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From Phocine Distemper to Avian Influenza: A Study of Immunogenetic Diversity in Two Sympatric Pinniped Species

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By

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FROM PHOCINE DISTEMPER TO AVIAN INFLUENZA: A STUDY OF IMMUNOGENETIC DIVERSITY IN TWO SYMPATRIC PINNIPED SPECIES

By Alayna K. Gigliotti

Thesis Advisor: Dr. Kristina Cammen

An Abstract of the Thesis Presented
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Gray (*Halichoerus grypus*) and harbor (*Phoca vitulina*) seals are sympatric species that inhabit the North Atlantic and have been subject to mortality events from disease outbreaks, particularly phocine distemper and avian influenza virus. Across mortality events, gray seals tend to exhibit a higher survival rate, which could be explained by various ecological factors impacting rates or direction of selection in parts of the genome related to the immune system. These factors could include haul-out site density, habitat, and degree of inter/intraspecies interaction. This research aims to compare genetic diversity within the Major Histocompatibility Complex (MHC) class I gene complex among gray and harbor seals sampled in the Northwest Atlantic to investigate how they have evolved in the face of shared natural stressors. MHC genes encode immune system receptors that recognize foreign pathogens, with class I responding to viral pathogens in particular. Possessing greater genetic diversity at MHC-I can be tied to greater immunocompetence. Due to high levels of gene duplication and polymorphism, MHC class I diversity has been traditionally challenging to evaluate at a population scale, but recent advances in sequencing technology enable high-throughput MHC genotyping. In this study, amplicon sequencing was used to characterize diversity in exons 2 and 3 of MHC-I, which encode

the peptide binding region. Analyses were performed on tissue biopsy samples from harbor seals by-caught in the Northeast US (n = 30), live harbor seal pups sampled in the Gulf of St. Lawrence (n = 30), and live gray seal pups sampled in Massachusetts (n = 30), Sable Island (n = 30), and the Gulf of St. Lawrence (n = 30). I compared the total number of MHC alleles, average number of alleles per individual, and sequence diversity among populations within species, as well as between species. My findings highlight the extent of allelic diversity and gene duplication that is present in MHC-I across Northwest Atlantic pinniped populations despite historical population bottlenecks. The presence of shared alleles between species and the lack of significant differences found for comparisons intra- or inter-specific MHC-I diversity suggest a shared selection regime in the MHC-I region for harbor and gray seals in the Northwest Atlantic. Overall, this study emphasizes the value of next-generation sequencing approaches to characterize multiple MHC loci given its polymorphic and duplicated nature. As gray seal populations expand, and sympatric harbor seal populations decline, a better understanding of the role of immunogenetic diversity in gray seal disease resistance will provide important insights into their role as disease reservoirs in coastal ecosystems.

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1. INTRODUCTION

As a result of changing climates and species distributions the potential for interspecific disease transmission is increasing. Studies that characterize factors affecting differences in disease susceptibility between species are therefore increasingly relevant to disease ecologists, conservation biologists, evolutionary biologists and protected resources managers. Such studies are particularly salient in sentinel species, such as marine mammals. Due to their high trophic level and long lifespans, the health of wild marine mammal populations closely reflects, and can act as an early indicator, of coastal ocean ecosystem health (Bossart 2011).

Harbor and gray seals in the Northwest Atlantic represent an ideal study system in which to investigate factors underlying interspecific differences in disease susceptibility. These two sympatric pinniped species share many aspects of their ecology, including common diseases, but differ significantly in their disease resistance. Historically, both seal populations in the Northwest Atlantic were drastically reduced by human-induced practices such as bounty hunting (Lelli & Harris 2006, Bowen & Ligard 2013). Since the passing of the United States Marine Mammal Protection Act of 1972, both pinniped populations have increased (Gilbert et al. 2005, Waring et al. 2010, Waring et al. 2015, Hayes et al. 2018). Yet, while celebrating this recovery, we must also acknowledge the potential for lasting effects of historical bottlenecks, like reductions in genetic diversity that can have significant consequences on species' capacity to adapt to natural stressors in their environment.

Examples of such stressors include the phocine distemper virus (PDV) and influenza A virus (IAV), which have both contributed to large-scale mortality events in gray and harbor seal populations across the North Atlantic. Disease transmission within seals are typically linked to seal haul-out sites, where the animals aggregate on land and both inter- and intraspecies

interactions occur. Therefore, seasonal, sex and age-related differences in haul-out patterns can affect viral transmission (Hall et al. 1992). Cross-species transmission of avian influenza is not widely studied, but could potentially come from sharing land-based haul-out sites and/or feeding on similar food sources as wild bird species (Fereidouni et al. 2016). It is suggested that Arctic phocid seals such as the harp seal (*Phoca groenlandica*) act as a primary host for these viruses while gray seals act as reservoirs, carrying both diseases to more susceptible harbor seal populations (Markussen & Have 1992, Hall et al. 2006, Puryear et al. 2016, Jo et al. 2018).

Mortality events associated with these viral outbreaks can have large impacts on pinniped populations. In 1988, European populations of harbor seals experienced a death rate from PDV of 10-60% (that varied among regions) affecting approximately 18,000 individuals over a 16-week span (Dietz et al. 1989, Thompson & Miller 1992, Heide-Jørgensen et al. 1992, Harding et al. 2002, Härkönen et al. 2006). A second European outbreak occurred in 2002, following similar patterns to that of 1988 (Harding et al. 2002, Jensen et al. 2002). Gray seals were also affected in the 1988 epidemic but to a much lesser extent totaling about 10% of total reported deaths, with most gray seals testing seropositive for the virus but remaining asymptomatic (Pomeroy et al. 2005).

Similar trends have been documented in the United States where PDV outbreaks have occurred on the coasts of Long Island, New York in 1988 (Duignan et al. 1993), and Massachusetts and Maine in 2006 (Earle et al. 2011). Several avian influenza outbreaks have occurred in the Northeast US and similarly in 2014 in the North Sea (Webster et al. 1981, Callan et al. 1995, Anthony et al. 2012, Zohari et al. 2014, Bodewes et al. 2015, Krog et al. 2015). Most recently, from July of 2018 through 2019, outbreaks of both PDV and avian influenza occurred in the Northeast US from the coast of Maine to Virginia. Although there were some instances of

co-infection, PDV was designated as the primary cause of a mass mortality event for gray, harbor and some species of ice seals. The total number of reported deaths during this period approximated over 3000 individuals, primarily harbor seals (NOAA 2019). These observations across many outbreaks and geographic regions of the world led to the hypothesis that gray seals appear be more resistant to PDV and IAV than harbor seals (Harwood et al. 1989, Harwood 1990, Hall et al. 1992).

Immunogenetic diversity at the Major Histocompatibility Complex (MHC) is one well-studied factor that affects disease resistance across diverse host and pathogen systems. The MHC is a polymorphic, multi-gene family that encodes cell-surface receptors integral in antigen presentation. Foreign bodies such as viruses become broken down into peptides upon entering the cell and are transported to the cell surface for MHC recognition. A series of biochemical reactions triggers an immune response to neighboring lymphocyte receptors upon antigen-presentation (Piertney & Oliver 2006). Extensive polymorphism has been documented in MHC proteins, in particular within the peptide binding region (PBR) where antigens bind to the MHC receptor. This polymorphism creates variation in peptide binding grooves which, in turn, allows for a diverse array of immune responses to foreign bodies (Hughes & Nei 1989, Neefjes et al. 2011). Numerous studies across vertebrate taxa have demonstrated the insights that can be gained in understanding disease resistance and susceptibility through investigating the polymorphic loci that encode the PBR.

Across many systems, researchers have identified evidence for heterozygote advantage shaping MHC diversity (Doherty & Zinkerngel 1975, Osborne et al. 2015). Heterozygote advantage, a form of balancing selection, can briefly be described as an increase in fitness that results from having two different alleles at a given locus. The degree of sequence variation

between alleles can also vary among individuals and is critical to immune performance, particularly sequence variation in the PBR. Natural selection for variation in this gene family has furthermore resulted in frequent gene duplications, that provide the potential benefit of expressing more than two variants of MHC receptors (e.g., *DQ*, *DR* and *DO* MHC-II loci in most mammals). Intraspecific variation in copy number variation, reflecting the extent of duplication within a given genomic region, has been identified and linked to disease resistance in some populations.

The MHC gene family is divided into three classes: I, II and III. Class I molecules are involved in facilitating an immune response to intracellular pathogens such as viruses whereas class II molecules recognize extracellular pathogens with overlap to class I. Class III molecules are often associated with macroparasite recognition (Janeway et al. 2004). Studies in pinnipeds have primarily focused on MHC-II, and report variable levels of genetic diversity across species and populations. Factors that can affect MHC diversity among pinnipeds include population structure, demographic history of species (i.e. population bottlenecks), habitat substrate and pathogen presence. Species that have experienced historical bottlenecks, such as the New Zealand sea lion (*Phocarctos hookeri*), Northern elephant seal (*Mirounga angustirostris*) and Australian sea lion (*Neophoca cinereal*), show similar trends of low allelic diversity across some MHC-II loci suggesting low potential for diverse immune response in these populations (Hoelzel et al. 1999, Weber et al. 2004, Lau et al. 2015). Within species, breeding colony genetic structure and habitat substrate have been shown to influence MHC-II DQB diversity among populations (Cammen et al. 2011). Within species, levels of diversity can also vary across loci; for example, there is little variation in the *DQB* locus but high variation in the *DRB* locus of the New Zealand sea lion, which may be explained by compensatory effects (Osborne et al. 2013 & 2015).

Relatively fewer studies have focused on MHC-I in pinnipeds, and those that do have reported low genetic diversity in classes I and II compared to terrestrial mammals (Slade 1992). For example, the Hawaiian monk seal (*Monachus schauinslandi*), a species that has experienced severe population declines, exhibits low sequence variation and number of alleles for MHC-I (Aldridge et al. 2006). A preliminary study of gray and harbor seals suggests gray seals exhibit more MHC-I variation in terms of number of copies present, sequence diversity and allelic diversity than harbor seals, finding up to 6 copies in harbor and 12 in gray seals (Hammond et al. 2012). This prior study characterized the full MHC-I gene sequence, but in a very small sample size of individuals, and therefore could only posit preliminary conclusions about this interspecific difference in MHC diversity.

In this study, we used high-throughput amplicon sequencing to compare levels of MHC-I diversity between gray and harbor seals on a population-scale in the Northwest Atlantic. Due to the high level of genetic variation and duplication, traditional sequencing methods such as Sanger sequencing are limited in their ability to resolve MHC-I genetic diversity. Cloning methods have been successful in multiple studies (Cammen et al. 2011, Hammond et al. 2012) but can be tedious and time-consuming. Next-generation high throughput sequencing methods provide a promising new approach to sequencing MHC loci and have become a widely-used method in genomic studies involving this region (Pearson et al. 2016, Palmer et al. 2016, Tarasyan et al. 2019). With this approach, we assessed multiple forms of genetic diversity within an individual's MHC-I loci. We compared (a) the total number of alleles identified in multiple gray and harbor seal populations in the Northwest Atlantic; (b) the average number of alleles per individual as a proxy for copy number variation (i.e., the extent of duplication within MHC-I); and (c) sequence diversity and imputed evolutionary relationships among alleles. We interpret

our findings within the context of previously described historical demography, contemporary genetic population structure, and apparent differences in disease resistance between species.

2. MATERIALS AND METHODS

2.1. Study Site and Sample Collection

Tissue samples were provided by the National Marine Fisheries Service (NMFS) Northeast Fisheries Science Center (NEFSC), Tufts University, and Department of Fisheries and Oceans Canada (DFO). The collection of samples in US waters and import of seal samples from Canada were conducted under NOAA Permit No. 17670-03 issued to the NMFS NEFSC. Gray seal samples were collected during the winter breeding seasons of 2013 to 2016 on Muskeget Island (N = 30) in Cape Cod, MA, USA, (41.334 N, 70.293685 W), Sable Island (N = 30) in Nova Scotia, Canada (43.9337° N, 59.9149° W) and the Gulf of St. Lawrence (N=30) in Canada (Table 2.1). Field sampling protocols followed Puryear et. al (2016) and all samples were tissue biopsies from weaned pups.

Table 2.1. List of gray seal samples by collection site and year. All DNA samples were good to excellent quality. Asterisk denotes samples sequenced in duplicate for replicate analysis.)

Sample ID	Collection Site	Year
Hg822*	Muskeget Island	2016
Hg824*	Muskeget Island	2016
Hg845*	Muskeget Island	2016
Hg808*	Muskeget Island	2016
Hg847*	Muskeget Island	2016
Hg802*	Muskeget Island	2016
Hg803*	Muskeget Island	2016
Hg805*	Muskeget Island	2016
Hg806	Muskeget Island	2016
Hg812	Muskeget Island	2016
Hg814	Muskeget Island	2016
Hg846	Muskeget Island	2016
Hg849	Muskeget Island	2016
Hg848	Muskeget Island	2016
Hg801	Muskeget Island	2016
Hg809	Muskeget Island	2016
Hg810	Muskeget Island	2016

Table 2.1. (continued) List of gray seal samples by collection site and year.

Hg811	Muskeget Island	2016
Hg813	Muskeget Island	2016
Hg820	Muskeget Island	2016
Hg355	Muskeget Island	2015
Hg356	Muskeget Island	2015
Hg358	Muskeget Island	2015
Hg371	Muskeget Island	2015
Hg372	Muskeget Island	2015
Hg373	Muskeget Island	2015
Hg377	Muskeget Island	2015
Hg378	Muskeget Island	2015
Hg379	Muskeget Island	2015
Hg363	Muskeget Island	2015
S04*	Sable Island	2015
S07*	Sable Island	2015
S28*	Sable Island	2015
S30*	Sable Island	2015
S31*	Sable Island	2015
S36*	Sable Island	2015
S38*	Sable Island	2015
S42*	Sable Island	2015
S97	Sable Island	2015
S84	Sable Island	2015
S82	Sable Island	2015
S81	Sable Island	2015
S77	Sable Island	2015
S73	Sable Island	2015
S71	Sable Island	2015
S70	Sable Island	2015
S79	Sable Island	2015
S51	Sable Island	2015
S46	Sable Island	2015
S24	Sable Island	2015
S19	Sable Island	2015
S10	Sable Island	2015
S91	Sable Island	2015
S89	Sable Island	2015
S88	Sable Island	2015
S69	Sable Island	2015
S75	Sable Island	2015
S74	Sable Island	2015
S43	Sable Island	2015
S72	Sable Island	2015
HgSg16-00*	Gulf of St. Lawrence	2016
HgSg16-01*	Gulf of St. Lawrence	2016
HgSg16-02*	Gulf of St. Lawrence	2016
HgSg16-03*	Gulf of St. Lawrence	2016

Table 2.1. (continued) List of gray seal samples by collection site and year.

HgSg16-04*	Gulf of St. Lawrence	2016
HgSg16-05*	Gulf of St. Lawrence	2016
HgSg16-06*	Gulf of St. Lawrence	2016
HgSg16-07*	Gulf of St. Lawrence	2016
HgSg16-10	Gulf of St. Lawrence	2016
HgSg16-11	Gulf of St. Lawrence	2016
HgSg16-12	Gulf of St. Lawrence	2016
HgSg16-13	Gulf of St. Lawrence	2016
HgSg16-14	Gulf of St. Lawrence	2016
HgSg16-15	Gulf of St. Lawrence	2016
HgSg16-16	Gulf of St. Lawrence	2016
HgSg16-18	Gulf of St. Lawrence	2016
HgSg16-19	Gulf of St. Lawrence	2016
HgSg16-20	Gulf of St. Lawrence	2016
HgSg16-21	Gulf of St. Lawrence	2016
HgSg16-22	Gulf of St. Lawrence	2016
HgSg16-23	Gulf of St. Lawrence	2016
HgSg16-25	Gulf of St. Lawrence	2016
HgSg16-26	Gulf of St. Lawrence	2016
HgSg16-27	Gulf of St. Lawrence	2016
HgSg16-28	Gulf of St. Lawrence	2016
HgSg16-29	Gulf of St. Lawrence	2016
HgSg16-30	Gulf of St. Lawrence	2016
HgSg16-31	Gulf of St. Lawrence	2016
HgSg16-32	Gulf of St. Lawrence	2016
HgSg16-33	Gulf of St. Lawrence	2016

Live harbor seal pups were similarly sampled in the Gulf of St. Lawrence (N = 30) in 2016.

Harbor seal samples from the Northeast US (N = 30) were collected by the Northeast Fisheries Observer Program from individuals bycaught in commercial fisheries between 2013-2015 (Table 2.2).

Table 2.2. List of harbor seal samples by collection site and year. All DNA samples were good to excellent quality. Asterisk denotes samples sequenced in duplicate for replicate analysis.

Sample ID	Collection Site	Year
D09377*	Northeast US	2015
D00579*	Northeast US	2015
D00820*	Northeast US	2015
D00827*	Northeast US	2015
D05711*	Northeast US	2015
D09365*	Northeast US	2015

Table 2.2. (continued) List of harbor seal samples by collection site and year.

DOA0029*	Northeast US	2015
DOA0031*	Northeast US	2015
D05148*	Northeast US	2015
D06125*	Northeast US	2015
DO7866*	Northeast US	2015
D09893*	Northeast US	2015
D09894*	Northeast US	2015
DOA0001*	Northeast US	2015
D09719*	Northeast US	2015
D06114*	Northeast US	2015
D05144*	Northeast US	2014
D00673*	Northeast US	2014
D00672*	Northeast US	2014
D00601*	Northeast US	2014
D00429*	Northeast US	2014
D00279	Northeast US	2014
D09500*	Northeast US	2013
D07091	Northeast US	2013
D05712	Northeast US	2013
D00298*	Northeast US	2013
D09500	Northeast US	2013
D07091	Northeast US	2013
D05712	Northeast US	2013
D00298	Northeast US	2013
MMF0338	Gulf of St. Lawrence	2016
MMF0345	Gulf of St. Lawrence	2016
MMF0344	Gulf of St. Lawrence	2016
MMF0343	Gulf of St. Lawrence	2016
MMF0340	Gulf of St. Lawrence	2016
MMF0339	Gulf of St. Lawrence	2016
MMF0336	Gulf of St. Lawrence	2016
MMF0335	Gulf of St. Lawrence	2016
MMF0330	Gulf of St. Lawrence	2016
MMF0333	Gulf of St. Lawrence	2016
MMF0331	Gulf of St. Lawrence	2016
MMF0327	Gulf of St. Lawrence	2016
MMF0328	Gulf of St. Lawrence	2016
MMF0326	Gulf of St. Lawrence	2016
MMF0325	Gulf of St. Lawrence	2016
MMF0324	Gulf of St. Lawrence	2016
MMF0323	Gulf of St. Lawrence	2016
MMF0322	Gulf of St. Lawrence	2016
MMF0320	Gulf of St. Lawrence	2016
MMF0319	Gulf of St. Lawrence	2016
MMF0318	Gulf of St. Lawrence	2016
MMF0317	Gulf of St. Lawrence	2016
MMF0316	Gulf of St. Lawrence	2016

Table 2.2. (continued) List of harbor seal samples by collection site and year.

MMF0315	Gulf of St. Lawrence	2016
MMF0314	Gulf of St. Lawrence	2016
MMF0312	Gulf of St. Lawrence	2016
MMF0311	Gulf of St. Lawrence	2016
MMF0306	Gulf of St. Lawrence	2016
MMF0305	Gulf of St. Lawrence	2016
MMF0304	Gulf of St. Lawrence	2016

2.2. Genomic DNA Extraction, PCR & Amplicon Sequencing

Genomic DNA was extracted from ~10-20 mg of seal skin from each individual using the Qiagen DNeasy Blood & Tissue Extraction kit and the manufacturer's spin column protocol for Purification of Total DNA from Animal Tissues, with minor modifications. 20 µl of 1M DTT was added during the digestion phase and tissues were incubated at 56 °C and 850 rpm overnight to fully digest. Following digestion, 4 uL of RNAse (100 mg/mL) was added to remove RNA from the targeted genomic DNA.

Independent PCRs were performed to amplify exons 2 and 3 of MHC-I, which span the peptide binding region. PCR primers were designed from an alignment of gray and harbor seal MHC-I sequences (GenBank Accession No: JX218867-JX218936 from Hammond et al. (2012)) and evaluated using primer3web v4.1.0 (Untergasser et al. 2012; Koressar and Remm, 2007; Koressar et al. 2018). Our custom reverse primer PvLAex2Rb (5'-GKCCTCGCTYTGGTTGTAG-3') in combination with primer PvLAex2F (5'-GGCTCCCACTCCMTGARGT-3') from Hammond et al. (2012) amplified a 272 bp fragment of exon 2. Primers PvLAex3F (5'-GGCGGGGCCAGGGTCT-3') and PvLAex3R (5'-CCGCGGCCCCTGGTA-3') amplified a 304 bp fragment of exon 3. Illumina Nextera adapter sequences were added onto the 5' end of the primers to facilitate downstream sequencing.

PCRs were conducted in a total volume of 20 μL that contained 2 μL of DNA, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dGTP, 0.3 μM of each primer, 3% DMSO, 2 units of Phusion polymerase (New England Biolabs), and 1X Phusion HF Buffer (7.5 mM $MgCl_2$ [1.5 mM at 1X dilution]). At 1X concentration, Phusion Master Mix provides 1.5 mM MgCl2 and 200 μM of each dNTP in the final reaction. Phusion cannot incorporate dUTP and is not recommended for use with uracil-containing primers or template (containing 1.5 mM MgCl2 and 200 uM of each dNTP). The PCR profile for exon 2 consisted of an initial denaturation step at 98 °C for 1 minute followed by 28 cycles of 98 °C for 10 s, 68 °C for 25s, 72 °C for 10s, and a final extension step of 72 °C for 10 min. The PCR profile for exon 3 consisted of an initial denaturation step of 100 °C for 2 min, followed by 28 cycles of 100 °C for 10s, 70 °C for 25s, 72 °C for 10s, and a final extension step of 72 °C for 10 min.

Following PCR, the KAPA Pure Beads (KR1245-v3.16) magnetic bead protocol was performed on all samples using Beckman Counter Agencourt AMPure XP beads to remove any PCR impurities such as small, unwanted fragments and primer-dimers. A 1.8X bead-to-sample volumetric ratio was used for size selection of our ~300 bp products. Agarose gel electrophoresis was performed on all samples to confirm size of final product and approximate concentration prior to sequencing. Dilutions were performed to products as necessary to achieve uniform concentration. Samples were sent to the Hubbard Center for Genome Studies in New Hampshire, USA for rapid, paired-end sequencing (2x250) on an Illumina HiSeq2500.

2.3. Read Filtering Pipeline to Determine Alleles

Sequencing reads were processed using the program USEARCH (Edgar 2010) and a pipeline adopted from Sommer et al. (2013) and implemented in R to generate a list of alleles per individual. Within USEARCH, the UPARSE-OTU algorithm was used to identify clusters of

identical sequences (i.e., putative alleles) in order of least to most frequent, and UNOISE was used to denoise the filtered set of amplicon reads. Paired reads were first merged using the fastq_mergepairs command. Any reads that did not contain the target primer were identified using the search_oligodb command and removed from further analysis. Primers and flanking intronic regions were trimmed using the fastx_truncate command, and reads were then oriented using the orient command and a reference sequence of exon 2 and 3 from Hammond et al. (2012). A quality filter was applied using the fastq_filter command and an Emax threshold of 1 to filter for a Phred score of 30 or higher. Remaining identical reads were clustered and sorted by frequency using the fastx_uniques command. All clusters with fewer than 8 reads were removed from further analysis.

The unoise command was then implemented to identify putative chimeras (unnatural fusion of two or more alleles in one sequence), shifted sequences (sequences being one base-pair mis-aligned), and sequences with putative PCR or sequencing point errors. The latter were identified by considering both sequence abundance (i.e., number of identical reads) and sequence distance (d, the number of differences including both substitutions and gaps) from the most similar, more abundant sequence cluster. A sequence was identified as containing putative errors if the ratio of its abundance to the abundance of the most similar, more abundant sequence was less than or equal to $1/2^{\alpha d+1}$, a model of error abundance distributions generated by Edgar (2016). The unoise command was run with $\alpha = 2$, the default parameter setting which was found to provide the best filtering approach for our dataset upon visual inspection of the unoise output at multiple α levels.

Guided by the unoise output, reads were further processed in a custom R script to identify a list of putative alleles, putative artefacts, and unclassified variants for each exon using a protocol modified from Sommer et al. (2013) (Figure 2.1). The most frequent cluster of identical

sequences for each sample was assumed to be a putative allele. All shifted variants were removed. Thereafter, the sequences from each sample were evaluated by comparison to other samples. All sequences identified as having putative point errors were discarded as a putative artefact unless they were identified as a putative allele in other samples (because they were the most frequent cluster within a sample, or because they were more frequent than any putative artefact within a sample, as described in the final steps of the pipeline below). All sequences identified as putative chimeras in multiple samples were discarded as putative artefacts. Sequences identified as a putative chimera in only a single sample were considered unclassified variants, unless called a putative allele in another sample. The unclassified variant category was used when there was no additional information in the dataset to confirm or refute the chimera designation. Of the remaining sequences for a given sample, any sequence whose frequency was not lower than any putative artefact was added to the list of putative alleles. Sequences with a frequency lower than that of any putative artefact for that sample were considered an unclassified variant unless it was identified as a putative allele in other samples. Unclassified variants and putative artefacts were removed from the dataset. Finally, a 1% filter was implemented on the entire set of reads remaining from the pipeline so that only alleles present in at least 1% of the reads per individual were retained for subsequent analyses.

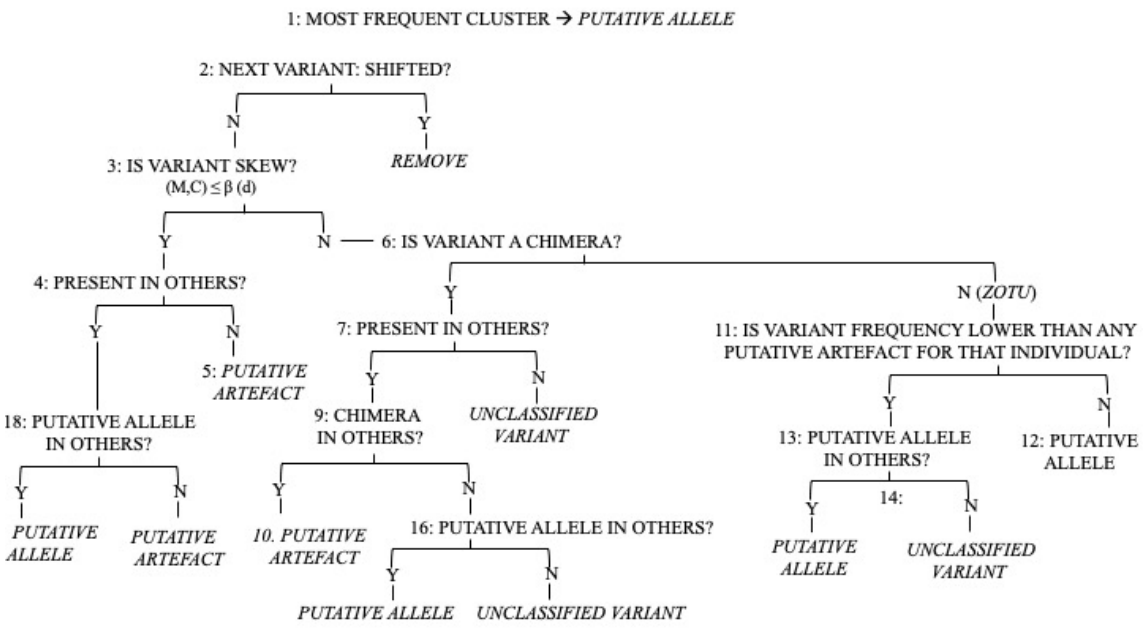

Figure 2.1. Flowchart illustrating the series of steps taken in allele filtering pipeline.

2.4. Technical Replicate Analysis

Technical replicates were sequenced to evaluate the consistency of the read filtering and allele calling pipeline described above. A subset of individuals from both species (24 bycaught N.E. U.S. harbor seals; 8 Muskeget Island, 8 Sable Island, and 8 Gulf of St. Lawrence gray seals) were amplified and sequenced twice in independent reactions. A custom script in R was used to compare both sequence overlap among replicates and weighted sequence overlap following Wen et al. (2017). These metrics were calculated as:

1. Sequence Overlap between replicates $= \frac{2\,x\,(number\ of\ shared\ sequences)}{(sequences\ of\ replicate\ A)+(sequences\ of\ replicate\ B)}$
2. Weighted Sequence Overlap between replicates $= \frac{(number\ of\ sequences\ of\ shared\ from\ replicate\ A)+(number\ of\ sequences\ of\ shared\ from\ replicate\ B)}{total\ number\ of\ sequences\ within\ replicate\ A\ +\ replicate\ B}$

If necessary, we were prepared to test other read filters (5%, 10%) if the analysis showed low read overlap between each sample and its respective replicate. It was determined, however, that high overlap was observed with the 1% filter.

2.5. Allele Discovery Saturation Curves

To test if the read depth was sufficient to reach saturation in allele discovery, saturation curves were generated using a custom R script. We calculated the average number of alleles detected in at least 1% of total reads for several subsamples of each individual, as well as a simulated individual with 240,000 reads and equal read proportions representing 24 alleles (greater than the maximum number of alleles observed per individual). We tested subsamples of 2^{-x} times the number of reads up to x = 11 for all locations except harbor seals at the Gulf of St. Lawrence, and up to x = 15 for this latter population, which had a significantly larger number of reads than all other locations. One hundred subsamples of each size were randomly drawn with replacement from the total set of reads using a custom R script. The curve from simulated data was compared to the individual curves from each sample to determine the minimum read depth for saturation of allele discovery. An additional filter for read depth was applied to exon 3 based on this analysis; any sample with fewer than 400 reads was removed from the dataset.

2.6. Analyses of Genetic Diversity

Following the filtering pipeline described above, the total number of alleles for every population and each species was calculated in R. Shared alleles were identified through intra and interspecific comparisons. The average number of alleles per individual was calculated as a proxy for copy number variation. MHC-I allele sequences we identified in gray and harbor seals sampled in the Northwest Atlantic were compared to sequences from other regions and other species using MEGA. Phylogenetic trees were built using the Maximum Likelihood Method and Tamura-Nei model (Tamura and Nei, 1993).

3. RESULTS

3.1. Assessing the Genotyping Pipeline

In this study, technical replicates and simulations were used to test the efficacy of our genotyping pipeline and to determine if additional filtering thresholds were needed.

3.1.1. Technical Replicate Analysis

We found that all populations showed high overlap in both exon 2 and exon 3 genotypes between replicates in weighted and unweighted comparisons of alleles (Figures 3.1), supporting precision of the genotyping pipeline. There was no significant difference in the average weighted or unweighted allele overlap for exon 2 between populations of gray seals (weighted: $F(2,21) = 0.90$, $p = 0.42$; unweighted: $F(2,21) = 0.53$, $p = 0.59$) or between harbor and gray seals (weighted: $t(46) = 0.89$, $p = 0.38$; unweighted: $t(46) = 1.66$, $p = 0.10$). For exon 3, there was no significant difference in either metric between harbor and gray seals (weighted: $t(33) = 0.05$, $p = 0.96$; unweighted $t(33) = -0.95$, $p = 0.35$). There was a significant difference in average unweighted exon 3 allele overlap between populations of gray seals ($F(2,20) = 4.58$, $p < 0.05$), but no significant difference in average weighted allele overlap ($F(2,20) = 3.18$, $p = 0.06$).

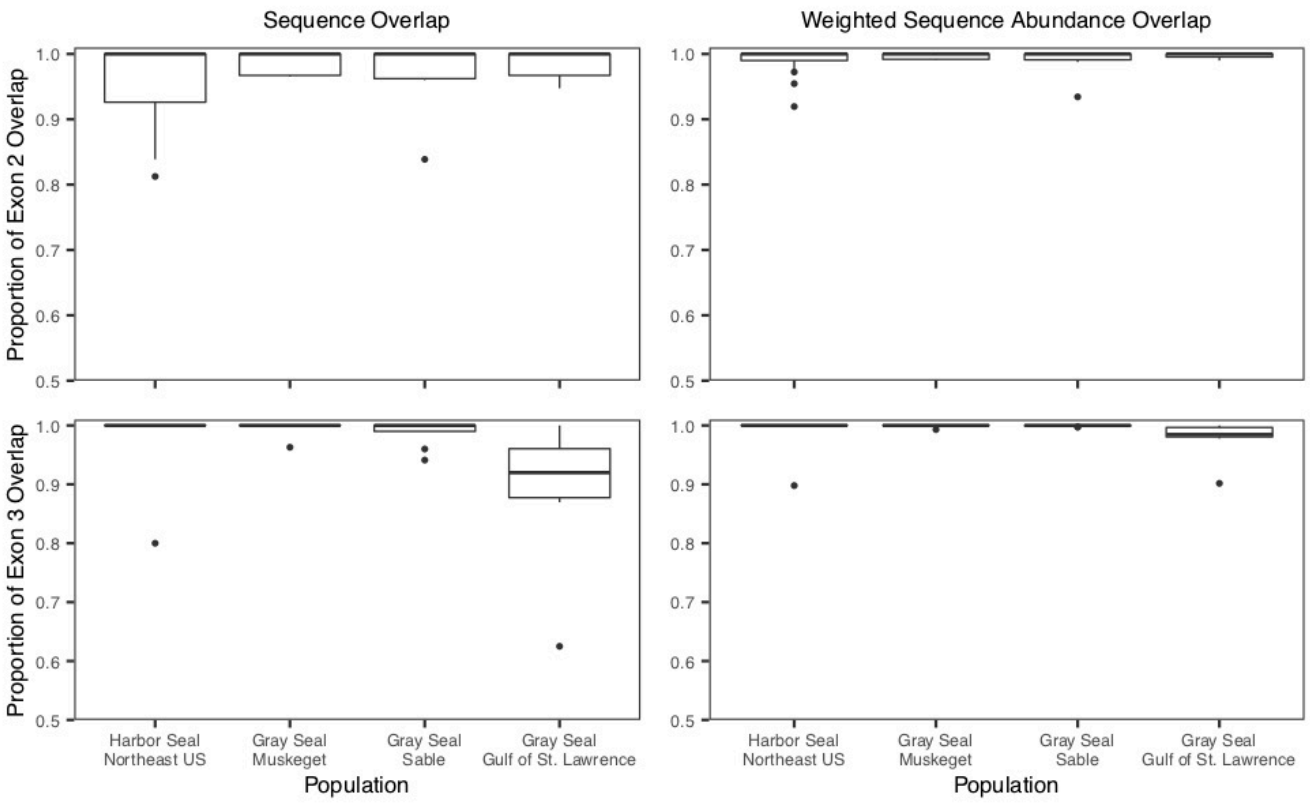

Figure 3.1. Proportion of sequence overlap and weighted sequence abundance between replicates**.** The exon 2 technical replicate analysis included 24 bycaught N.E. U.S. harbor seals; and 8 Muskeget Island, 8 Sable Island, and 8 Gulf of St. Lawrence gray seals that were amplified twice in independent reactions and successfully sequenced. Due to low read depth resulting in exclusion of some samples, the exon 3 technical replicate analysis included 12 bycaught N.E. U.S. harbor seals; and 7 Muskeget Island, 8 Sable Island, and 8 Gulf of St. Lawrence gray seals. Boxplots show the median and 25th and 75th quartiles, and whiskers represent 1.5x the interquartile range beyond the bounds of the box.

3.1.2. Allele Discovery Saturation Curves

The effect of read depth on allele discovery was evaluated through down sampling a simulated dataset with 24 alleles (greater than the maximum number of alleles observed per individual) and 240,000 reads, as well as the actual sequencing data generated for each individual that passed initial filtering steps. The simulated individual appeared to reach saturation in allele discovery by 400 reads (Figure 3.2).

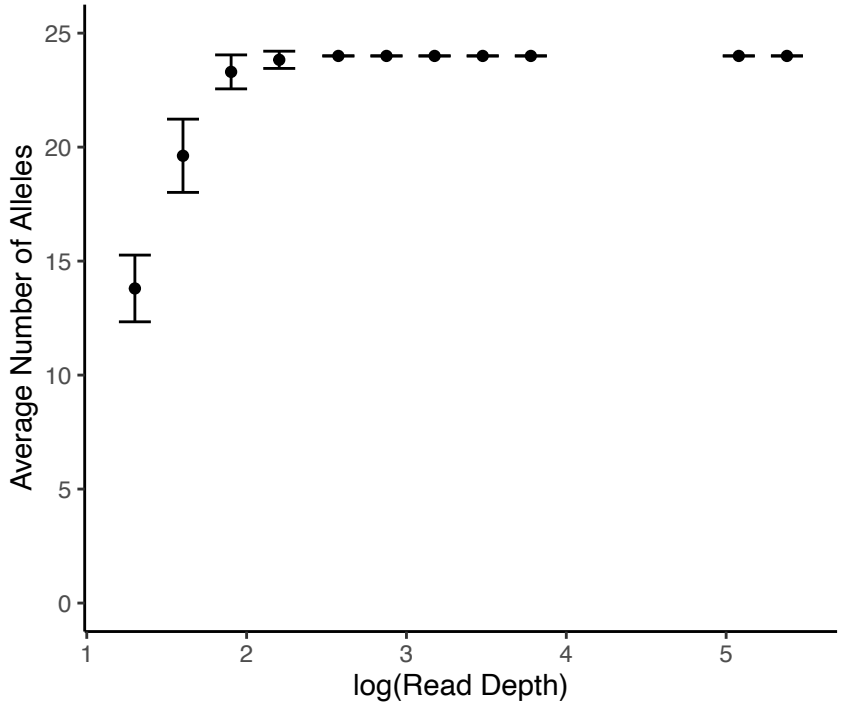

Figure 3.2. Simulated data saturation curve for allele discovery**.** Average number of alleles detected in 100 random subsamples per read depth of a simulated individual with 240,000 reads and equal read proportions representing 24 alleles.

This saturation point was also observed in the actual sequencing data for most individuals for both exon 2 (Figure 3.3) and exon 3 (Figure 3.4). A read depth of 400 was therefore used as a minimum threshold for inclusion in subsequent analyses, and 15 exon 3 samples with a read depth below 400 reads were excluded.

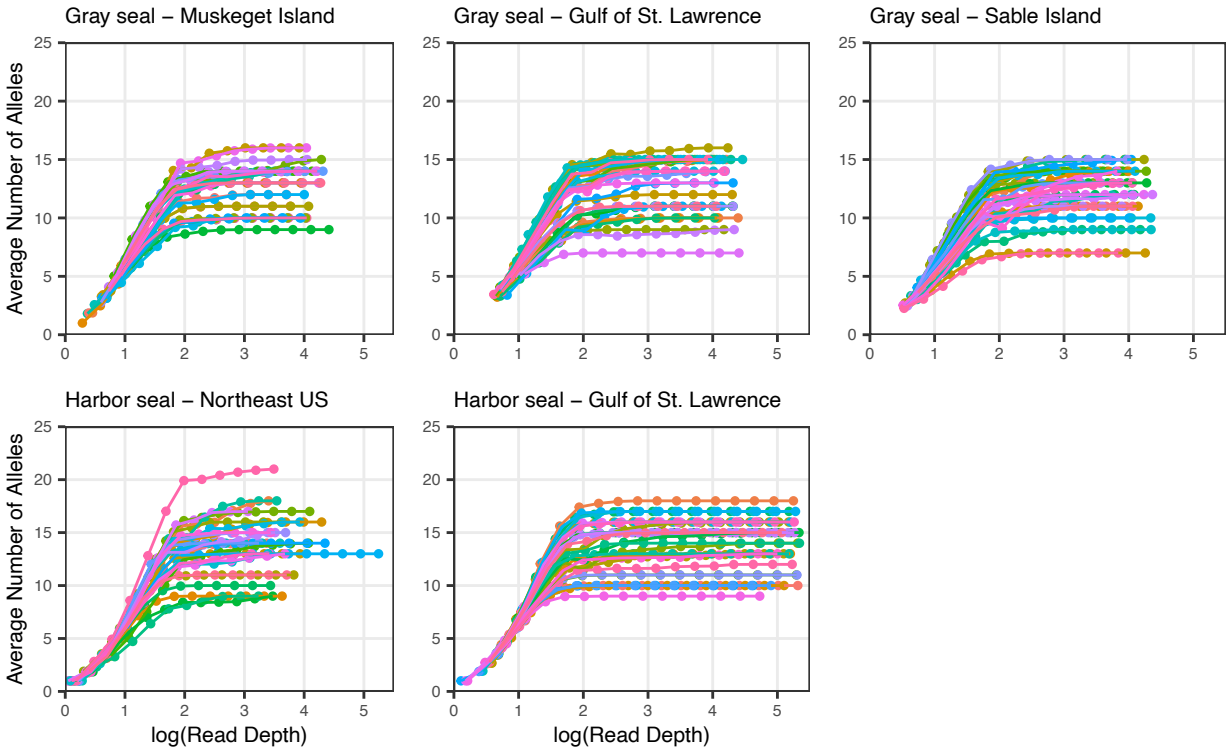

Figure 3.3. Saturation curves for exon 2 allele discovery across gray and harbor seal populations. Average number of alleles detected in 100 random subsamples per read depth for all individuals, with each colored line representing an individual.

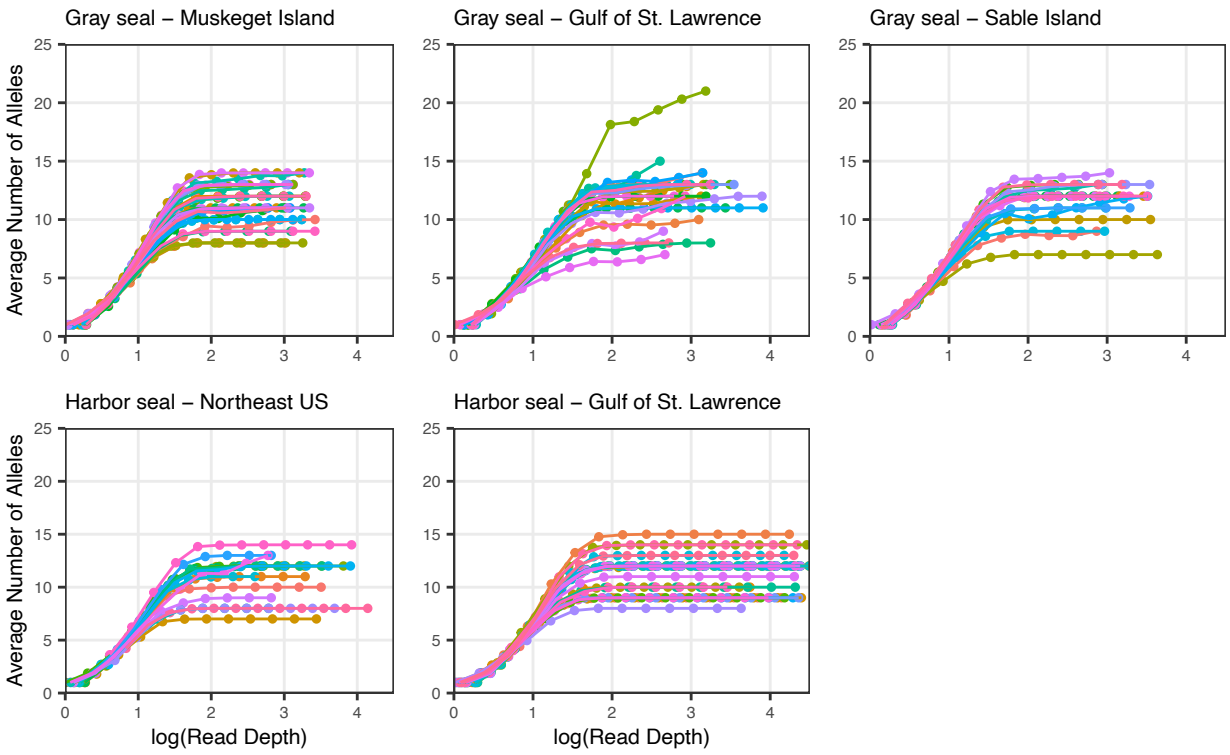

Figure 3.4. Saturation curves for exon 3 allele discovery across gray and harbor seal populations. Average number of alleles detected in 100 random subsamples per read depth for all individuals.

3.1.3. Read Depth

Sequencing resulted in variable read depth across seal sampling locations and between species. Harbor seals had a significantly greater average number of reads per individual than gray seals for both exon 2 ($t(139) = -7.78$, $p = 1.47 \times 10^{-12}$) and exon 3 ($t(116) = -7.36$, $p = 2.80 \times 10^{-11}$). Harbor seals from the Gulf of St. Lawrence had significantly higher average read depth than harbor seals from the Northeast US (exon 2: $t(54) = 11.34$, $p =$. 6.61×10^{-16}; exon 3: $t(43) = 5.07$, $p = 8.17 \times 10^{-6}$). Gray seals also showed a significant difference in average read depth among populations for exon 2 ($F(2,82) = 4.76$, $p < 0.05$), but not exon 3 ($F(2,70) = 0.66$; $p = 0.52$). Yet, the technical replicate analysis and assessment of saturation curves described above suggest that our pipeline is robust to differences in read depth, above a minimum read depth of 400, and support our ability to compare allelic diversity among populations and between species.

3.2. Analysis of Allelic Diversity

3.2.1. Total Number of Alleles

We identified a total of 93 alleles in exon 2 and 100 alleles in exon 3 among gray and harbor seals (Figure 3.5). For exon 2, 53 alleles were unique to harbor seals (n=56), 47 alleles were unique to gray seals (n=86) and 7 alleles were observed in both species. For exon 3, 65 alleles were unique to gray seals (n=73), 36 alleles were unique to harbor seals (n=45) and 1 allele was observed in both species.

The majority of alleles were shared among populations within species. Among harbor seals, for exon 2, we found 2 unique alleles in the Gulf of St. Lawrence population (n=29), 21 unique alleles in the N.E. U.S. (n=27), and 37 alleles were shared between the two populations. For exon 3, 2 unique alleles were found in the Gulf of St. Lawrence harbor seal population (n=28), 7 unique alleles were found in the N.E. U.S. population (n=17) and 27 alleles were observed in both populations. Among gray seals, for exon 2, 4 alleles were unique to the Gulf of St. Lawrence population (n=29), 4 alleles unique to Sable Island (n=30) and 0 alleles unique to Muskeget Island (n=27). A total of 41 alleles were shared between all gray seal populations with fewer than 2 additional alleles shared between any two populations. For exon 3, we found 1 unique allele in the Gulf of St. Lawrence gray seal population (n=25), 19 unique alleles in Sable Island (n=20), and 1 unique allele in Muskeget Island (n=28). A total of 37 alleles were shared amongst all three populations with fewer than 3 additional alleles shared between any two populations.

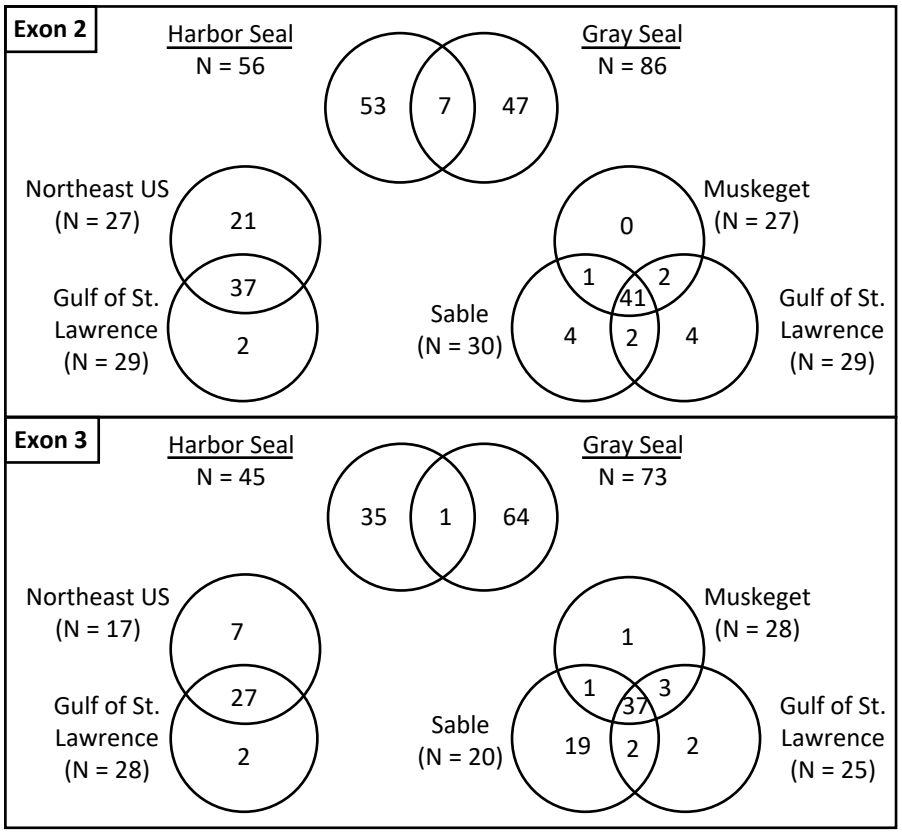

Figure 3.5. Venn diagram of total number of exon 2 alleles discovered in gray and harbor seals.

3.2.2. Copy Number Variation

We used the number of alleles per individual as a proxy of copy number variation. The number of alleles observed in a single individual ranged from 9 to 21 for exon 2 and 7 to 15 for exon 3 in harbor seals, and 7 to 16 for exon 2 and 7 to 21 for exon 3 in gray seals. These values suggest a minimum of 4 to 11 MHC-I copies in both harbor and gray seals.

Harbor seals were observed to have a significantly greater average number of exon 2 alleles per individual than gray seals (harbor: 13.93 ± 0.37 SE; gray 12.48 ± 0.28 SE; $t(139) = -3.40$, $p < 0.001$) (Figure 3.6), though no interspecific difference was noted for exon 3 (harbor: 11.07 ± 0.28 SE; gray: 11.64 ± 0.25 SE; $t(116) = 1.48$, $p = 0.14$) (Figure 3.7). There was no significant difference in the average number of alleles per individual observed among populations of harbor or gray seals for either exon 2 (harbor: $t(54) = -0.19$, $p = 0.85$; gray:

F(2,82) = 0.29, p = 0.75) (Figures 3.6) or exon 3 (harbor: t(43) = 0.66, p = 0.51; gray: F(2,70) = 1.43, p = 0.25) (Figures 3.7).

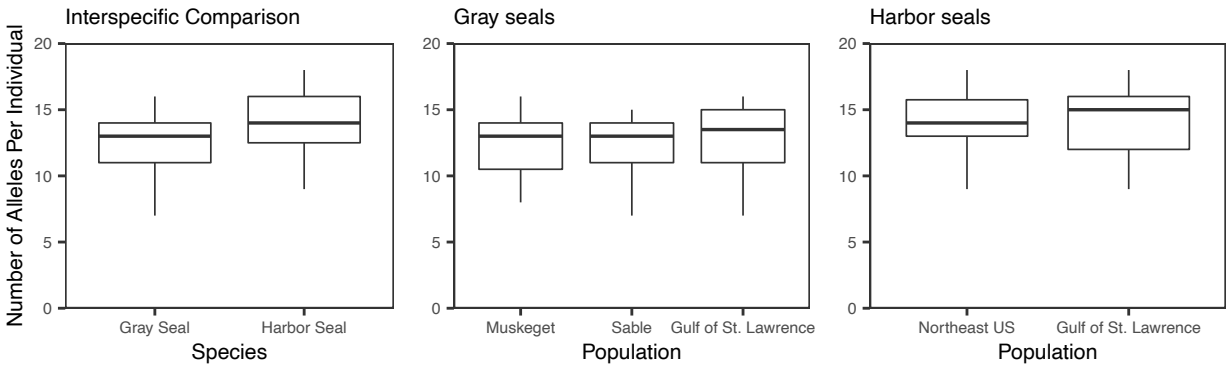

Figure 3.6. Inter- and intra-specific comparisons of average number of exon 2 alleles per individual in harbor and gray seals. Boxplots show the median and 25th and 75th quartiles, and whiskers represent 1.5x the interquartile range beyond the bounds of the box. Sample sizes as in Figure 3.5.

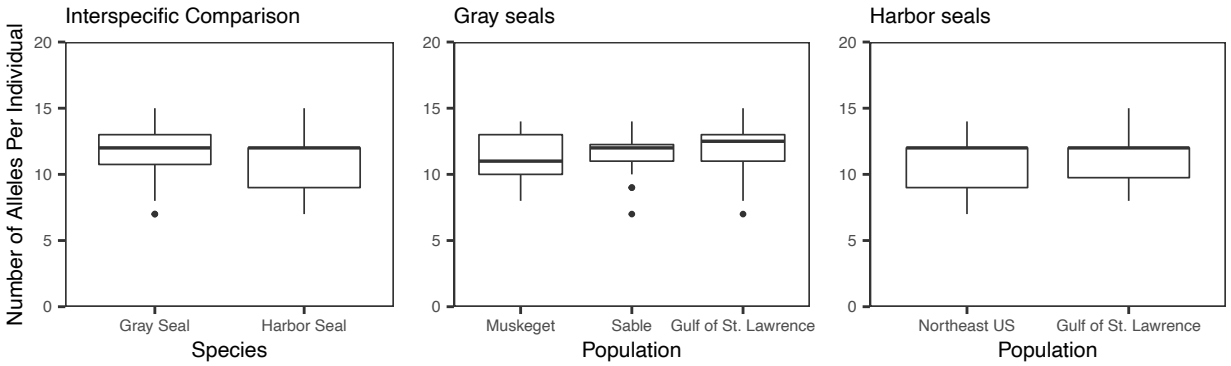

Figure 3.7. Inter- and intra-specific comparisons of average number of exon 3 alleles per individual in harbor and gray seals. Boxplots show the median and 25th and 75th quartiles, and whiskers represent 1.5x the interquartile range beyond the bounds of the box. Sample sizes as in Figure 3.5.

3.2.3. Phylogenetic Analysis

The phylogenetic analysis showed evidence of alleles that are strongly suggested by their relationships on the tree to be part of lineages previously described in MHC-I gray and harbor seal work (Hammond et al. 2012) (Figure 3.8).

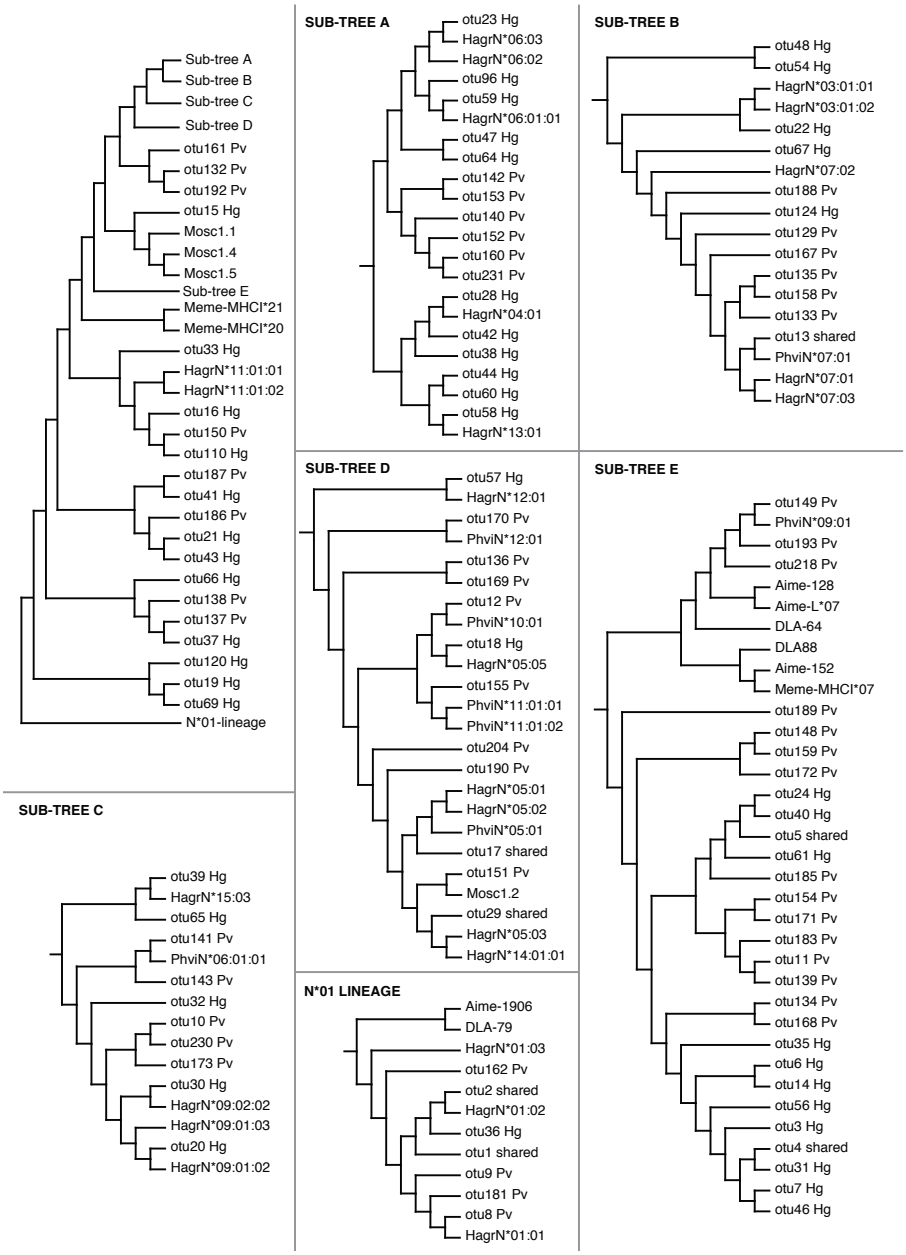

Figure 3.8 Phylogenetic tree showing allele relatedness for exon 2 using the maximum-likelihood method and Tamura-Nei model. Sequences discovered in this study are referred to with their temporary identifiers, composed of “otu" followed by Pv indicating an allele only

observed in harbor seals, Hg indicating an allele only observed in gray seals, or shared indicating the allele was observed in both species. These sequences will be renamed following MHC naming conventions prior to publication and submission to NCBI GenBank. MHC I sequences characterized in this study are compared with gray (HagrN) and harbor (PhviN) MHC I sequences from Hammond et al. (2012), as well as MHC I sequences from other carnivores including Hawaiian monk seal (*Monachus schauinslandi*) translated from Aldridge et al. (2006), giant panda (*Ailuropoda melanoleuca*, Aime) (GenBank: EU162656-57, JX987023, EU162661), dog (*Canis lupus*, DLA) (GenBank: NM001014378, NM001014767), and European badger (*Meles meles*, Meme) (LC350080-81, JQ425446).

4. DISCUSSION

The purpose of this study was to characterize immunogenetic diversity at MHC-I loci in gray and harbor seals in the Northwest Atlantic, two species in an environment with shared natural stressors. We present a high-throughput pipeline to detect alleles in a highly polymorphic, duplicated gene region on a population-scale despite varying read depths using amplicon sequencing. Overall, we found high MHC-I diversity in both of these historically bottlenecked species, and little evidence of a significant difference in diversity metrics between species, despite their observed difference in resistance to viral pathogens.

We found that the total number of alleles unique to each species did differ in that harbor seals had more than gray seals at exon 2, but vice versa for exon 3 (Figure 3.5). Several exon 2 and few exon 3 alleles were shared between species. For exon 2, two of the alleles that were shared between the two species were closely related to alleles previously characterized as ancestral alleles (N*01 lineage, Figure 3.8), while the remaining shared alleles were distributed across the divergent lineages. Alleles shared between species, or trans-species polymorphism (TSP) are consistent with balancing selection (Klein et al. 1998) acting on MHC-I. The phylogenetic analysis pointed to new alleles discovered that were part of lineages previously described in gray and harbor seal MHC-I work (Hammond et al. 2012). It is important to note that we did not have a method of testing for functionality of alleles, so it may be that some alleles are pseudogenes. Important next steps for these data include calculating dN/dS ratios for PBR loci and assessing translated amino acid sequences to further characterize selection on this genomic region. If a premature stop codon is found in an allele, that could give insight into functionality. Although the dN/dS ratio was not calculated in this study, our data are consistent with other studies that show high levels of MHC-I diversity and balancing selection acting on

this region. Linking pairwise sequence divergence to our phylogeny in the future will allow characterization of MHC "supertypes" (or clusters of MHC alleles) that could allow for characterization of MHC diversity per individual, species and location on a difference scale.

We found that most of the alleles were shared between populations, though the Northeast US harbor seal population and the Sable Island gray seal population had many unique alleles at exon 2 and exon 3, respectively (Figure 3.5). A prior study of MHC-II in Northeast Atlantic gray seals did report evidence of local adaptation, finding a significant difference in allele frequencies among colonies (Cammen et al. 2010). Further analysis of our MHC-I data should investigate allele frequencies among the sampled areas in the Northwest Atlantic. It could become possible to then link a specific allele having a stronger selection pressure than others from PDV and IAV, through directional selection. This could also help explain the lack of significant differences between gray and harbor seals that we see in diversity estimates found while considering the difference in disease resistance between the two species.

Our results indicate that MHC-I loci are highly diverse compared to their class II counterparts, as multiple studies characterizing MHC-*DQB* in pinnipeds have found relatively few alleles (Hoelzel et al. 1999; Bowen et al. 2002; Lento et al. 2003; Weber et al. 2004; Cammen et al. 2010). In addition, our results show evidence of high number of gene copies in both species for MHC-I. In fact, the number of gene copies may exceed our estimates, as it is not possible to determine homozygosity from our data and so we assume that each allele that we see is only present once. We found that within each species populations are similarly diverse in the average number of alleles per individual. On an interspecific level, we found that harbor seals had a significantly greater average number of exon 2, but not exon 3, alleles per individual than harbor seals. This finding is in contrast to our expectations of higher diversity in gray seals,

which have a higher survival rate and remain mostly asymptomatic during outbreaks of phocine distemper virus and avian influenza.

We should note that a greater number of samples were lost from the analysis for exon 3 during the filtering process, due primarily to low individual read depth. It is unclear what contributed to these data being poorer quality, but noted issues in the first stages of PCR. Agarose gel photos showed exon 3 primer-dimers (and adapter-dimers once Illumina adapters were added in library prep) that were brighter than our target band. By magnetic bead size-selection, we were able to successfully isolate our targeted region and remove dimers, but it seems something could be wrong in the biochemistry of Illumina adapters and our exon 3 genomic composition. Nextera adapters gave the best results, but some data were still lost. This issue, in addition to the differences between exons in some of our comparisons of MHC-I diversity, highlight the importance of evaluating more than one exon for a given gene. Traditional MHC approaches have focused on a single exon, but next-generation high-throughput sequencing enables efficient genotyping of multiple exons. Future studies such as this could also attempt to use long-read sequencing rather than amplicon sequencing. While amplicon sequencing is limited by read length to determine unphased alleles at individual exons, long-read sequencing could allow multiple exons to be phased into an MHC gene haplotype, as in Hammond et al. (2012) but on a population scale.

This study provides a first step in investigating the role of immunogenetic diversity in variable disease resistance that is observed among pinnipeds. To further link the MHC-I diversity described here with disease resistance would require a direct comparison of cases and controls, or seals that died due to disease exposure and those that survived. Identifying such individuals in natural populations is challenging. However, samples collected during the recent PDV-associated

mortality event of primarily harbor, and some gray and harp seals, in the Northeast US provides an opportunity for future study. As gray seal populations expand, and sympatric harbor seal populations decline, a better understanding of the role of immunogenetic diversity in gray seal disease resistance could provide important insights into their role as disease reservoirs in coastal ecosystems.

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