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MHC class II β diversity as a correlate of neutral-locus similarity and diversity, and a predictor of overwinter return, in song sparrows (*Melospiza melodia*)

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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Abstract

The major histocompatibility complex (MHC) is a family of genes involved in recognizing pathogens and mounting an immune response. Parasite-mediated selection often favours heterozygosity at MHC because MHC-diverse individuals recognize a wider range of pathogens. Because migratory birds encounter many pathogens, I hypothesized that MHC diversity predicts overwinter and juvenile survivorship in song sparrows (*Melospiza melodia*). I found no correlation between MHC diversity and neutral-locus (microsatellite) heterozygosity, suggesting that measures of neutral and adaptive genetic diversity provide complementary rather than redundant information. However, pairwise similarity at MHC predicted pairwise similarity at microsatellite loci. In contrast to my hypothesis, MHC diversity did not predict overwinter return rates (interpreted as survivorship). However, age cohort comparisons showed that adults were more MHC-diverse than nestlings in one of the two years examined. MHC-diverse individuals in this population may thus be more likely to survive to adulthood in some years, but not in others.

Keywords

Major histocompatibility complex, song sparrow, *Melospiza melodia*, survivorship, overwinter return, microsatellites, genetic diversity

Co-Authorship Statement

My supervisor, Beth MacDougall-Shackleton, and my collaborators, Joel Slade, and Dr. Greg Gloor will be co-authors on any publications resulting from this thesis. Dr. Beth MacDougall-Shackleton contributed to the development of the project, aided in the collection of field data, and funded and oversaw the genetic analysis. Joel Slade aided in the collection of field data, development of genetic techniques and assisted with lab work. Dr. Greg Gloor developed the bioinformatics pipeline used to sort and analyze the genetic data.

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List of Abbreviations

AICc – Akaike information criterion

HFC – Heterozygosity-fitness correlation

HWE – Hardy-Weinberg Equilibrium

MHC - Major histocompatibility complex

MLH - Multilocus heterozygosity

ASY- After second year

N - Nestling

SY - Second year

1 Introduction

1.1 *Genetic diversity*

Genetic diversity is critically important for the adaptive potential and persistence of populations and also for the fitness of individuals. Populations with higher levels of genetic diversity are often more resilient to environmental stressors and changes, and as a result are more likely to persist over evolutionary time (Hughes et al. 2008). For example, populations of fruit flies (*Drosophila melanogaster*) with larger amounts of genetic diversity were better able to survive temporally varying ethanol conditions compared to less diverse populations (Mackay 1981). In particular, genetic diversity plays a key role in disease and pathogen resistance. For example, in Italian agile frogs (*Rana latastei*), populations with lower levels of genome-wide diversity were more susceptible to emergent pathogens and suffered higher mortality rates due to infections compared to more genetically diverse populations (Pearman and Garner 2005). At the individual level, more genetically diverse (outbred) individuals often outperform less diverse (inbred) individuals (Keller and Waller 2002). For example, in the California sea lion (*Zalophus californianus*), individuals that had higher infection loads typically had higher than normal parental relatedness, indicating that more inbred individuals were more susceptible to infections (Acevedo-Whitehouse et al. 2003). Similarly, increased neutral-locus genetic diversity is associated with reduced incidence and load of bloodborne parasites in white-crowned sparrows (*Zonotrichia leucophrys oriantha*) (MacDougall-Shackleton et al. 2005).

Many factors may influence levels of genetic diversity in populations. Spatial heterogeneity in the environment tends to maintain high levels of genetic diversity (Hedrick 1986; Schmidt et al. 2000), because different traits and different alleles are favoured in different local areas.

Similarly, environmental conditions often vary over time and such varying conditions can help maintain genetic diversity of populations (Ellner and Hairston 1994).

In addition to spatial and temporal variation in the environment, mate choice can also affect levels of population genetic diversity. If individuals mate disassortatively (that is, prefer mates that are phenotypically and genetically dissimilar to themselves), and/or if they avoid mating with close relatives, heterozygosity increases and rare alleles are more likely to persist in a population over time (Penn and Potts 1998; Penn et al. 1999). For example, in scale-eating cichlids (*Perissodus microlepis*), individuals with left-facing mouths mate primarily with individuals with right-facing mouths, and the ratio of left vs. right facing mouths in offspring is approximately 1:1. This suggests that disassortative mating in this species contributes to the maintenance of variation for this trait (Takahashi and Hori 2008). Conversely, assortative mating (preferentially mating with genetically similar individuals) and inbreeding (preferentially mating with close relatives) tend to decrease heterozygosity (Wright 1921; Charlesworth and Charlesworth 1999). For example, in blue tits (*Parus caeruleus*), individuals prefer mates with ultraviolet chroma patterns on their crown ornament that are similar to their own (Andersson et al. 1998). This preference for similar chroma patterns correlates to preference that are more genetically similar and thus increases homozygosity of offspring and overall decreases population heterozygosity.

Population size and history can also influence genetic diversity. For example, bottleneck events (defined as a significant decrease in population size) typically reduce population genetic diversity (Eimes et al. 2011). With a decrease in population size, rare alleles are often lost,

meaning that population genetic diversity decreases (Miller and Lambert 2004a). Population bottlenecks may be caused by human activity or by extreme weather events. For example, in the 1920's, human influence on Australian ecosystems drastically decreased population sizes of koalas (*Phascolarctos cinereus*). Koala populations that were most heavily affected now harbour the lowest levels of genetic diversity (Houlden et al. 1996).

Finally, an increasing body of evidence indicates the relationship between individual genetic diversity and fitness is a key contributor to levels of population genetic diversity. Inbred individuals often have low fitness relative to their more outbred counterparts (e.g. Keller 1998), due in part to increased expression of deleterious recessive alleles (Dudash and Carr 1998). In many taxa, positive relationships have been documented between various aspects of fitness and individual genetic diversity (Reed and Frankham 2003). For example, in European alpine marmots (*Marmota marmot*), individuals that are more heterozygous at three microsatellite loci have higher rates of juvenile survival compared to less heterozygous individuals, and this pattern is even stronger in years where the environmental conditions are more stressful (Silva et al. 2005). Such relationships are termed heterozygosity-fitness correlations (HFC). Heterozygosity can help mask deleterious recessive alleles and thus increase fitness (Hansson and Westerberg 2002). Additionally HFC can arise when there is a selective advantage for heterozygous individuals. An example of heterozygote advantage can be seen in the implication of increased heterozygosity in disease resistance. In multiple species of leaf-cutter ants (*Acromyrmex* spp.), as well as in rice (*Oryza* spp.), more genetically diverse populations and individuals are more resistant to pathogens and are more likely to survive infections (O'Brien and Evermann 1988; Zhu et al. 2000; Hughes and Boomsma 2004).

In the past, measuring genetic diversity of populations was difficult and could often only be accomplished through pedigree analysis (Cox et al. 1985). However, developing pedigrees is logistically challenging, especially when working with free-living organisms (Liberg et al. 2005). One approach for studying genetic diversity involves genotyping individuals at multiple neutral loci, such as microsatellites. Microsatellites are short tandem repeat DNA sequences that usually do not code for proteins, and as a result are generally selectively neutral (Schlötterer and Pemberton 1994). By assessing heterozygosity at multiple microsatellite loci and using this as a proxy of genome-wide genetic variation, researchers can assess genetic diversity of individuals (e.g. proportion of genotyped loci at which the individual is heterozygous) and of populations (e.g. proportion of individuals that are heterozygous at a given locus) (Ellegren 2004). Microsatellites are an excellent tool for studying genetic diversity and population genetic structure, in part because of their high levels of variation. For example, one study on Canadian polar bears (*Ursus maritimus*) found that even when genetic diversity of populations is low, microsatellites can be hypervariable, which permits detecting subtle population genetic structure (Paetkau et al. 1995). Microsatellites are thus a useful tool for assessing neutral-locus diversity of populations, which can offer information indicating levels of inbreeding, population structure, and other features relevant to the conservation and management of free-living populations (Dow and Ashley 1998; Liu et al. 2003). However, recent evidence has shown in populations that have undergone bottleneck events that neutral-locus diversity does not always correlate with adaptive-locus diversity (Holderegger et al. 2006; Vásquez-Carrillo et al. 2014), indicating that collecting information about both neutral and adaptive loci is important for predicting adaptive potential of populations.

1.2 Neutral- vs adaptive-locus diversity

Measures of neutral-locus diversity, based on microsatellite genotyping (Hauser et al. 2002) or the detection of single nucleotide polymorphisms (SNPs; Consortium 2009) are widely used when monitoring the genetic diversity of populations. Although such tools are effective for parentage analysis (Kellogg et al. 1995) and may even help to predict the likelihood of developing certain genetic diseases (Forghanifard et al. 2016), neutral-locus diversity may not always correlate with diversity at coding loci experiencing selection (that is, adaptive-locus diversity). For example, a bottleneck event in greater prairie chicken (*Tympanuchus cupido*) resulted in diversity of immunogenes decreasing by 44%, while microsatellite diversity only decreased by 8% (Eimes et al. 2011). In this case, using the loss of microsatellite diversity to estimate the loss of diversity at coding loci would underestimate the severity of the loss in adaptive genetic diversity, and potentially hinder conservation efforts. In contrast, low levels of heterozygosity at microsatellites might occur despite substantial adaptive genetic diversity, potentially leading to inaccurate assessment of genome wide genetic diversity in populations with high levels of adaptive loci diversity. For example, the San Nicolas Island fox (*Urocyon littoralis dickeyi*) population shows almost no variation at microsatellite loci that are hypervariable in other populations, but contains substantial variation at multiple MHC loci (Aguilar et al. 2004).

The genetic diversity of species, particularly species at risk of extinction, is key in developing management and breeding strategies for conservation efforts. A study on African wild dogs (*Lycaon pictus*) highlights this point by showing that consideration of both adaptive and neutral-

locus diversity, rather than one or the other, provides the clearest signal of population structure and allows a better management strategy to be implemented for breeding programs (Marsden et al. 2013). Thus, especially for populations of conservation concern or that have undergone bottleneck events, measuring both neutral and adaptive locus diversity can be important.

1.3 Parasites as a selective force and the major histocompatibility complex

Parasites are organisms that live on or within a host individual at some point in their lifecycle, with a detrimental effect on host fitness (Anderson and May 1978). Parasites are very taxonomically diverse (Poulin and Morand 2000), with representatives in groups including bacteriophages (Vos et al. 2009), fungi (Gworgwor and Weber 2003), invertebrates (Johnson and Klemm 1977), and vertebrates (Breault 1991). With approximately 40 % of described species exhibiting some type of parasitic behaviour, parasitism is a tremendously widespread lifestyle (Dobson et al. 2008).

By definition, parasites reduce the fitness of their host individual. Some potential consequences of infection include reduced expression of secondary sexual characteristics (e.g. Wedekind 1992; Mougeot et al. 2009). For example, male red grouse (*Lagopus scotica*) infected with parasitic nematodes (*Trichostrongylus tenuis*) show reduced carotenoid pigment levels in their supra-orbital combs, which are used as sexual ornaments in mate attraction (Martínez-Padilla et al. 2007). Parasites can also reduce host survival, as in cliff swallows (*Hirundo pyrrhonota*) where individuals with higher ectoparasite loads have reduced survivorship compared to less infested individuals (Brown et al. 1995), and in *Drosophila melanogaster*, where individuals infected with parasites are more susceptible to desiccation and starvation (Hoang 2001). Another fitness

consequence of parasitic infections is reduced reproductive output. In Arctic char (*Salvelinus alpinus*), males infected with nematodes have significantly reduced sperm quality, which reduces their ability to secure fertilizations in the presence of intense sperm competition (Liljedal et al. 1999). In house martins (*Delichon urbica*), treating females with medication to reduce blood parasites (*Haemoproteus progeneri*) increases their fecundity (clutch size) relative to that of untreated and parasitized individuals (Marzal et al. 2004). Thus, parasites constitute an important selective pressure on their hosts.

Vertebrate animals have evolved a diverse set of immune defenses to avoid and combat the negative fitness consequences of parasitic infections, including a family of genes collectively termed the major histocompatibility complex (MHC). The MHC has been highly conserved over evolutionary time with gene maps of birds, reptiles, amphibians, fish and mammals all showing many similarities in genetic structure (Kronenberg et al. 1994). MHC gene products are cell-surface receptors that recognize and bind to antigens derived from parasites, allowing these antigens to be presented to T cells and elicit an immune response (Trowsdale 1995).

MHC gene products comprise two main classes: class I bind antigens from intracellular parasites, such as viruses, and are found on all nucleated cells, and class II bind antigens from extracellular parasites, such as bacteria, and are commonly found only on macrophages, B-cells and dendritic cells (Campbell and Trowsdale 1993; Newell et al. 1994). MHC molecules consist of either one (class I) or two (class II) transmembrane anchor proteins, plus two peptide binding proteins that create a peptide binding groove where amino acid chains can be presented to T-cells (Norment et al. 1988). The β subunit of MHC class II encodes the peptide-binding region and is particularly

polymorphic (Kaufman 2000). A study on zebrafish (*Danio rerio*) found that the species contained three MHC class I loci and six MHC class II loci, indicating that in zebrafish there is greater variation at MHC class II loci than MHC class I loci (Bingulac-Popovic et al. 1997). Another study conducted in red jungle fowl (*Gallus gallus*) found up to nine MHC class I loci and ten MHC class II loci (Worley et al. 2008). In passerine birds, MHC class I and class II are both polymorphic, but class II appears to be more variable. For example, in the great reed warbler (*Acrocephalus arundinaceus*) as many as seven different class I alleles have been found in a single individual, suggesting a minimum of four class I loci in this species (Westerdahl et al. 1999), but in the common yellowthroat (*Geothlypis trichas*) as many as 39 different class II alleles have been found in a single individual, suggesting a minimum of 20 class II loci in this species (Bollmer et al. 2010). A study conducted on song sparrows found a maximum of 4 class I alleles in song sparrows (suggesting at least two class I loci) but up to 26 class II alleles (suggesting at least 13 loci) (Slade et al. 2016).

The high level of polymorphism at MHC shown by most natural populations is likely explained through a combination of historical gene duplication events, mutation, balancing selection (e.g. heterozygote advantage), and mate choice (Hughes 1994; Jordan and Bruford 1998). Throughout evolutionary history, there have been multiple gene duplication events at MHC loci resulting in the formation of multiple MHC loci (Miller and Lambert 2004b). Mutations can give rise to new MHC alleles, which are often favoured by selection. The peptide binding region of MHC appears to be under strong positive selection pressure in many species (Eizaguirre et al. 2012a). Thus, when mutations at this region occur, the new alleles formed can bind to new peptide chains thus

diversifying the range of parasites that can be presented to T-cells (Nikolić-Žugić and Carbone 1990).

Balancing selection at MHC is widely thought to be another important factor contributing to diversity being maintained at these loci. For example, evolutionary arms races between hosts and parasites may impose frequency-dependent selection favouring rare MHC alleles. In this situation, parasites will adapt to evade the most common MHC alleles in the host population, such that individual hosts with rare alleles have a selective advantage in defending against the most common parasite genotypes (Kaltz and Shykoff 1998). Thus, rare MHC alleles may be most effective in resisting infections in three-spined sticklebacks (*Gasterosteus aculeatus*), and individuals bearing such rare alleles may be preferred as mates (Eizaguirre et al. 2009).

Previously-rare MHC alleles will then spread through the host population until parasites adapt to them, resulting in new host alleles becoming advantageous (Kaltz and Shykoff 1998). This pattern of negative frequency-dependent selection preserves rare MHC alleles in the population and thereby prevents the loss of rare alleles, ultimately maintaining genetic variation at MHC (Judson 1995).

Heterozygote advantage at MHC may also maintain genetic variation within host populations, because MHC-diverse individuals can recognize and defend against a broader set of parasites (e.g. Messaoudi et al. 2002; Acevedo-Whitehouse et al. 2005, Evans and Neff 2009). In mice (*Mus musculus*), individuals with greater MHC class II diversity are better able to clear multi-strain infections, compared to less diverse individuals (Penn et al. 2002). Similarly, a study on song sparrows (*Melospiza melodia*) found that individuals with more MHC class I alleles had

reduced infection risk from *Plasmodium* (Slade et al. 2016). In systems in which heterozygote advantage operates at MHC, mate choice may also help maintain genetic diversity through disassortative mating: by choosing mates with dissimilar MHC profiles, parents can increase the likelihood of producing MHC-diverse offspring that should be defended against a wide variety of parasites. For example, in great frigatebirds (*Fregata minor*), mated pairs are more different in their MHC amino acid profiles than would be predicted under random mating, a pattern that should maximize MHC allele diversity in the resultant offspring (Juola and Dearborn 2011).

1.4 Research objectives and predictions

Monitoring adaptive-locus diversity in addition to neutral-locus diversity can provide a more complete picture of genome-wide diversity than neutral-locus measures alone (Marsden et al. 2013). In particular, diversity at MHC loci may affect the ability of individuals and populations to defend against parasitic infections. In this thesis I focus on MHC class II, because of its greater variability in birds compared to MHC class I (Westerdahl et al. 2004; Worley et al. 2008; Bollmer et al. 2010; Slade et al. 2016). I first examined the relationship between microsatellite heterozygosity and MHC class II β diversity, in a free-living population of song sparrows (*Melospiza melodia*). I hypothesized that MHC diversity is maintained through balancing selection despite fluctuations and reductions in population sizes (Aguilar et al. 2004; Schuster et al. 2016), therefore I predicted that individual diversity at MHC (number of alleles) does not correlate with diversity at microsatellites (multilocus heterozygosity). In addition, I examined the relationship between pairwise genetic distance at neutral markers (microsatellite distance) between individual birds, and pairwise genetic distance at MHC. Because MHC is presumably subject to selection pressures that microsatellites are not, I predicted that pairwise distance at

MHC distance should be independent of pairwise distance at microsatellites. Alternatively, if neutral-locus distance and MHC-related distance are correlated, this would suggest that birds could potentially infer MHC similarity from genome-wide similarity. If so, choosing mates that are MHC-dissimilar might also help to avoid inbreeding.

My second major objective was to determine the relationship between MHC class II β diversity and survivorship. Because migratory birds such as song sparrows are likely to encounter a wide range of parasites during their annual cycle, I hypothesized that individual MHC class II β diversity should predict overwinter survivorship. Specifically, I predicted that birds with higher diversity at MHC should be more likely than less-diverse individuals to return to the breeding grounds the following year (interpreted as MHC-diverse individuals being more likely to survive from one year to the next). Furthermore, if individuals with lower MHC class II diversity are less likely to survive from year to year, average diversity at MHC class II β should differ among age cohorts. Specifically, younger age cohorts should have lower average diversity at MHC, and greater variation around this average, relative to older age cohorts from which individuals with low diversity at MHC have already been culled.

2 Methods

2.1 Study system, site, and field methods

I examined overwinter survivorship, MHC diversity, and microsatellite diversity in a breeding population of song sparrows (*Melospiza melodia*). Song sparrows are a common migratory songbird across North America, showing considerable geographic variation in morphology and in mitochondrial control-region allele frequencies (Fry and Zink 1998). The study population breeds near Newboro, Ontario, Canada (44°28'38.77"N, 76°20'4.86"W), on land owned by the Queen's University Biological Station. The field site harbours a mixture of small ponds, wetlands, fields, and forest edge, making it suitable habitat for breeding song sparrows. The site supports 20-60 breeding pairs of song sparrows each year and is not physically isolated from similar surrounding habitat. Up to 50% of the adults breeding at the site each year return to the site to breed the following year, indicating a high level of adult philopatry (MacDougall-Shackleton et al. 2009).

Field data and samples were collected in 2014, 2015, and 2016 from April to June, which corresponds to the breeding season for this population. Members of the research team, along with myself, captured and collected blood samples from a total of 102 song sparrows (69 adults and 33 nestlings) in 2014, and 259 song sparrows (115 adults, 33 of which had been captured the previous year, and 144 nestlings) in 2015. In 2016, the only data collected for this thesis involved noting which individuals present in 2015 returned for the 2016 breeding season.

We captured adult song sparrows between 06:30 and 11:00 EST using seed-baited Potter traps. Upon capture, members of the research team equipped each bird with a numbered leg band from

the Canadian Wildlife Services, along with a unique combination of plastic coloured leg bands for future field identification (Environment Canada Capture and Banding Permit 10691B). I then recorded the colour band combination and corresponding identification number from the CWS band. We searched for nests within the field site by walking transects looking for adult song sparrows to flush out of the nest. Once the nest was located a marker was placed near the nest for future reference. The nests were then observed for lay-date and hatch date. Once the eggs hatched I, along with other members of the research team, collected blood samples from nestlings by using tarsus venipuncture. Once the nestlings reached an age of 12 days we returned to the nest and attached CWS bands to the nestlings. For birds captured in 2014 and 2015, ~ 25 μL of blood was collected via brachial venipuncture in adults, and metatarsal venipuncture in nestlings. Blood was blotted onto high wet-strength filter paper, then saturated with a drop of 0.5 M Na EDTA, pH 8.0. Blood blots were dried and stored at room temperature awaiting DNA extraction.

2.2 Genetic analysis

DNA Extraction

I extracted DNA from blood blots using ammonium-acetate precipitation to salt out proteins, using a protocol modified from (Laitinen et al. 1994). Using bleach-sterilized scissors, I cut the blood blots into sections ($\sim 1 \text{ mm}^2$) and placed the sections into a 1.5 mL microcentrifuge tube. I added cell lysis buffer (400 μL) and proteinase K (5 mM) to each sample, then incubated at 60°C for two hours. After incubation, I added ammonium acetate (400 μL) to precipitate proteins out of solution, centrifuged at 12000 RPM, and retained the supernatant that contained the dissolved DNA. Using ice-cold 99% isopropanol, I precipitated the DNA out of solution then centrifuged

the samples to generate a pellet of DNA at the bottom of the microcentrifuge tubes. After discarding the supernatant, I washed the pellet with 70% ice-cold ethanol, centrifuged, and retained the pellet. I allowed the tube to dry until all excess ethanol had evaporated. Using 50 μ L 1X TE (Tris buffer and EDTA), I dissolved the DNA pellet. I quantified DNA concentration of each sample using a NanoDrop 2000 Spectrophotometer (Thermo Scientific), then diluted with 1X TE to create 20 ng/ μ L working aliquots.

Microsatellite genotyping

Song sparrows captured in 2014 were genotyped at 12 microsatellite loci previously developed for use in song sparrows and other Emberizid species (Table 2.1). One of the primers from each pair was fluorescently labeled (Life Technologies), and I multiplexed up to 4 loci in the same polymerase chain reaction (PCR) for each individual. I conducted PCR in a total volume of 10 μ L, including 10 mM Tris-HCl, 0.2 mg/mL BSA, 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0.1 mM of each primer (except SOSP 14, for which I used 0.4 mM of each primer), 0.5 U *Taq* polymerase (Invitrogen, Cat. No. 18038-042), and 25 ng of genomic DNA. Thermal cycling conditions included an initial step of 180 s at 94°C; followed by 28 cycles of 30 s at 94°C, 90s at the annealing temperature, and 60s at 72°C; and a final step of 270 s at 72°C. Annealing temperatures were 57°C for Mme 1, Mme 12, Pdo μ 5, Sosp 2, 3, 4, 9, 13, and 14; and 55°C for Sosp 1, 5, and 7.

Microsatellite PCR products were separated by capillary electrophoresis on an Applied Biosystems DNA Analyzer (ABI 3730) at the London Regional Genomics Centre (Robarts Research Institute, University of Western Ontario). I used Genemapper 4.0 software to manually

identify and analyze the fragment length on alleles by referencing the internal size standard LIZ. I tested for deviations from Hardy-Weinberg equilibrium that might indicate null (non-amplifying) alleles, and for linkage disequilibrium, using Genepop on the Web (<http://genepop.curtin.edu.au>), with a dememorization number of 1000, and 1000 iterations. After applying a Bonferroni correction (critical $\alpha = 0.0041$, based on 12 loci tested), I found evidence of deviation from Hardy-Weinberg equilibrium in four of the 12 loci. These loci were excluded from my calculations of multilocus heterozygosity (MLH; see section 2.3) because they may harbour null alleles and would thus underestimate heterozygosity for individuals with null alleles. After applying a Bonferroni correction to the 2014 data (critical $\alpha = 0.00075$, based on 66 pairwise comparisons), I found no evidence for linkage disequilibrium between any pairs of loci. Some individuals had missing genotypes at a single locus due to PCR failure. For these individuals I calculated MLH based on heterozygosity at the remaining loci.

MHC sequencing

I amplified MHC class II β of song sparrows captured in 2014 and 2015 using the degenerate forward primer *SospMHCint1f* (Slade et al. 2016) together with the conserved primer *Int2r.1* (Edwards et al. 1995) developed for sequencing class II MHC in other passerine species. This primer combination amplifies part of introns 1 and 2, and all of exon 2, the peptide binding region of MHC class II β in passerines (Aguilar et al. 2006). Due to the high level of polymorphism that has been reported for MHC class II β in passerines (e.g. Bollmer et al. 2010), I characterized this locus using next generation sequencing implemented in the Illumina MiSeq platform. Thus, in addition to the priming sequence, each primer also contained the Illumina MiSeq adaptor sequence, four wobble bases, and a unique eight-base barcode sequence. I

conducted PCR in a total volume of 30 μL , including 12.5 μL GoTaq® hot start colourless master mix (Promega), 0.2 μM of each primer, 25 ng of gDNA, and nuclease free water to 30 μL . The thermocycling profile consisted of 180s at 94°C following 28 cycles of 30 s at 94°C, 30 s at 62°C, 45 s at 72°C and ending with 600 s at 72°C.

To confirm that some or all of the MHC class II β alleles amplified from genomic DNA (gDNA) are transcribed and expressed, I amplified MHC class II β from cDNA from two individual song sparrows and compared the resultant profiles to the same individuals' gDNA-derived profiles. These individuals were randomly selected from a group of captive song sparrows at the Advanced Facility for Avian Research. Each individual's spleen and liver tissue was homogenized in 1000 μL of TRIzol (Thermo Scientific), and I isolated total RNA according to the manufacturer's protocol. I quantified RNA concentration of each sample using a NanoDrop 2000 Spectrophotometer (Thermo Scientific), diluted with nuclease-free water to a working concentration of 0.2 $\mu\text{g}/\mu\text{L}$, and reverse-translated RNA to cDNA using a Quanta cDNA synthesis kit (Quanta Biosciences®). I used primers *Songex1F.2* and *SongEX3R.1* (Aguilar et al. 2006), which bind to exon 1 and 3 respectively, to amplify MHC class II β from cDNA samples. PCR conditions for cDNA were otherwise identical to the conditions for gDNA described above.

I ran a portion of each individual's PCR product on an agarose gel to confirm amplification, then pooled the remaining PCR products to form a library. All PCR products from birds captured in 2014 (n = 102, plus four PCR products cloned from a single individual to assess error rates; see section 2.3) were pooled into one library and all PCR products from birds captured in 2015 (n = 259, plus nine individuals from 2014 that were re-sequenced, four PCR products cloned from a

single individual to assess error rates, and gDNA and cDNA samples from two individuals) were pooled into a second library. Each library was sequenced using 300 bp paired-end reads on a single flowcell of an Illumina MiSeq at the London Regional Genomics Centre.

I tested repeatability of sequencing by randomly selecting nine individuals from the 2014 field season for re-sequencing (in the same library as the individuals from the 2015 field season). I then compared the two sets of sequences for each of the nine individuals. I used the same working aliquot of extracted DNA for the repeatability test that I used for the initial sequencing reaction.

2.3 Data Analysis

Microsatellite data

Microsatellite heterozygosity: I calculated multi-locus heterozygosity (MLH) for each individual by dividing the number of loci at which the individual was heterozygous, by the total number of loci genotyped (usually 12). I created a Q-Q plot for the MLH scores using base R (version 3.2.3) and found them to be normally distributed.

I also calculated heterozygosity-heterozygosity correlations (Alho et al. 2010) to determine if the microsatellite loci used were representative of genome-wide genetic diversity. Heterozygosity-heterozygosity correlation compares the correlation between multilocus heterozygosity estimates from randomly selected loci. Using the R package RHH (Alho and Välimäki, 2012), I generated 1000 permutations using standardized heterozygosity (proportion of heterozygous loci/mean heterozygosity of typed loci) for all individuals genotyped. Standardized heterozygosity was

used for this analysis because some individuals were missing data for specific loci and standardized heterozygosity ensures that all individuals were measured on an identical scale.

MHC sequence data

To identify very rare sequences likely to represent PCR or Illumina sequencing error, I calculated a sequencing error threshold. I amplified MHC II β from a single song sparrow, using the conditions outlined above, and cloned the PCR products into bacterial cells (Promega pGEM-T Easy Vector System) to generate multiple colonies, containing a single allele, confirmed by Sanger sequencing (Slade et al. 2016). I included PCR products from four colonies in the 2014 flow-cell run. Without errors in PCR or sequencing, each colony should yield only one sequence, so the frequency of rare sequences within each colony provides an estimate of error rates. Based on the observed frequencies of such secondary sequences across the four colonies (median = 0.0082), I established a threshold error rate of 1%. Thus, sequences making up fewer than 1% of an individual's reads were considered likely to have resulted from errors in PCR or sequencing, and were discarded (Slade et al. 2016).

The amplicons were found to be approximately 354 base pairs long. I aligned the song sparrow MHC class II β sequences in MEGA 7.0 (Kumar et al. 2016) against MHC class II β sequences from common yellowthroats (Getr-DAB*1089) using the BLAST algorithm (Altschul et al. 1990) implemented in GenBank. Sequences were aligned, trimmed and clustered based on 100% sequence identity, then translated into amino acid sequences using ExPASy (<http://web.expasy.org/translate/>). I generated an initial maximum likelihood tree based on amino acid sequences for all clustered sequences (Appendix B). Next, I clustered alleles (518) into

“superalleles” (161), based on well-defined clade membership and terminal branch length being shorter than internal branch length (Appendix B). Alleles clustered into superalleles are functionally similar based on shared amino acid sequence and clustering was done to determine the total number of functionally unique alleles. This was done because sequences with near identical amino acid profiles would be functionally similar and as a result likely identify and bind similar antigens. Since I am interested in assessing MHC diversity as a predictor of overwinter survivorship I am interested in assessing the functional diversity of MHC. Clustering alleles into superalleles was done independently by myself and by a colleague, with 92% similarity in the final superalleles identified. I generated a final (maximum likelihood) tree by combining the analyses conducted by the colleague and myself to determine the optimal group structure. I collapsed any repetitive superalleles for every individual to give a final number of unique MHC class II β superalleles for each individual. I confirmed that the number of MHC class II β alleles per individual was normally distributed by visually checking a Q-Q plot generated in base R.

Microsatellite-MHC correlations: To determine whether neutral-locus heterozygosity (MLH) is associated with MHC class II β diversity (number of superalleles), I ran a Pearson’s correlation in base R for all individuals represented in the 2014 dataset.

I also investigated whether pairwise similarity estimates derived from microsatellite genotypes were associated with pairwise similarity estimates derived from MHC class II β genotypes, again for all individuals in the 2014 dataset. I calculated neutral-locus genetic distance between all pairwise combinations of individuals based on the method of Kosmas and Leonard (2005), using

the R package PopGenReport (Adamack and Gruber, 2014). This method works by calculating genetic distance of diploid organisms by determining number of shared alleles at each locus and number of non-shared alleles. Additionally, this method measures the distance of a homozygous individual to a completely dissimilar heterozygous individual and completely dissimilar homozygous individuals as the same distance as opposed to Jaccard's index which does not. This analysis included all 12 microsatellite loci, but the neutral-adaptive diversity correlation analysis did not because deviations from HWE could indicate that there is selection causing allele frequencies to change. In the diversity correlation analysis we are interested in comparing neutral vs. adaptive loci and if selection is acting on microsatellite alleles that would indicate they are not entirely selectively neutral. However in this analysis I am interested in determining if microsatellite genotypes and MHC genotypes predict similar genetic distance between individuals, rather than comparing levels of diversity. I calculated unweighted genetic distance at MHC between all pairwise combinations of individuals using the R package GUniFrac (Chen, 2012). Using the model selection tool in MEGA 7.0, I determined that the most appropriate model to use for the GUniFrac analysis was a Jones-Taylor-Thornton maximum likelihood tree with five gamma distributions. After generating pairwise matrices of microsatellite distances and MHC distances, I ran Mantel tests (Pearson method) in XLSTAT 2014 (Addinsoft) to determine whether pairwise genetic distances derived from microsatellites are associated with those derived from MHC genotypes.

Age cohort comparisons of MLH and MHC diversity: I compared both measures of genetic diversity (microsatellite MLH and MHC class II β diversity) across three age classes: nestlings (N), second-years (SY), and after-second years (ASY). For the MLH calculation for comparing

age cohorts all microsatellite loci were included. In a few cases, second-year birds had been banded as nestlings or juveniles the previous year, and their age was thus known with certainty. Similarly, individuals that had been captured and banded as adults in previous years were known to be older than second-year (after-second year). Birds that were captured for the first time as adults were assumed to be one year of age (second-year) upon first capture. Supporting this assumption, birds in this population classified as second-year have shorter wings than do birds known to be after-second year (Kelly et al. 2016), suggesting that the former have likely not undergone their first basic moult (Smith et al. 1986). Moreover, high rates of adult philopatry (~50% return rate) and high intensity trapping effort (~95% of breeding adults each year are captured and banded) further imply that birds classified as second-years were indeed yearlings breeding for the first time.

I compared MHC diversity across the three age groups using an ANOVA in base R. I ran two ANOVAs, one for 2014 and one for 2015. Similarly, I compared neutral-locus diversity (MLH) across the three age groups using an ANOVA in base R. For each measure of genetic diversity (MHC diversity, MLH) I also conducted an F test in base R to compare the variances of the three age groups.

MHC diversity as a predictor of overwinter survivorship: I estimated overwinter survivorship of adults by noting which individuals banded in a given year returned to the field site the following year. Because adult song sparrows in this population are highly philopatric to their breeding grounds, usually returning to the same territory (MacDougall-Shackleton et al. 2009, Potvin et al.

2015), I used overwinter return as a proxy for survivorship. That is, individuals not returning were considered to have died.

I constructed generalized linear mixed models to predict overwinter return for the combined 2014 (i.e. returning in 2015) and 2015 (i.e., returning in 2016) datasets. I used an information-theoretic approach to compare a set of alternative models predicting overwinter return. All candidate models were fit with binomial error distribution, and included individual ID as a random effect. Candidate models differed in the presence versus absence of the following predictors: MHC diversity (number of superalleles), year (i.e, 2014-15 vs 2015-16), MHC diversity-by-year interaction, age class (i.e., SY or ASY), and sex. The candidate set of models also included a null model (return $\sim 1|ID$). I ranked models by AICc (Table 3.3) and calculated parameter estimates by model averaging of all models within 2 AICc units of the best model, using the R package `glmulti` (Calcango, 2013).

Table 2.1. Microsatellite loci at which song sparrows (2014 dataset) were genotyped, including repeat motifs, product size range, estimated frequency of null alleles for each locus and observed heterozygosity. Asterisks indicate loci that were found to be out of Hardy-Weinberg equilibrium even after Bonferroni correction for multiple tests.

| Locus Name | Repeat Motif | Product Size Range (bp) | Estimated Frequency of Null Alleles | Source |
|------------|---|-------------------------|-------------------------------------|-------------------------------------|
| *Mme 1 | (TG) _n TC(TG) _n | 130-172 | 0.301 | Jeffery et al., 2001 |
| Mme 12 | (CCCACA) _n | 180-250 | -0.030 | Jeffery et al., 2001 |
| Pdoμ 5 | (CA) _n | 221-269 | 0.021 | Griffith et al., 1999 |
| Sosp 1 | (GGAT) _n G (GGAT) _n GCAT | 225-280 | -0.016 | Sardell et al., 2010 |
| Sosp 2 | (CTGT) _n (GT) ₃ | 130-182 | -0.014 | Sardell et al., 2010 |
| *Sosp 3 | (CTGT) _n | 178-226 | 0.023 | L. Keller, pers. comm. to E.A. M-S. |
| Sosp 4 | (TGTC) _n | 155-210 | 0.055 | Sardell et al., 2010 |
| *Sosp 5 | (GACA) _n G (GACA) _n ACT | 80-139 | 0.252 | Sardell et al., 2010 |
| Sosp 7 | (GACA) _n | 60-120 | -0.037 | Sardell et al., 2010 |
| Sosp 9 | (GACA) _n | 80-130 | 0.049 | Sardell et al., 2010 |
| *Sosp 13 | (GATA) _n | 172-228 | 0.027 | Sardell et al., 2010 |
| Sosp 14 | (CTAT) _n | 223-299 | 0.013 | Sardell et al., 2010 |

3 Results

3.1 MHC class II β diversity and MLH

I pooled all allele sequences for MHC class II β from both the 2014 and 2015 flow cell runs and found a total of 518 unique alleles in the population, based on nucleotide sequences. I then generated a maximum likelihood tree based on amino acid sequences (Appendix B), and grouped alleles together into ‘superalleles’ based on a criterion of internal branch length being greater than the terminal branch length. This resulted in a total of 161 superalleles being identified. The average (\pm SE) number of superalleles per individual was 13.15 ± 0.26 for birds genotyped in 2014, and 12.03 ± 0.21 for birds genotyped in 2015. 2014 has significantly higher levels of MHC superallele diversity compared to 2015 (t-test, $t_{359} = -3.06$, $p = 0.002$). Males and females did not differ in number of superalleles (t-test, 2014: $t_{57.4} = -0.361$, $p = 0.719$; 2015: $t_{70.2} = -1.333$, $p = 0.186$). The average MLH for all individuals that I genotyped in 2014 was 0.69 ± 0.01 , and this measure did not differ between males and females (t-test, $t_{94.8} = -0.273$, $p = 0.785$)

For the two individuals with both cDNA and gDNA sequences available, all superalleles amplified from each individual’s cDNA were also detected in its gDNA-derived sequences (Table 3.1). However, not all alleles detected in an individual’s gDNA were detected in its cDNA (Table 3.1). For the nine birds that were sequenced in two separate flow cell runs (2014 and 2015), I found substantial but not total overlap between flow cell runs (average percentage of superalleles detected by both runs = 57.0%; Table 3.2).

Table 3.1. Comparison of cDNA alleles and gDNA alleles for two individual song sparrows. The table shows the total number of alleles recovered across both spleen and liver tissues, the number of alleles found only in cDNA, the number of alleles found only in gDNA, total number of alleles found in both gDNA and cDNA, and the percentage of alleles found in both gDNA and cDNA.

| Individual | Total Number of Alleles | cDNA Alleles only | gDNA Alleles only | Shared Alleles | % Shared |
|------------|-------------------------|-------------------|-------------------|----------------|----------|
| TK1 | 10 | 0 | 7 | 3 | 30.0 |
| TK2 | 14 | 0 | 11 | 3 | 21.4 |

Table 3.2 Repeatability of MHC class II β genotypes, inferred from comparing genotypes of nine individuals that were sequenced in two separate flow cell runs. The table shows the total number of alleles detected across the two flow cell runs combined, the number of alleles found only in the 2014 run, the number of alleles found only in the 2015 run, the number of alleles shared between both runs, and the percentage of alleles shared.

| Individual | Total Number of Alleles | 2014 Alleles | 2015 Alleles | Shared Alleles | % Shared |
|------------|-------------------------|--------------|--------------|----------------|----------|
| MW02 | 16 | 2 | 4 | 10 | 62.5 |
| MW11 | 17 | 2 | 3 | 13 | 76.5 |
| MW27 | 17 | 3 | 3 | 11 | 64.7 |
| MW38 | 18 | 2 | 1 | 15 | 83.3 |
| MW57 | 19 | 12 | 2 | 5 | 26.3 |
| MW72 | 14 | 1 | 2 | 11 | 78.6 |
| MW86 | 14 | 2 | 1 | 11 | 78.6 |
| MW94 | 22 | 9 | 8 | 5 | 22.7 |
| MW97 | 20 | 8 | 7 | 5 | 20.0 |
| | | | | Average % | 57.0 |
| | | | | Average | |

3.2 Diversity and distance at neutral versus adaptive loci

For birds genotyped in 2014 and thus having both microsatellite and MHC information available, I found no correlation between an individual's MLH score (proportion of microsatellite loci that were heterozygous) and its MHC diversity (number of class II β superalleles; Pearson's $r_{1,67} = 0.166$, $p = 0.171$). However, pairwise genetic distance based on microsatellite genotypes was positively correlated with pairwise genetic distance based on MHC (Mantel test, $r_{17020} = 0.0789$, $p < 0.0001$; Figure 3.1). Finally, for the set of microsatellite markers examined, heterozygosity was not significantly correlated across loci (heterozygosity-heterozygosity correlation, average $r = -0.024$; 95% CI = $-0.078 - 0.026$) indicating that the panel of microsatellite loci I used are not representative of genome-wide genetic diversity. Observed heterozygosity varied from 0.30-0.93 between loci (Table 3.3).

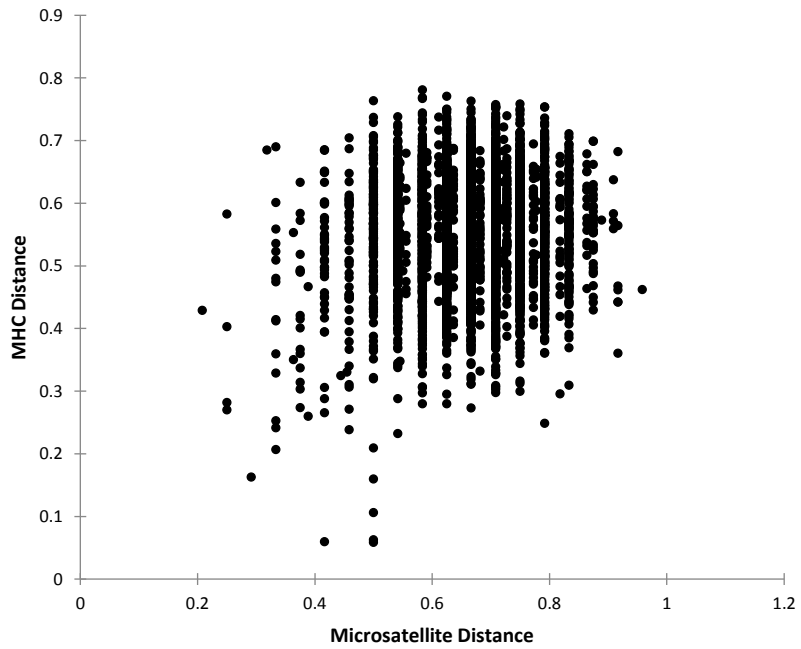


Figure 3.1. Pairwise genetic distance at MHC class II β (unweighted Unifrac method) increased with increasing pairwise distance at 12 microsatellite loci (Kosman and Leonard method), for song sparrows from the 2014 dataset (n = 102 individuals, 5202 pairwise comparisons).

Table 3.3. Observed heterozygosity at 12 microsatellite loci at which song sparrows (2014 dataset) were genotyped.

| Locus Name | Observed Heterozygosity |
|-------------|-------------------------|
| *Mme 1 | 0.47 |
| Mme 12 | 0.30 |
| Pdo μ 5 | 0.88 |
| Sosp 1 | 0.93 |
| Sosp 2 | 0.59 |
| *Sosp 3 | 0.90 |
| Sosp 4 | 0.75 |
| *Sosp 5 | 0.46 |
| Sosp 7 | 0.72 |
| Sosp 9 | 0.38 |
| *Sosp 13 | 0.80 |
| Sosp 14 | 0.88 |

3.3 Age cohort MHC comparison

For birds captured in 2014, I found no significant difference between age cohorts in the average number of MHC superalleles per individual (ANOVA, $F_{2,99} = 0.260$; $p = 0.775$; Figure 3.3).

Similarly, I detected no significant difference in variance of MHC diversity between any age classes (F-test, N vs SY: $F_{32,61} = 1.458$, $p = 0.204$; N vs ASY: $F_{32,6} = 2.132$, $p = 0.347$; SY vs ASY: $F_{61,6} = 1.462$, $p = 0.673$). However, for birds captured in 2015, MHC diversity (number of superalleles per individual) differed among age cohorts (ANOVA, $F_{2,256} = 4.751$, $p = 0.009$; Figure 3.3). Specifically, nestlings (N) were significantly less diverse at MHC than SY individuals (Tukey's test, $p = 0.019$), although the difference between N and ASY only approached significance ($p = 0.087$) and SY and ASY age classes were not significantly different ($p = 0.989$). Similarly, variance in MHC diversity was significantly greater for N than for the SY (F-test, $F_{143,81} = 1.802$, $p = 0.004$) or ASY age classes (F-test, $F_{143,33} = 2.811$, $p < 0.001$), but SY and ASY age classes did not differ significantly in variance (F-test, $F_{81,33} = 1.559$, $p = 0.154$).

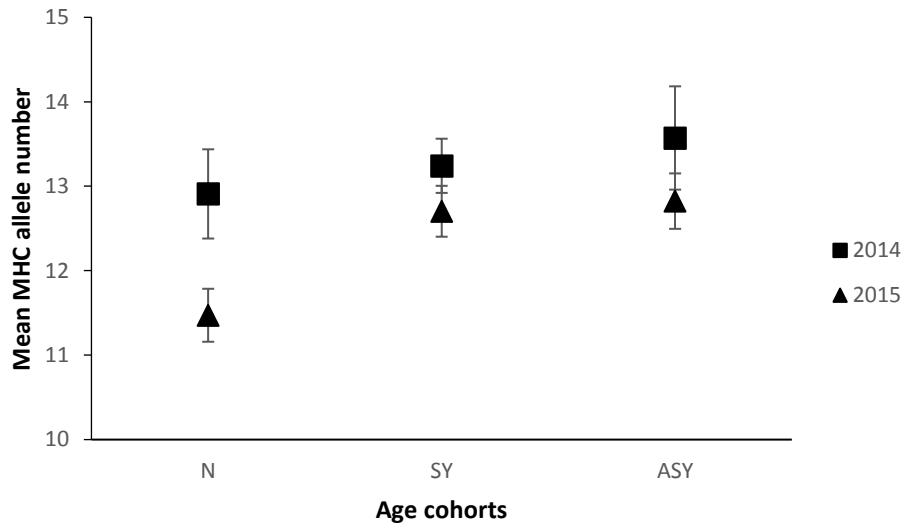


Figure 3.2. MHC diversity for each age cohort (N= nestlings, SY = second year, ASY = after second year) for song sparrows captured in 2014 and 2015. Error bars represent standard errors. 2014 sample sizes for N, SY, and ASY are 33, 62 and 7 respectively. 2015 sample sizes for N, SY, and ASY are 144, 81, and 34 respectively. In 2014, age classes did not differ significantly in MHC diversity, but in 2015 nestlings were less diverse at MHC than adults.

For birds captured in 2014, I found no significant difference among age cohorts in MLH scores (ANOVA, $F_{2,99} = 1.405$, $p = 0.250$; Figure 3.4), nor did I detect any difference among age cohorts in variance of MLH (F test, N and SY: $F_{32,61} = 0.756$, $p = 0.394$; N vs ASY: $F_{32,6} = 1.629$, $p = 0.565$; SY and ASY: $F_{61,6} = 0.464$, $p = 0.336$).

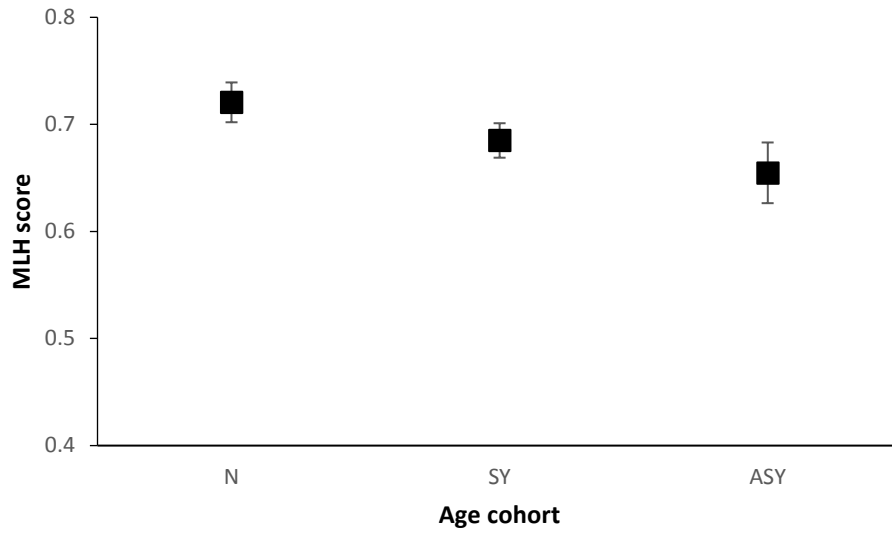


Figure 3.3. Multi Locus Heterozygosity scores did not differ across age cohorts for song sparrows captured in 2014. Sample sizes for nestlings (N), second-year (SY), and after-second-year (ASY) are 33, 62 and 7 respectively. Error bars represent standard errors.

3.4 *Overwinter return*

Of the 16 candidate models predicting overwinter return, five top models were identified that were within 2 AICc units of the most competitive model (Table 3.4). Model averaging of these top five models indicated that age and sex were important predictors of returning to the breeding grounds the following year (Table 3.5). Specifically, ASY birds had relatively higher return rates compared to SY (95% Confidence Interval: -1.741 - -0.207) and males had higher return rates compared to females (95% Confidence Interval: 0.371 – 1.815). The predictors MHC, Year, and MHC*Year interaction contributed to some models but were not important predictors of overwinter survivorship (Table 3.5).

Table 3.4. Ranked candidate set of models predicting overwinter return (interpreted as survival) of adult song sparrows. All models were fit with binomial error distribution and included a random term for bird ID. The top five models had AICc values within two units of the best model, and were used for model averaging of parameter estimates.

| Candidate Model | K | Log Likelihood | AICc | Δ AICc | Weight |
|--|---|----------------|-------|---------------|--------|
| Survived~ Age+Sex | 4 | -113.2 | 234.7 | 0 | 0.33 |
| Survived~MHC+Age+Sex | 5 | -112.5 | 235.4 | 0.7 | 0.23 |
| Survived+Year+Age+Sex | 5 | -112.8 | 235.9 | 1.2 | 0.18 |
| Survived~MHC+Year+Age+Sex | 6 | -112 | 236.4 | 1.7 | 0.14 |
| Survived~MHC+Year+Age+Sex +Year*MHC | 7 | -111 | 236.7 | 2.0 | 0.12 |
| Survived~Sex | 3 | -116 | 238.2 | 3.5 | - |
| Survived~MHC +Sex | 4 | -115.3 | 238.9 | 4.2 | - |
| Survived~Year+Sex | 4 | -115.9 | 240.1 | 5.4 | - |
| Survived~MHC+Year+Sex | 5 | -115.2 | 240.7 | 6.0 | - |
| Survived~Age | 3 | -118 | 242.1 | 7.4 | - |
| Survived~Year+Age | 4 | -117.4 | 242.9 | 8.2 | - |
| Survived~MHC+Age | 4 | -117.6 | 243.5 | 8.8 | - |
| Survived~MHC+Year+Age | 5 | -116.9 | 244.1 | 9.4 | - |
| Null – Survived~1 | 2 | -122.4 | 248.9 | 14.2 | - |
| Survived~MHC | 3 | -122.1 | 250.4 | 15.7 | - |
| Survived~Year | 3 | -122.3 | 250.8 | 16.1 | - |
| Survived~MHC+Year | 4 | -122 | 252.2 | 17.5 | - |

Table 3.5. Model-averaged predictors of overwinter return for song sparrows breeding in 2014 and/or 2015.

| Predictor | Relative Variable Importance | Beta | z value | SE | Confidence Interval | |
|-------------|------------------------------|---------|---------|--------|---------------------|--------|
| | | | | | 2.5% | 97.5% |
| Age (SY) | 1.00 | -0.9746 | 2.49 | 0.3887 | -1.741 | -0.207 |
| Sex (male) | 1.00 | 1.0939 | 2.97 | 0.3658 | 0.371 | 1.815 |
| MHC | 0.48 | -1.0163 | 0.61 | 1.5065 | -3.978 | 1.945 |
| Year (2015) | 0.44 | -0.1085 | 0.39 | 0.0963 | -0.298 | 0.081 |
| Year*MHC | 0.12 | 0.1892 | 0.29 | 0.1414 | -0.089 | 0.468 |

4 Discussion

4.1 *Summary of findings*

The two measures of genetic diversity I examined, multilocus heterozygosity (MLH) for microsatellite loci and number of superalleles for MHC class II β , were not correlated. As predicted, this suggests that adaptive locus diversity is not always correlated with neutral measures of genetic diversity. These results are consistent with other studies showing that diversity at adaptive loci, such as MHC, does not correlate with microsatellite heterozygosity (Loiseau et al. 2009; Worley et al. 2010). However, these previous studies were conducted on small, isolated populations that have often undergone a bottleneck event (Loiseau et al. 2009; Worley et al. 2010): my findings demonstrate that even in populations that are open to immigration and have not been recently bottlenecked, diversity at MHC does not correlate with measures of neutral-locus diversity. However, the set of microsatellite loci used did not reflect genome-wide heterozygosity (heterozygosity-heterozygosity correlation was not significant), and the use of a much larger panel of microsatellites might have generated a stronger relationship between microsatellite heterozygosity and diversity at MHC.

In contrast to the lack of relationship between neutral-locus heterozygosity and MHC diversity, I found an unexpected positive relationship between microsatellite-derived and MHC-derived estimates of pairwise genetic distance. This result contradicts my original prediction that MHC distance and microsatellite distance would not be associated, and suggests that if individuals do possess a mechanism allowing them to assess similarity at MHC, they might also be able to obtain information on genome-wide similarity and relatedness. Conversely, if individuals in this

population are capable of kin recognition, this information may also provide correlated information on a potential mate's similarity at MHC.

In 2014, I found no significant differences between age cohorts in diversity of either MHC or neutral markers. The following year, however, age cohorts differed in MHC diversity, with older age cohorts having more MHC alleles than nestlings. Moreover, variance in MHC diversity decreased with increasing age. Together, these findings suggest that individuals with lower genetic diversity at MHC were less likely to survive than more MHC-diverse individuals, but that this pattern may be year-specific. That is, MHC-diverse individuals may be more likely to survive in some years but have no advantage in other years. However, in contrast to my prediction that MHC-diverse individuals should be more likely to return to the breeding grounds the following year, I found that MHC diversity did not significantly predict survivorship. Overall, the relationship between MHC and survivorship appears to be more complex than originally hypothesized.

4.2 Adaptive vs. neutral-locus diversity and distance

As predicted, microsatellite heterozygosity did not correlate with MHC class II β diversity. This result suggests that selective pressures on the MHC results in different levels of genetic diversity being maintained than at selectively neutral loci such as microsatellites. Similar to my findings, in the common frog (*Rana temporaria*), microsatellite measures of genetic diversity do not correlate with MHC diversity (Zeisset and Beebee 2010). Collectively, these findings suggest that neutral markers are not necessarily a good proxy for overall genetic diversity, and that ideally, studies should also investigate variation at adaptive (coding) markers.

Although microsatellites serve as an excellent method for determining paternity, and in some cases for monitoring inbreeding, a growing body of evidence suggests that microsatellites alone cannot always give an accurate representation of genome-wide genetic diversity. Many captive breeding and management programs rely on microsatellites to determine overall genetic diversity of endangered species or populations (Alcaide et al. 2009). By neglecting to assess genetic diversity at selectively important loci such as MHC, such breeding programs could inaccurately estimate true levels of genetic diversity. However, my results also suggest that even though measures of neutral-locus diversity and MHC diversity are not correlated, measures of neutral-locus distance do predict pairwise distance at MHC reasonably well. Thus, captive breeding programs that select mating pairs based on neutral-marker distance may also enhance dissimilarity and thus compatibility at MHC. Conversely, in many species such as: three-spined sticklebacks, fat-tailed dwarf lemur (*Cheirogaleus medius*), and humans, individuals preferentially mate with partners that are relatively dissimilar at MHC (Aeschlimann et al. 2003; Schwensow et al. 2007; Lie et al. 2010) and my findings suggest that disassortative mating at MHC may have the correlated effect of minimizing overall relatedness between mates, thus reducing the potential for inbreeding.

4.3 Differences in MHC between age cohorts

MHC class II β diversity did not differ between age cohorts in 2014, but in 2015 adults had higher average MHC diversity, and lower variance around the average, than nestlings. The different patterns observed in different years could reflect variation in the strength of parasite-mediated selection. However, the difference in findings between years could also be influenced

by differences in sample size: in particular, only seven after-second year birds were sampled in 2014. Finally, MHC diversity has also been found to vary significantly with year in a wild population of Guppy (*Poecilia reticulata*) (Fraser et al. 2010). Yearly variations in MHC diversity resulting from potential variation in the strength of parasite mediated selection could be another factor that could have contributed to the difference in results between the 2014 and 2015 populations. Since it was found that there is a higher average number of MHC alleles in 2014 compared to 2015 there is likely some factor causing yearly variation that could be investigated further. In contrast to the difference in MHC diversity across age cohorts in 2015, when MLH scores were compared between age cohorts in 2014 (the only year for which MLH data were available) no significant difference was found, nor did age cohorts differ in variances in MLH scores. However this result is similar to the result found for MHC diversity compared among age cohorts for 2014.

In 2015, I found higher average MHC class II β allele diversity in adults than in nestlings, but older adults were not more diverse than younger (second-year) adults. This suggests that MHC diversity may primarily affect the ability of juveniles surviving to adulthood, and could be less important to adult survival. A large body of research supports the idea that individual genetic diversity, including diversity at MHC, is important to survivorship (Westerdahl et al. 2005; Charpentier et al. 2008; Knafler et al. 2012). For example, in water voles (*Arvicola terrestris*), individuals with heterozygous genotypes at MHC have significantly lower parasite burdens than do MHC homozygotes (Oliver et al. 2009), and in Italian agile frogs individuals that are heterozygous at MHC have higher survival when exposed to the fungal pathogen *Batrachochytrium dendrobatidis* (Savage and Zamudio 2011). Thus, MHC diversity is likely to

play an important role in disease resistance and survivorship to adulthood. Most likely, the birds with too few alleles at MHC don't survive to adulthood, which is why we see a mean increase in MHC diversity from nestlings to adults. This filter likely acts early in life, and later in life all individuals with lower MHC diversity have been filtered out. Therefore we would expect to see an increase in mean MHC diversity between nestlings and adults but not between SY and ASY age cohorts.

When gDNA and cDNA were compared for two individuals it was found that only three alleles from the gDNA were being expressed as cDNA. This could indicate that the number of true alleles per individual is being overestimated. Alternatively, it is possible that since the birds being sequenced were kept in captivity in a clean environment with very little chance of pathogen and parasite exposure that all MHC alleles are not being expressed. If individuals are not being immunologically challenged then there wouldn't be a need to express all alleles all the time and therefore we would see an underrepresentation of alleles in the cDNA compared to gDNA.

When repeatability was tested between the 2014 and 2015 flowcell runs on the Illumina MiSeq the average percentage of overlap between years was 57%. This could indicate that there is a large number of PCR or sequencing artifacts, which are being identified as alleles that aren't actually alleles. Alternatively, there could be a large number of alleles and the Illumina MiSeq is sequencing. If there is indeed more alleles that we are identifying, they would have very low flow cell reads and would be excluded from our analysis due to the error cutoff rate of 1%. If this were the case potentially lowering the error rate would increase the overlap in sequence identity

between years. Another possible method for overcoming this issue would be to lower the number of individuals sequenced on each flow cell run. This would allow a greater portion of reads to be allotted to each individual, which would increase the number of sequences identified per individual and would likely increase the overlap between flow cell runs.

4.4 *Overwinter survivorship*

In contrast to my original prediction, and in contrast to the age cohort differences in MHC diversity observed in 2015, MHC diversity did not predict overwinter survivorship as inferred from overwinter returns. Instead, age and sex were the only significant predictors of apparent survivorship: after second year birds had higher overwinter return rates than second year birds, and males had higher overwinter return rates than females.

Throughout their annual cycle, and particularly during migration, birds are exposed to a diverse set of parasites, which can have serious consequences for survival (Davidar and Morton 1993, Garvin et al. 2006). If being diverse at MHC confers song sparrows an advantage in resisting, clearing and/or recovering from multi-strain infections, as it does in other species, (Penn et al. 2002), then more MHC-diverse song sparrows should have had higher overwinter survival rates. However, there are at least two possible explanations as to why no relationship between MHC class II β diversity and overwinter survivorship was observed in this population.

First, whereas my study examined diversity at MHC class II, diversity at MHC class I (primarily involved in recognizing intracellular parasites) could be more important for overwinter survivorship in the study population. Among the most common infections that birds acquire on

migration are the intracellular protozoan parasites from genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* (Garvin et al. 2006). Therefore, it is possible that MHC class I diversity is more important for overwinter survivorship than MHC class II. However, there are still many extracellular infections that birds may acquire (*Salmonella spp.*, *Escherichia coli*, and *Pseudomonas aeruginosa*), suggesting that diversity at MHC class II β may still be important for survivorship.

Another possible explanation involves the finding that average MHC diversity only increased from nestlings to the second year age cohort, not from second-years to older birds, whereas variance in MHC diversity continued decreasing with age. Together, these patterns suggest that instead of survivorship increasing linearly with higher MHC diversity, there could be an optimal level of MHC diversity. In this case, individuals with very low diversity at a young age might be culled from the population, resulting in an increase in average MHC diversity between nestlings and adults. Then during adulthood there would be no increase in mean MHC diversity due to individuals with low levels of diversity already having been culled. This could help explain why MHC class II β diversity does not appear to influence overwinter survivorship during adulthood. Alternatively, MHC diversity might be linearly related to survivorship to adulthood, but less important to survivorship later in life.

My finding that males were more likely to return the following year than females is consistent with findings from other migratory bird species, such as black-throated blue warblers (*Setophaga caerulescens*) in which males have higher survival rates during both summer and winter than do females (Sillett and Holmes 2002). However, Sillett and Holmes (2002) note that different

apparent survival rates could reflect the fact that males show higher breeding site fidelity than females, such that females travel further distances on the breeding grounds between years and thus may be less detectable than males if they move off the study site. Thus, using return to the breeding grounds as an estimate of survivorship could underestimate rates of female survivorship. In my study population of song sparrows, males often return to within 30 meters of their previous year's territory (MacDougall-Shackleton et al. 2009), showing very high breeding site fidelity. In this population of song sparrows females were found to have much lower site fidelity compared to males, which means that females likely disperse further distances to find breeding territories on a yearly basis and as a result our sampling strategy may miss some returning females (Potvin et al. 2015). However, typical dispersing distance of females is approximately 200-400 m (Potvin et al. 2015) it is likely that our sampling strategy would likely capture the majority of returning females as well as males.

Another significant predictor of overwinter survivorship was age, with after second year birds being more likely to return the following year compared to second year birds. That is, younger age cohorts of song sparrows have lower relative overwinter survivorship than do older age cohorts. The oldest recorded song sparrow was 11 years old, but the typical lifespan among individuals who survive to adulthood in this species is two to five years (Klimkiewicz and Futcher 1987). It is possible that older birds have higher overwinter survivorship compared to younger birds due to the stress of breeding. Breeding season can be a very stressful period for individuals (Romero et al. 1997). Second-year birds not having previously undergone breeding may be more susceptible to infections and predation during migration, as their stress during the breeding season may be heightened by their inexperience. In contrast, older birds have

undergone breeding events previously and may be able to cope better with the stress of breeding, which could contribute to them having higher overwinter return rates. A study conducted on snow petrels (*Pagodroma nivea*) found that older birds were better able to manage their stress response during the breeding period compared to younger birds (Angelier et al. 2007). This indicates that experience with breeding plays a role in the ability to control an individual's stress response, and as a result of inexperience younger birds could be more strongly affected by their stress response during migration.

4.5 Conclusion

In conclusion, selection pressures that operate on adaptive loci such as MHC may result in different patterns of diversity at these loci than at neutral markers that are not under selection. In support of this I found no correlation between MHC diversity and microsatellite heterozygosity. This has important implications for management and conservation decisions requiring assessment of genetic diversity. However, it appears that genetic distance at MHC and microsatellite loci are correlated. This indicates that individuals could be assessing relatedness to other individuals based on a combination of genetic cues. Finally, the effects of MHC diversity on survival appear to be more complex than originally hypothesized. My findings indicate that there selection pressures on MHC could vary from year to year. Some years may potentially have higher parasite prevalence, placing greater selective pressure in favour of individuals with higher MHC diversity.

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Appendices

Appendix A: Animal Use Protocol

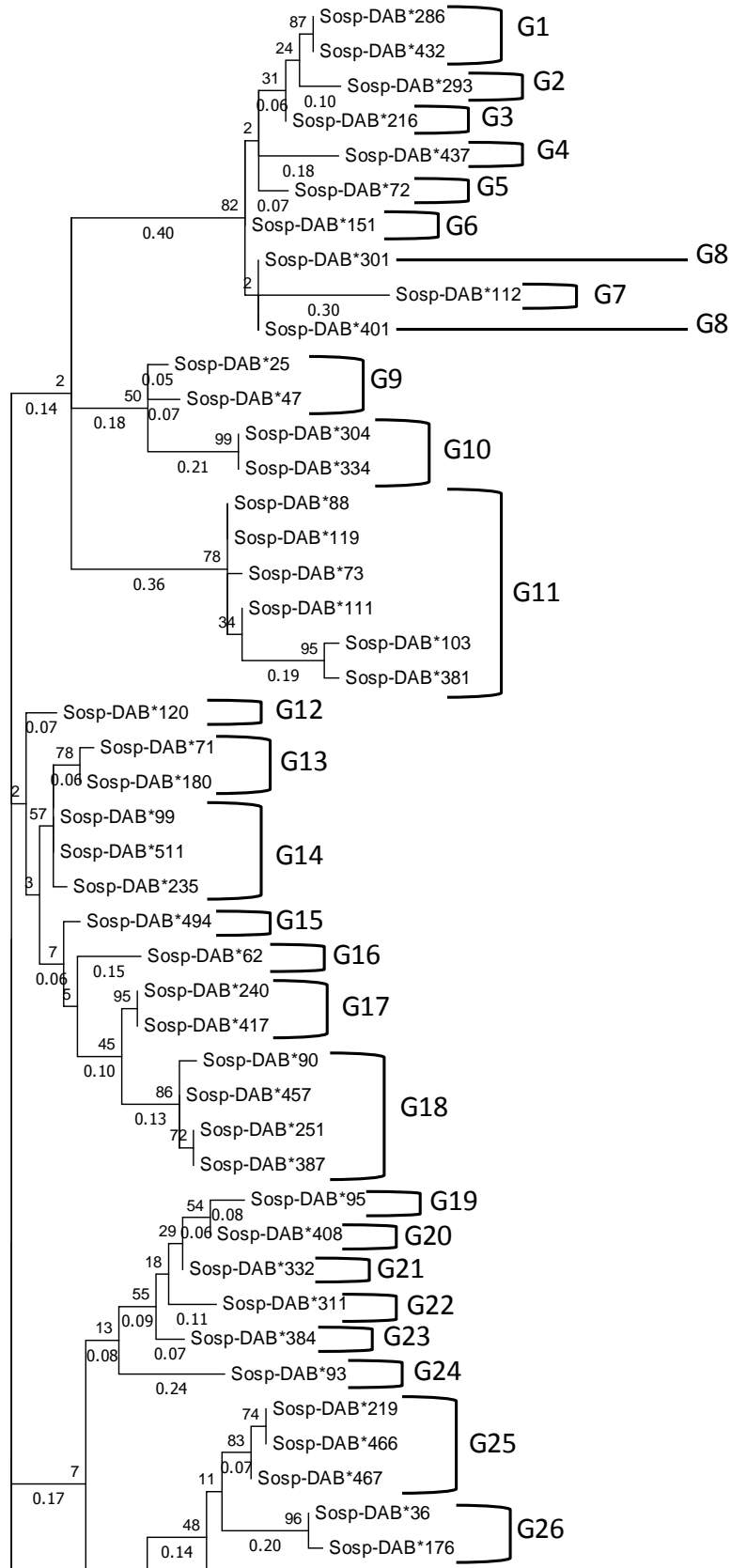
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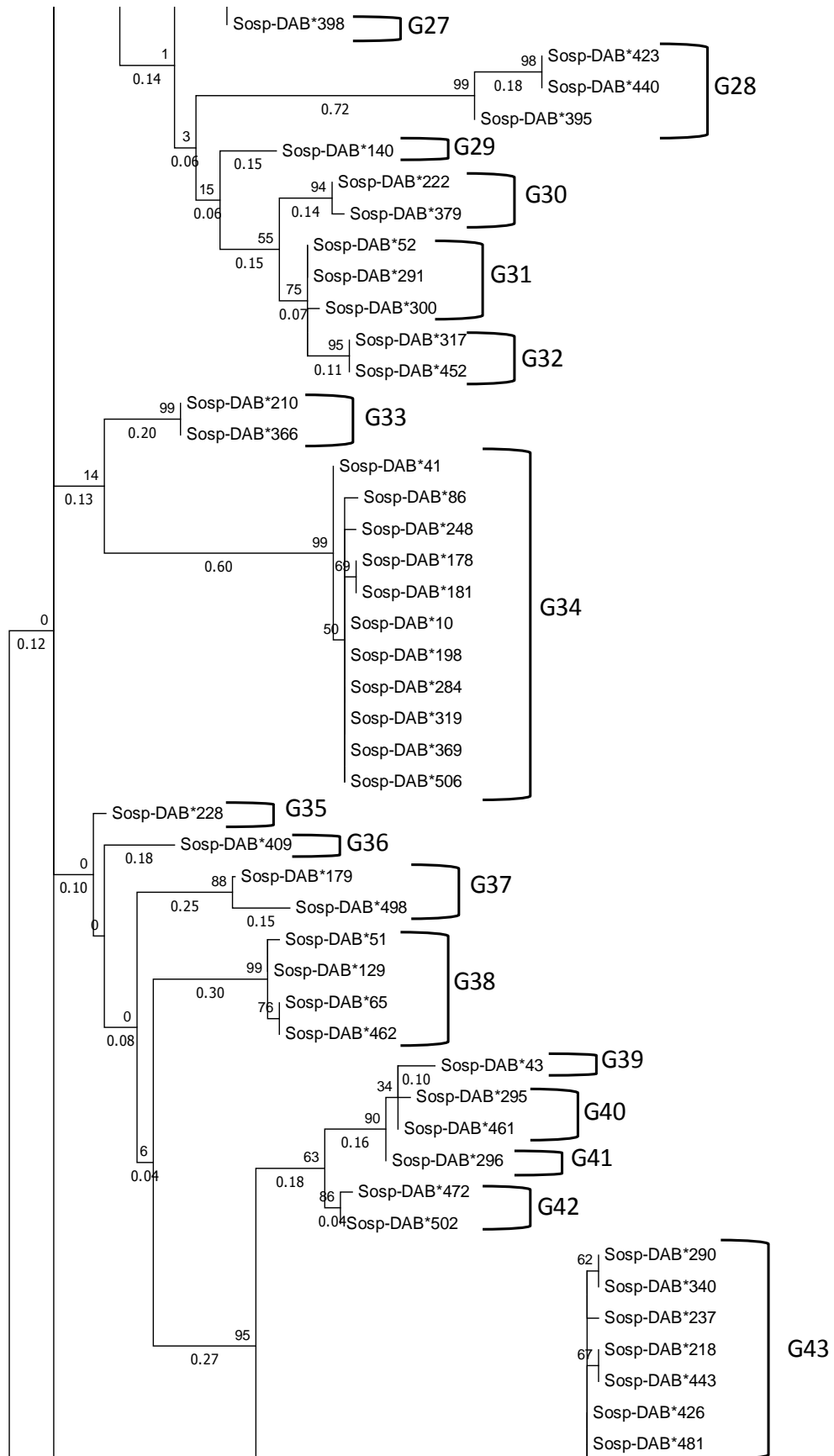
PI Name: Macdougallshackleton, Elizabeth

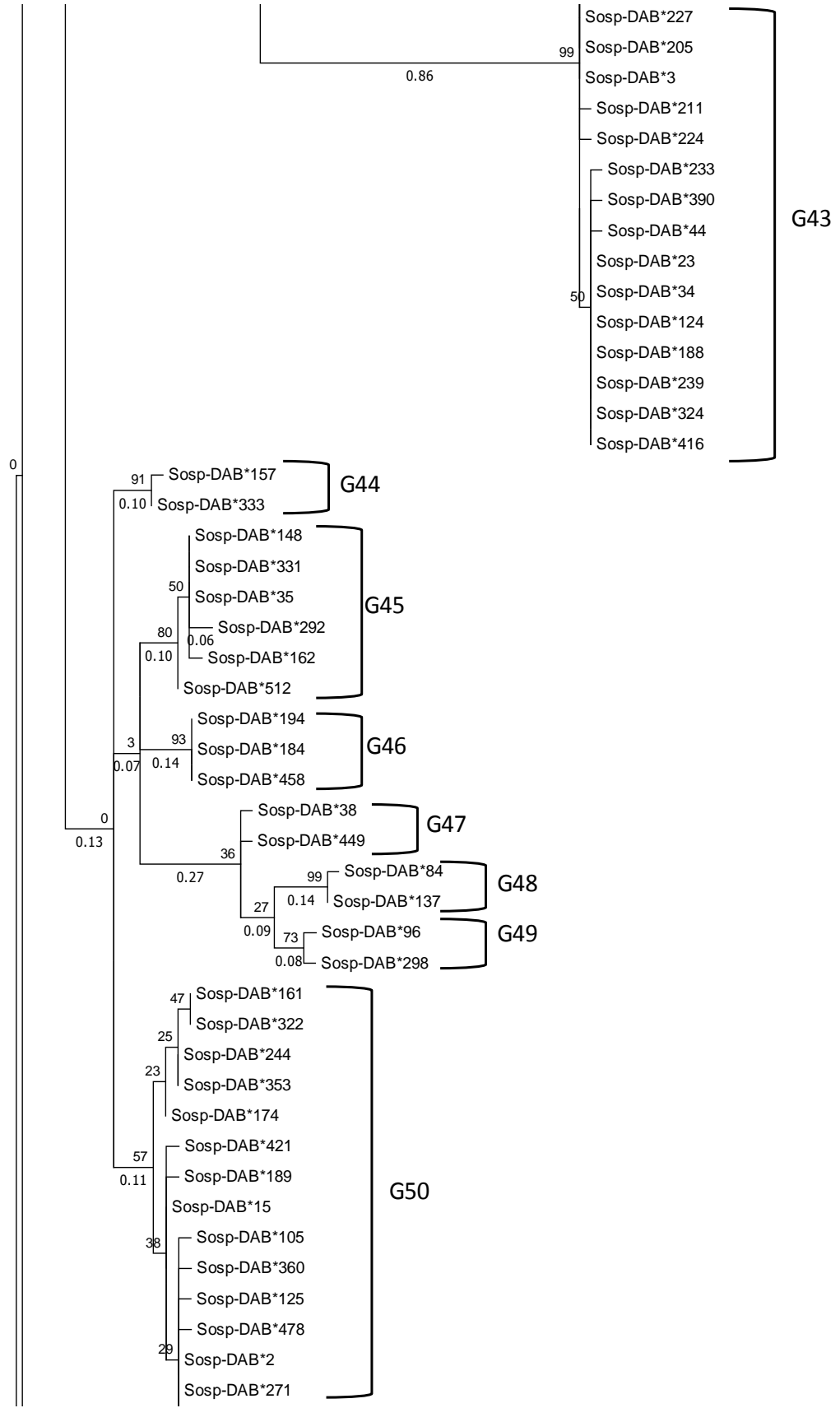
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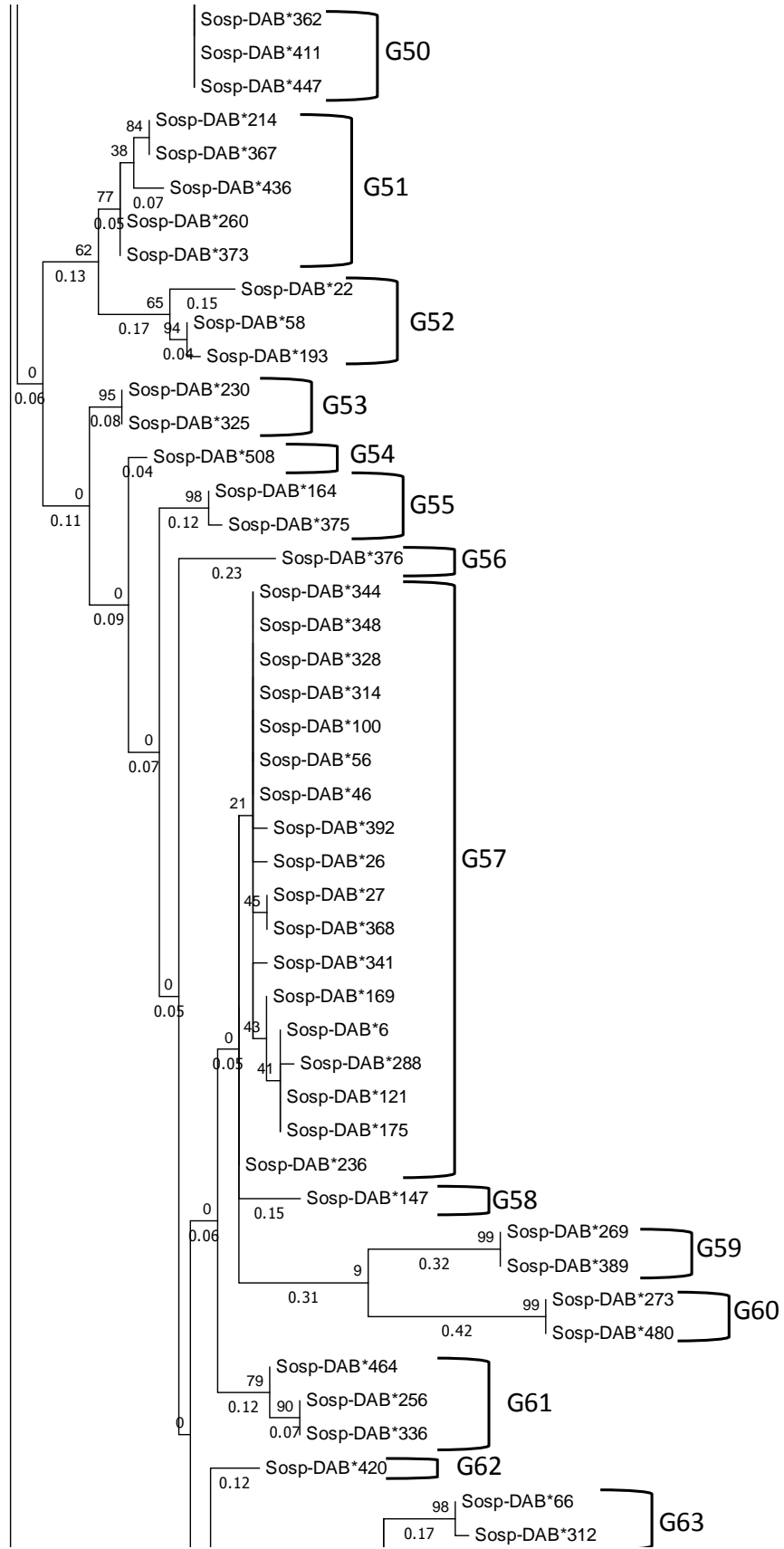
Approval Date: 06/09/2016

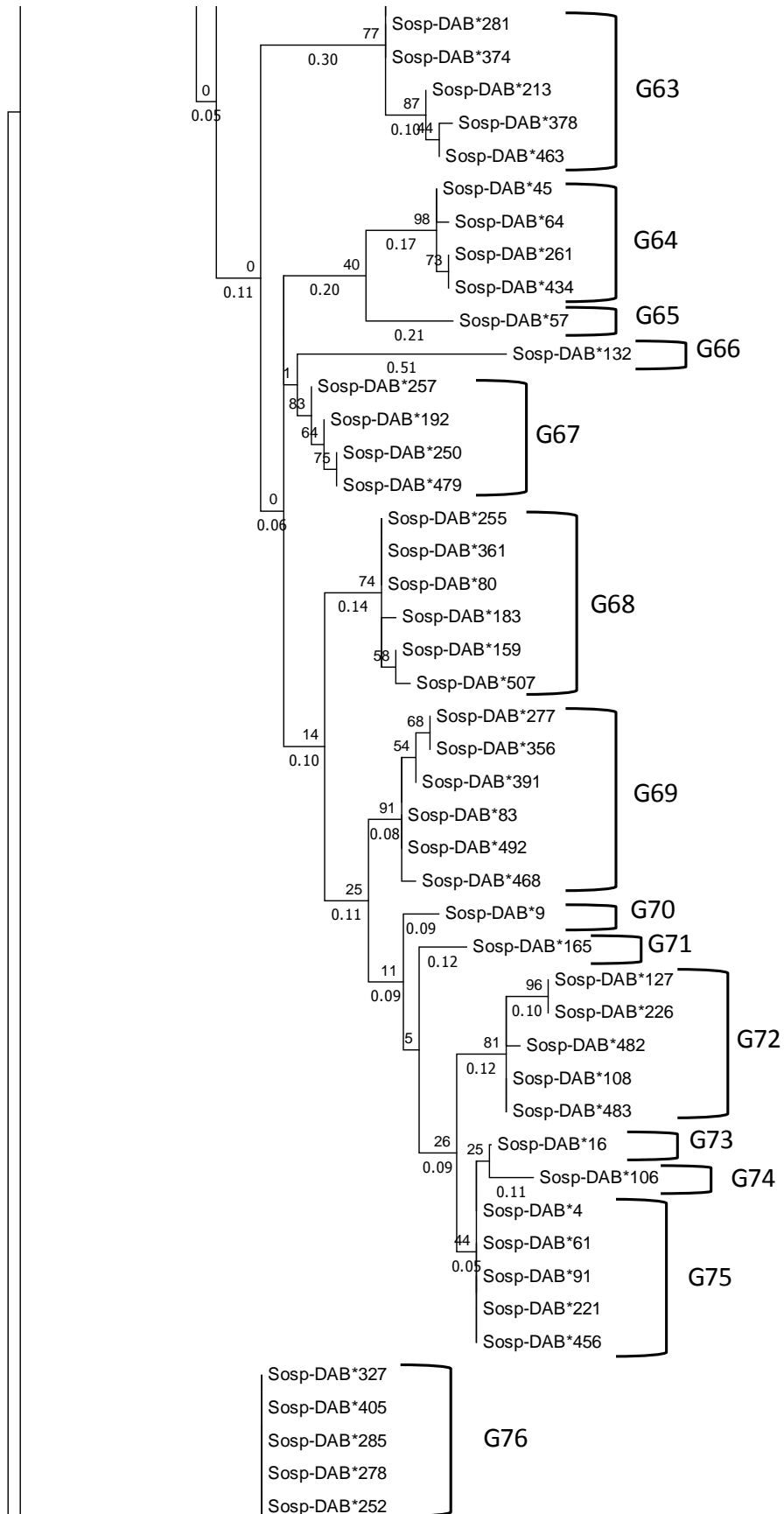
Appendix B: Maximum Likelihood Tree of MHC class II β alleles

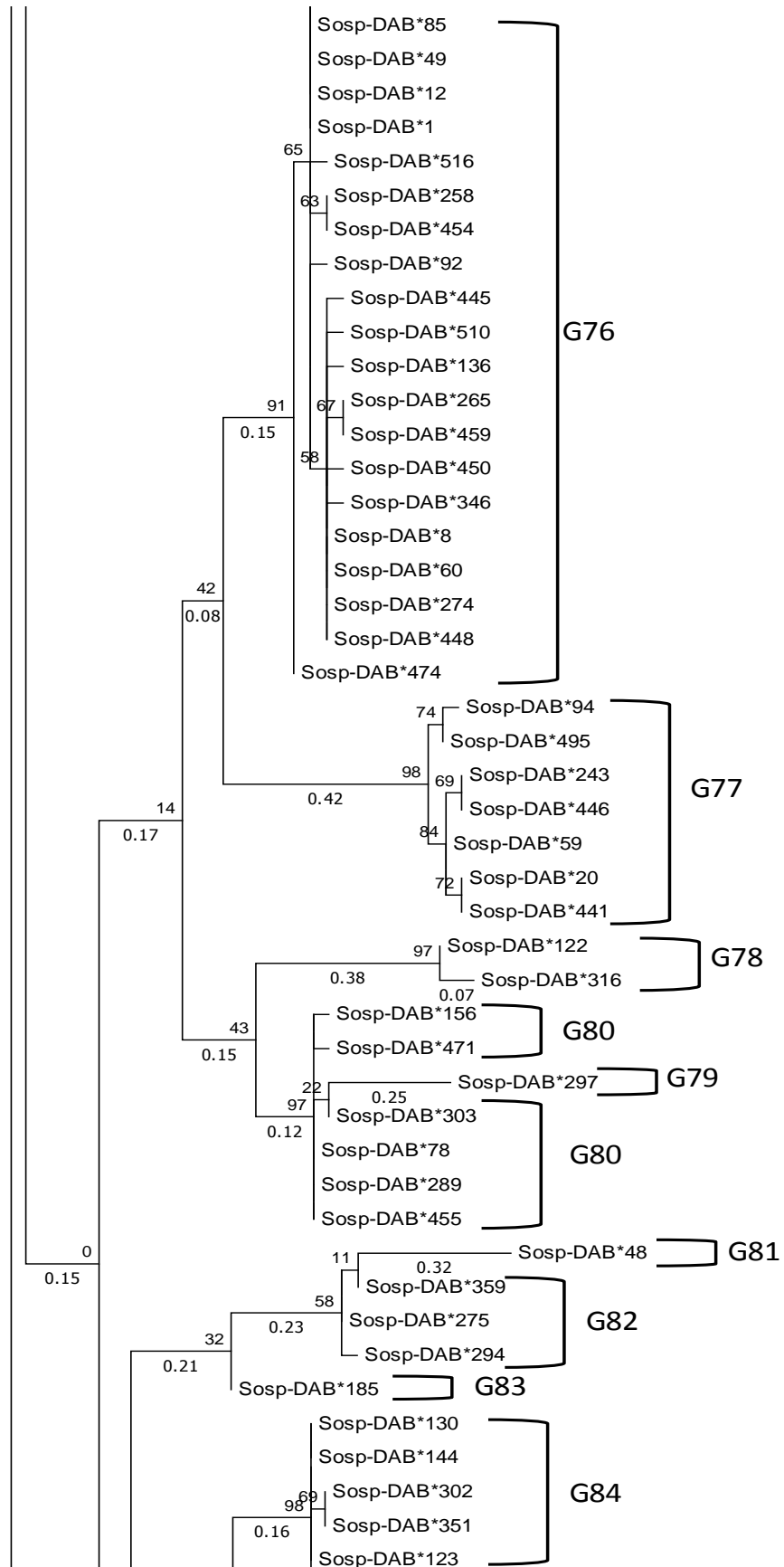


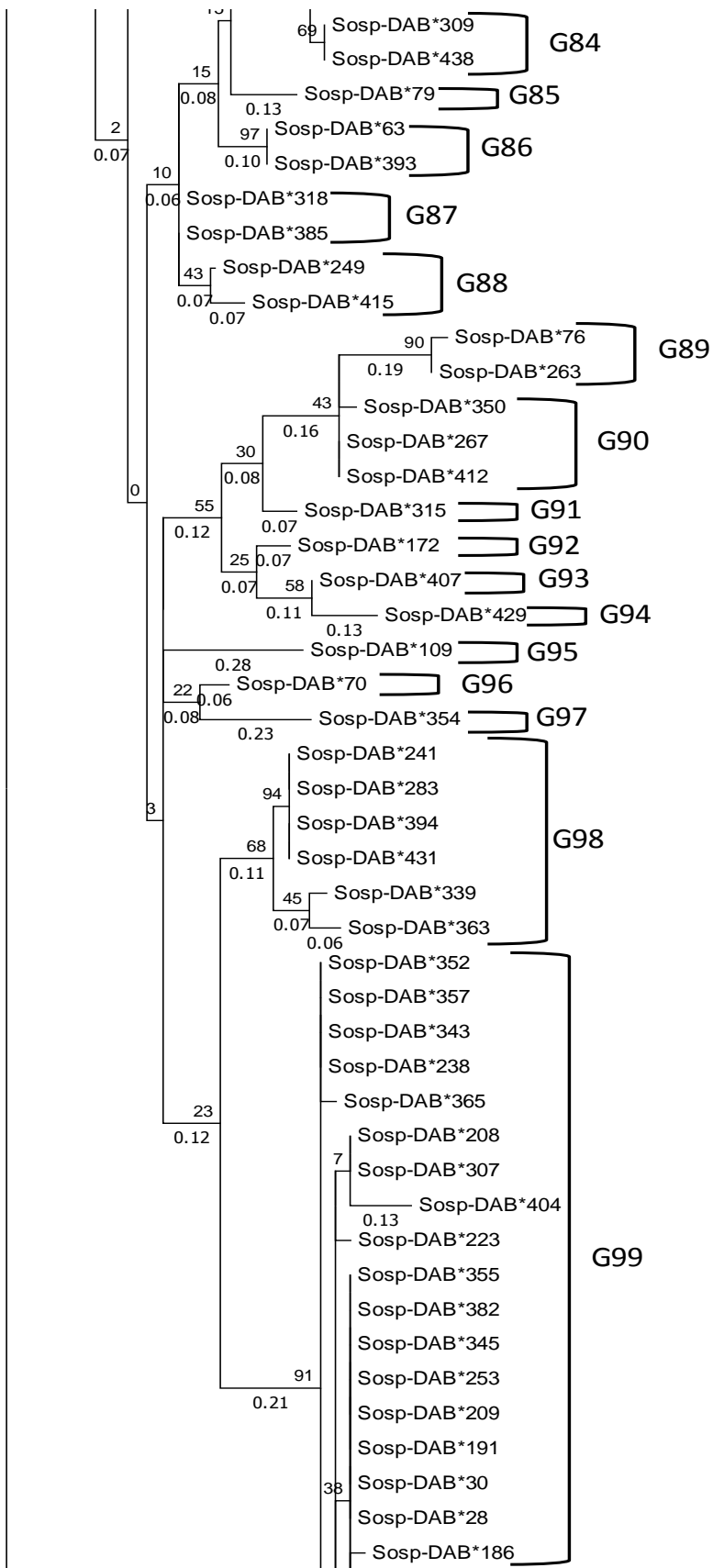


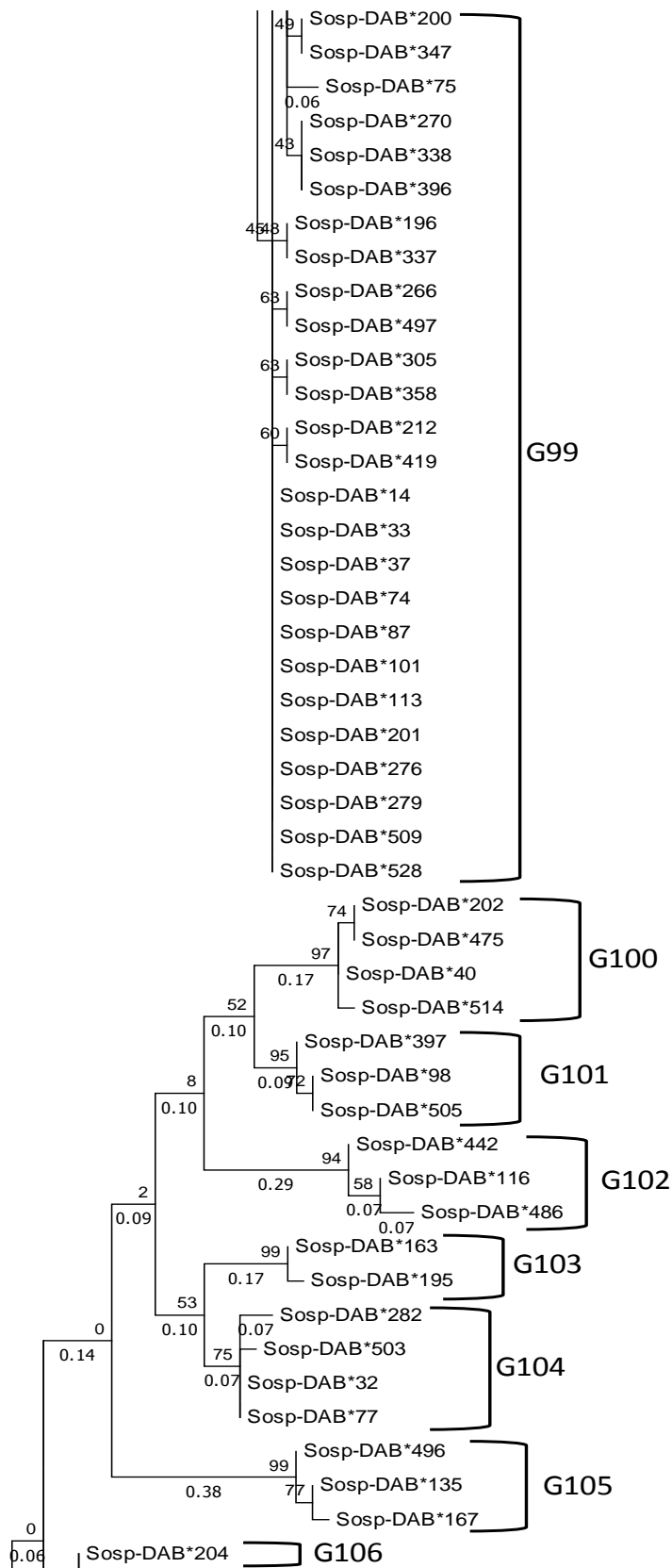


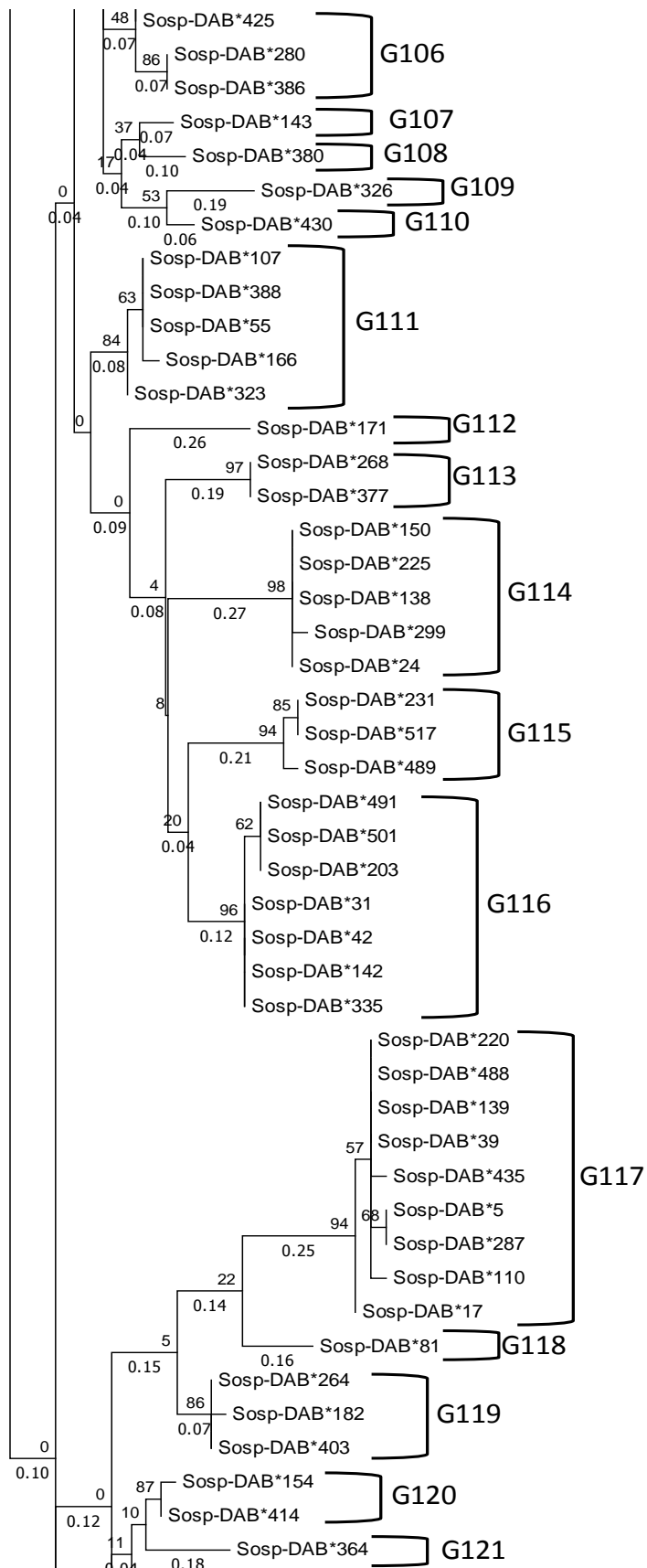


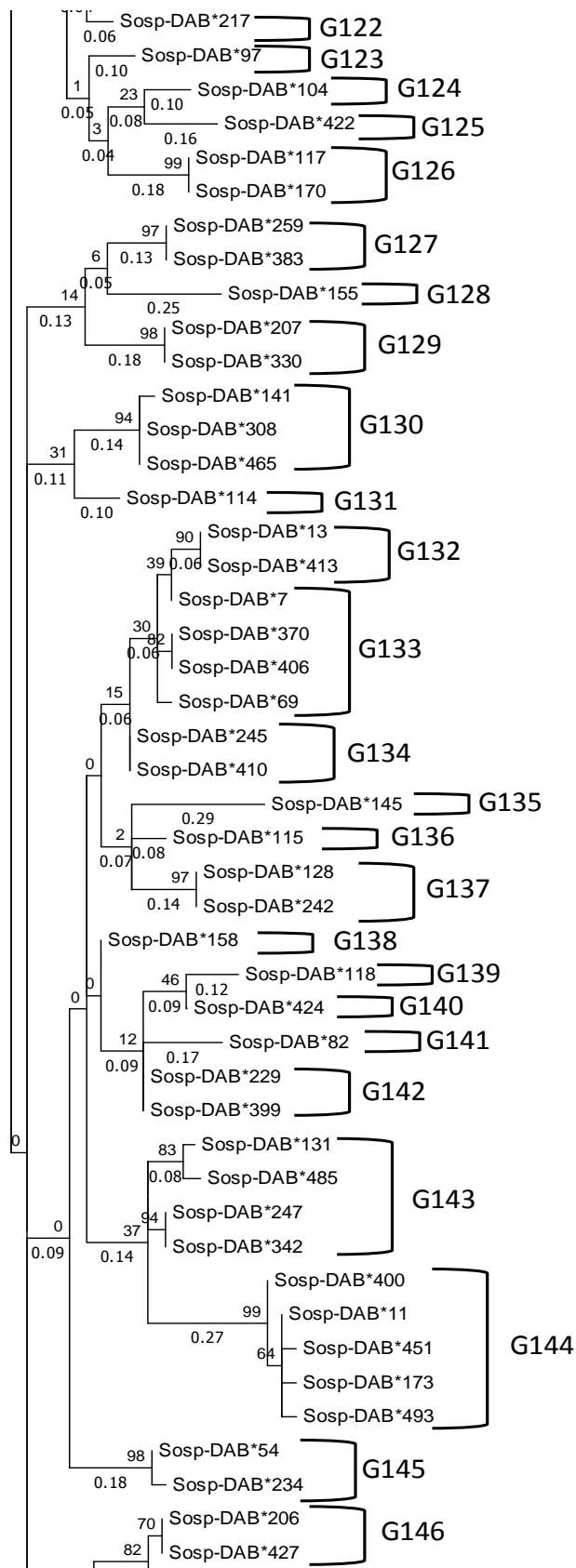


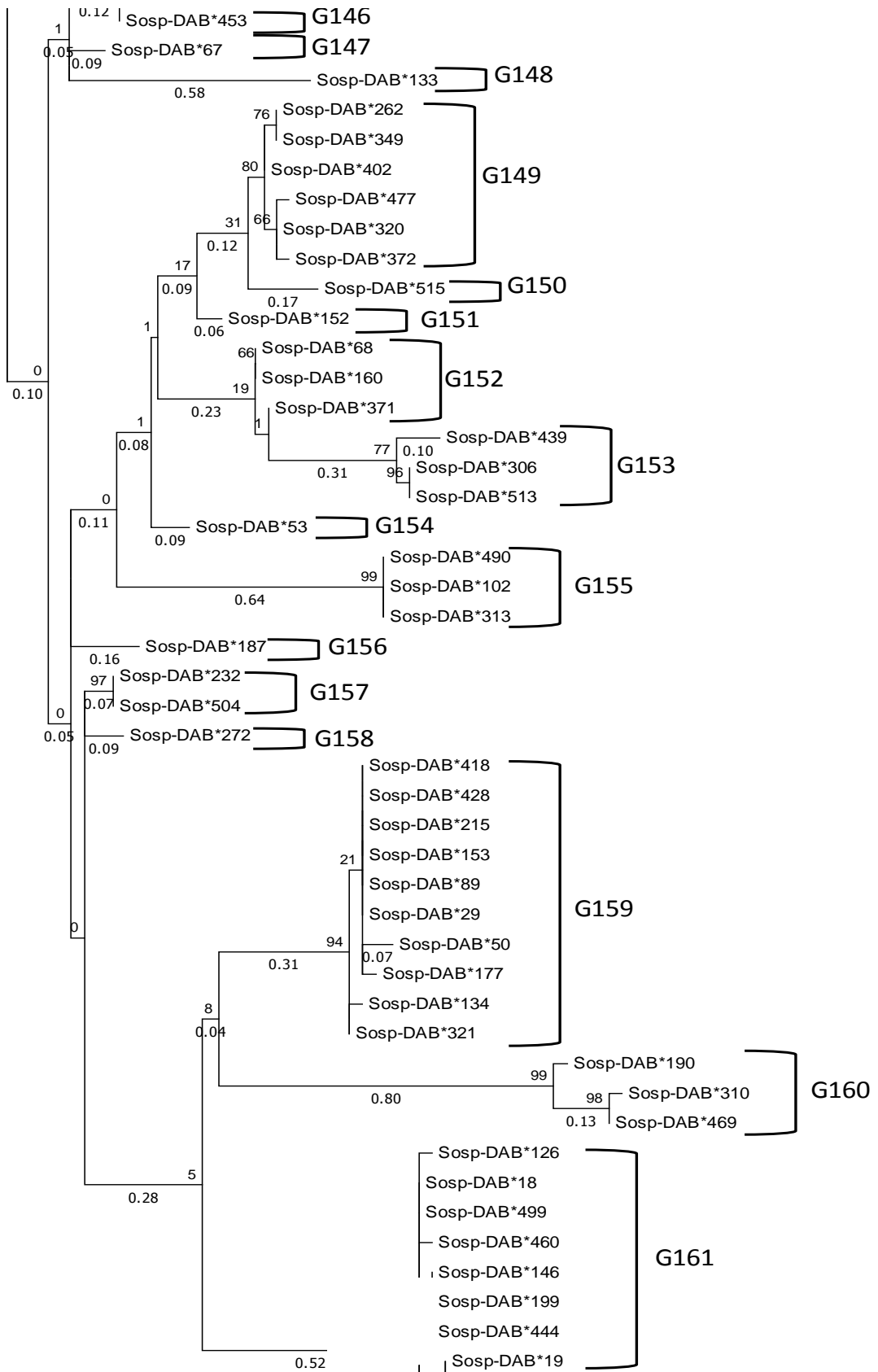












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Watson, M. *Anthropogenic light impacts nocturnal migration*. British Ornithologists Union (2016) (<http://www.bou.org.uk/watson-anthropogenic-light-migration/>)

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Slade, J., **Watson, M.**, Kelly, T., Gloor, G., Bernards, M., MacDougall-Shackleton, E. *Chemical composition of preen oil encodes information on MHC similarity in breeding songbirds*. Proceedings B (In Press)

Contributions to Research

Watson, M., Slade, J., Gloor, G., MacDougall-Shackleton, E. *Influence of MHC diversity on overwinter survivorship in song sparrows (*Melospiza melodia*)* – Canadian Society of Zoologists meeting (May 2016)

Slade, J., **Watson, M.**, Kelly, T., Gloor, G., Bernards, M., MacDougall-Shackleton, E. *Chemical composition of preen oil encodes information on MHC similarity in breeding songbirds* - Canadian Society of Zoologists meeting (May 2016)

Watson, M., Slade, J., Gloor, G., MacDougall-Shackleton, E. *Influence of MHC diversity on overwinter survivorship in song sparrows (*Melospiza melodia*)* – Biology Graduate Research Forum, Western University, London, Ontario, Canada (Sep 2015)

Watson, M., Slade, J., Gloor, G., MacDougall-Shackleton, E. *Influence of MHC diversity on overwinter survivorship in song sparrows (*Melospiza melodia*) and a correlate of neutral locus heterozygosity* - *Ornithology Meeting* - (American Field Ornithologists, Society of Canadian Ornithologists, Wilson's Ornithological Union) Wolfville, Nova Scotia, Canada (July 2015)

Watson, M., Wilson, D., Mennill, D. *Anthropogenic light influences the behaviour of nocturnally migrating birds* - Biology Undergraduate research forum, University of Windsor, Windsor, Ontario, Canada (April 2014)