062	137 / 137	0.024
Bmy18_1	83B1	0.049	0.112			0.699	0.892	127 / 127	0.031
Bmy42_1	96B11	0.038	0.077			0.769	0.744	182 / 182	0.056
Bmy18_1	96B8	0.049	0.082			0.817	0.541	117 / 117	0.052
Bmy18_1	05BpB12	0.049	0.077			0.838	0.479	125 / 125	0.054
Bmy18_1	92B3	0.049	0.076			0.842	0.466	117 / 117	0.052
Bmy42_1	02B6	0.038	0.056			0.874	0.406	162 / 162	0.08
Bmy18_1	05B5	0.049	0.068			0.882	0.348	131 / 131	0.052
Bmy42_1	03B13	0.038	0.055			0.884	0.374	162 / 162	0.08
Bmy42_1	90B8	0.038	0.051			0.904	0.311	180 / 180	0.103
Bmy42_1	04B15	0.038	0.051			0.904	0.310	176 / 176	0.131
Bmy42_1	97B18	0.038	0.051			0.905	0.308	180 / 180	0.103
Bmy18_1	02B7	0.049	0.062			0.919	0.240	133 / 133	0.066
Bmy18_1	03B12	0.049	0.061			0.923	0.227	109 / 109	0.13
Bmy18_1	03B4	0.049	0.061			0.926	0.220	119 / 119	0.144
Bmy18_1	92B5	0.049	0.061			0.926	0.218	119 / 119	0.144
Bmy18_1	05B23	0.049	0.060			0.929	0.211	109 / 109	0.13
Bmy18_1	92B2	0.049	0.059			0.936	0.188	119 / 119	0.144
Bmy18_1	96B11	0.049	0.057			0.945	0.162	109 / 109	0.13
Bmy18_1	96B7	0.049	0.057			0.947	0.157	119 / 119	0.144
Bmy18_1	99B1	0.049	0.056			0.956	0.130	119 / 125	0.144 / 0.05
Bmy18_1	05B27	0.049	0.053			0.974	0.078	115 / 117	0.2 / 0.052
Bmy18_1	04B2	0.049	0.053			0.974	0.077	115 / 115	0.2
Bmy18_1	97B17	0.049	0.053			0.978	0.066	115 / 129	0.2 / 0.075
Bmy18_1	96B20	0.049	0.052			0.978	0.065	115 / 115	0.2
Bmy18_1	04B5	0.049	0.052			0.979	0.063	115 / 125	0.2 / 0.054
Bmy18_1	97B29	0.049	0.052			0.979	0.063	113 / 115	0.05 / 0.2
Bmy18_1	84B4	0.049	0.052			0.981	0.055	115 / 115	0.2
Bmy18_1	97B18	0.049	0.052			0.983	0.049	119 / 135	0.14 / 0.047
Bmy18_1	97B20	0.049	0.051			0.986	0.042	121 / 131	0.026 / 0.05
Bmy18_1	05B20	0.049	0.051			0.987	0.040	119 / 131	0.144 / 0.05
Bmy18_1	88B9	0.049	0.051			0.987	0.037	109 / 135	0.13 / 0.047
Bmy18_1	05B26	0.049	0.051			0.988	0.035	121 / 135	0.026 / 0.05
Bmy18_1	97B11	0.049	0.051			0.988	0.035	115 / 119	0.2 / 0.144
Bmy18_1	96B5	0.049	0.051			0.989	0.031	131 / 135	0.052 / 0.05
Bmy18_1	97B19	0.049	0.051			0.991	0.025	109 / 133	0.13 / 0.066
Bmy18_1	03B6	0.049	0.050			0.995	0.015	119 / 119	0.144

Figure D-1. Frequency of the log-odds ratio of the Hardy-Weinberg *p*-values from the 40 jackknife replicates given in Table 1.



Discussion

There is no doubt that errors exist in all genetic data sets. A review of the literature and analysis of unintentionally replicated samples indicates that the observed 1% per allele error rate for the BCB bowhead microsatellite data is low and similar to the published observed error rates (e.g., ≤0.8% per genotype from several studies based

on DNA from tissue). A more accurate estimate of genotyping error rates in this data set will be possible when targeted replication is complete.

The effects of these various types of genotyping errors on analyses of population structure depend heavily on the type of error and the assumptions of the particular analysis being attempted. Analyses, such as F_{st} (Weir and Cockerham 1984), that are based on assessing differences among population allelic frequency distributions would tend to be affected by allele-specific errors. This is because the primary assumption underlying these analyses is that the allelic frequency distributions in the data are a random sample and therefore accurate reflection of the frequency distributions of the population. Therefore, any error that significantly alters the frequency distribution will produce results that do not correctly reflect the true degree of population differentiation.

It is not possible to determine the magnitude or direction of any potential bias of this nature a-priori as this is the product of the specific type of genotyping error and the allelic distributions within the strata under consideration. As an example, a laboratory condition that leads to allelic dropout of larger products could cause strata to be more similar if a large allele exists at high frequency in one stratum, but not in the other. Conversely, if the same allele is common in both populations, dropout would decrease its overall frequency and could lead to an assessment of significant differentiation due to secondary differences in the relative frequencies of other rarer alleles.

The results of our jackknife analysis have demonstrated how analyses that rely on estimates of homo- or heterozygote frequencies, such as the detection of Wahlund effect via Hardy-Weinberg disequilibrium, can be adversely affected by errors that modify these distributions. It is clear that the HWE test will be sensitive to individuals that are homozygous for rare alleles, which are, by definition, unlikely to exist in a sample from a single, randomly-mating population.

Once these individuals have been identified, it is imperative that their genotypes be replicated in the laboratory to ensure that they are valid prior to continuing with other analyses. In the example we have presented with the 213 samples from Barrow, this would mean verifying at most 27 genotypes for 25 samples. If the original genotype is determined to be correct, then it is left to the researcher to decide whether the sample should remain in the dataset based on the circumstances of its collection and what is known about genetic diversity overall. An alternative would be to eliminate that particular locus from analyses of population subdivision.

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APPENDIX E

SC/59/BRGWP4 REASSESSMENT OF GENOTYPES FOR 'PROBLEM' INDIVIDUALS

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Analyses for stock structure have shown that a few specific genotypes (Morin et al., 2007) or individuals (Jorde and Schweder, 2007) can lead to significant findings. In the first instance (Morin et al) a small number of individuals at specific loci possessed homozygous genotypes for rare alleles. In these cases, a single homozygous genotype for the specific individual caused the entire population to significantly differ from Hardy-Weinberg equilibrium. If the individual was removed, the deviation from HWE became non-significant for $p = 0.05$. In the second case of Jorde and Schweder (2007), heterogeneity between sampling years was identified. The specific year identified contained a small sample set of individual (n=8) and had three whales that were unusually homozygous (Givens, 2007).

In an effort to determine if these individuals were in fact homozygous at the loci previously reported, we regenotyped six individuals from Morin et al. (2007) for the single locus in question and the 4 individuals that led to the interannual heterogeneity (Jorde and Schweder, 2007; Givens, 2007). For the four individuals that were unusually homozygous, we regenotyped 12 loci for which the majority were homozygous in the first analysis. It is noted that all 33 loci were not rerun on the 4 individuals due to time constraints in the laboratory and for providing this information for the scientific committee meeting. For this analysis all samples were genotyped on an ABI 3730, whereas they were initially genotyped on an ABI3100. Samples were amplified individually and run individually to minimize the chance of generating miscalls.

For the Morin et al. individuals, two individuals were homozygous for the same locus. In the remaining four individuals it was caused by a different locus for each individual. The regenotyped allele scores are shown in table E.1. Three of the individuals remained homozygous after reanalysis. One of these individuals (99B3) was also included in the regenotyping of 12 loci because it was unusually homozygous. Upon reanalysis of the other 3 individuals, it was found that these animals were heterozygous at the specific locus in question. In the instance of 96B11 (Bmy42), the difference between analysis runs was due to poor PCR amplification in the first run.

The instances of TV14 and TV19 provided demonstrated issues of 'poor' amplification of one allele. In the case of TV14, the amplified intensity of the 101 allele was 10x less than the 93 allele. In this specific case, the fluorescence intensity levels of this allele

(101) would have been indistinguishable from background noise on the ABI 377. It is likely that the low level of detectability prevented the identification of the 101 allele in the first analysis. In the case of TV19, a poorly amplified allele was identified outside of the normal allele range for the locus (~20bp). Normally, the small allele in a microsatellite amplification is of equal or greater intensity when compared with the larger allele of a heterozygote. This however was not the case with regards to the 160bp allele. It was found to be less intense in all of the samples in which it was found. Additionally, the 160bp allele was found to be a rare allele (7 instances in the entire dataset). The compounding factors of being outside the normal size range for alleles, weaker amplification, and being a rare allele, caused these seven instances to be regarded as noise in the original dataset.

In the case of interannual heterogeneity, four individuals (92B3, 92B5, 92B6, & 99B3) were regenotyped for 12 loci. The data for the regenotyped individuals is shown in table E.2. For the reanalysis DNA was extracted via newer protocols than the original DNA used in the analysis. Additionally, the old 'original' DNA was diluted 100x for PCR reactions. It was noted that the old 'original' DNA contained a yellowish-brown coloration after it had been extracted (Phil Morin, personal communication). In the original analysis, the 'original' DNA was utilized in its undiluted form.

The individuals from 1992 (B3, B5, B6) all showed higher levels of heterozygosity for the 12 regenotyped loci. In analysis of the sample 92B3, it was noted a high level of errors between the first analysis and the reanalysis. In this instance, 92B3 provided inconsistent results at four of the loci that were reanalyzed. Another individual (92B5) was found to have a discrepancy at one locus (Bmy42) where the original genotype did not match alleles found in the reanalysis.

The remaining individual, 99B3, demonstrated that it was still homozygous at a substantial number of its loci. However, it exhibited two cases of allelic dropout within the reanalyzed samples. At Bmy26 and Bmy33 alleles were amplified in one DNA (original dilute), but not in the re-extracted DNAs. This brings into question the quality of the 99B3 sample and its ability to produce consistent results upon reamplification experiments.

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Table E-1. Allele calls for regenotyped individuals from whales identified by Morin et al. (2007).

Individual	Locus	Original Genotype	'New' Genotype
02B6	TV14	93/93	93/101
05B7	TV19	184/184	160/184
83B1	Bmy18	127/127	127/127
99B3	Bmy18	137/137	137/137
02B16	Bmy41	233/233	233/233
96B11	Bmy42	182/182	176/182

Table E-2. Allele calls for regenotyped individuals from whales identified in Jorde and Schweder (2007). Individuals listed as ###_new are alleles from the current DNA extraction method. ###_old are the new allele calls for diluted DNAs from the first DNA extraction. ###_original are the original genotypes that were submitted for the DAA. Missing data for an individual are shown as blank cells.

Individuals	Bmy1	Bmy1	Bmy10	Bmy10	Bmy11	Bmy11	Bmy14	Bmy14	Bmy16	Bmy16	Bmy18	Bmy18	Bmy19	Bmy19	Bmy26	Bmy26	Bmy33	Bmy33	Bmy42	Bmy42	Bmy55	Bmy55	Bmy57	Bmy57
92B3_new	243	263	216	226			179	181	210	210			124	126	162	174	134	148	174	178	219	221	154	162
92B3_old	243	263	234	246	226	232	179	181	210	210	115	117	124	126	162	174	134	148	178	180	219	221	154	162
92B3_original	**243**	**243**	**246**	**246**	**226**	**226**	**181**	**181**	**210**	**210**	**117**	**117**	**120**	**124**	**174**	**174**	**134**	**148**	**180**	**180**	**219**	**219**	**174**	**174**
92B5_new	251	261	236	238			179	179	214	220			106	114	164	166	138	138	174	180	219	223	154	160
92B5_old	251	261	236	238	226	230	179	179	214	220	109	119	106	114	164	166	138	138	174	180	219	223	154	160
92B5_original	**261**	**261**	**238**	**238**	**226**	**226**	**179**	**179**	**220**	**220**	**119**	**119**	**114**	**114**	**166**	**166**	**138**	**138**	**174**	**174**	**219**	**219**	**154**	**160**
92B6_new	253	253	224	240	220	236	175	179	212	212			118	124	152	168	138	148	162	180	217	223	160	160
92B6_old	253	253	224	240	220	236	175	179	212	212	109	115	118	124	152	168	138	148	162	180	217	223	160	160
92B6_original	**253**	**253**	**224**	**224**	**000**	**000**	**179**	**179**	**212**	**212**	**109**	**109**	**118**	**118**	**000**	**000**	**148**	**148**	**174**	**178**	**217**	**217**	**160**	**160**
99B3_new	243	243	226	226	220	230	179	179	212	212			114	122	154	154	134	134	178	178	219	219	160	160
99B3_old	243	243	226	226			179	179	212	212					142	154	134	148	178	178	219	219	160	160
99B3_original	**243**	**243**	**226**	**226**	**220**	**242**	**179**	**179**	**212**	**212**	**137**	**137**	**114**	**122**	**154**	**154**	**134**	**134**	**178**	**178**	**219**	**219**	**160**	**160**

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