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Variation in MHC class II B genes in marbled murrelets: implications for delineating conservation units

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Abstract

Conserving genetic variation is critical for maintaining the evolutionary potential and viability of a species. Genetic studies seeking to delineate conservation units, however, typically focus on characterizing neutral genetic variation and may not identify populations harboring local adaptations. Here, variation at two major histocompatibility complex (MHC) class II B genes was characterized in four populations of marbled murrelets *Brachyramphus marmoratus*, a threatened species in which little neutral genetic population structure has been detected. High diversity, as well as evidence of balancing selection, was detected in exon 2 of these genes. Genetic population structure based on MHC markers was uncorrelated to genetic structure estimated with neutral markers, suggesting that selection played a more important role in shaping population structure at these markers than genetic drift. A high proportion of alleles and inferred peptides were unique to a single population, with the Aleutian Islands and southeast Alaska having the highest richness of both. Murrelets sampled in Oregon had low MHC exon 2 allele and inferred peptide richness, and were significantly differentiated from individuals sampled in the Aleutian Islands based on the frequency of exon 2 alleles. In addition, murrelets sampled in Oregon were differentiated from murrelets in both the Aleutian Islands and southeast Alaska based on inferred peptide frequencies, suggesting that the Oregon population could be prioritized for conservation measures. More broadly, combining information from neutral and adaptive genetic markers can improve the delineation of conservation units in threatened species.

Introduction

Conserving adaptive genetic variation is critical for maintaining the evolutionary potential of a species, particularly in light of ongoing and rapid environmental change (Hedrick, Kim & Parker, 2001; Sommer, 2005). However, most population genetic studies seeking to define conservation units for threatened species focus on neutral, rather than adaptive, genetic variation due to the practicality of developing neutral markers (Schwensow *et al*., 2007; Landguth & Balkenhol, 2012). Delineation of conservation units based only on neutral genetic variation may not adequately identify populations harboring important adaptive traits because patterns of neutral variation generally reflect demographic history and connectivity rather than natural selection (Crandall *et al*., 2000; Piertney, 2003; Holderegger, Kamm & Gugerli, 2006). For instance, genetic structure was more pronounced at adaptive than at neutral genetic markers in both lesser kestrels *Falco naumanni* and great snipe *Gallinago media*, presumably because of spatial variation in selection regimes (Ekblom *et al*., 2007; Alcaide *et al*., 2008). Indeed, populations that have large effective sizes or that are highly connected may exhibit little or no population structure at neutral genetic markers, but could be reservoirs of adaptive genetic variation important for the evolutionary potential of the species (Limborg *et al*., 2012).

The marbled murrelet *Brachyramphus marmoratus* (hereafter 'murrelet') is a threatened species of seabird whose populations have been reduced extensively by the loss of old-growth forest nesting habitat and other factors (Ralph, Hunt & Piatt, 1995; Raphael, Mack & Cooper, 2002; Peery *et al*., 2004; Piatt *et al*., 2007). Patterns of population genetic structure based on neutral genetic markers (microsatellites, nuclear introns and mitochondrial DNA) support the delineation of three conservation units: (1) peripheral populations in central and western Aleutian Islands; (2) a large central region including populations from the eastern Aleutian Islands to northern California; (3) central California (Friesen *et al*., 2005; Piatt *et al*., 2007; Hall *et al*., 2009). However, lack of population genetic structure within the central portion of the murrelet's range based on neutral markers could be due to large effective

population sizes or high levels of gene flow. Indeed, the central unit may encompass individual populations possessing adaptations to local environments that are important to the evolutionary potential of the species. As a result, genetic markers that evolve in response to natural selection may provide more information for characterizing adaptive population genetic structure in this portion of the murrelet's range, and in conjunction with neutral markers, may improve the delineation of conservation units. Incorporating measures of adaptive genetic variation into decisions about conservation units in murrelets is by no means a purely academic exercise given recent proposals to remove murrelet populations in California, Oregon and Washington from the list of threatened species under the US Endangered Species Act (ESA), in part based on a lack of genetic differentiation between these and larger, non-threatened populations in the central part of the murrelet's range. To date, no adaptive markers have been screened in this species.

Major histocompatibility complex (MHC) genes have been used as markers to characterize adaptive genetic variation in several vertebrate species (e.g. Edwards *et al*., 2000; Landry & Bernatchez, 2001; Jarvi *et al*., 2004; Schwensow *et al*., 2007) because of their key role in the immune system response (Apanius *et al*., 1997; Hedrick, 1998). MHC class II B genes code cell-surface glycoproteins that recognize, bind and present exogenous antigens (i.e. peptides from bacteria, viruses and fungi) to immune system cells to elicit an appropriate immune response (Germain & Margulies, 1993). The outermost part of the glycoprotein is called the peptide-binding region (PBR) and is the most polymorphic portion of these molecules because it is in direct contact with antigens (Hughes & Hughes, 1995). High MHC diversity within populations is thought to be maintained by selection via heterozygote advantage (Doherty & Zinkernagel, 1975; A.L. Hughes & Nei, 1992), rare-allele advantage (Clarke & Kirby, 1966) and/or fluctuating selection (Hedrick, Ginevan & Ewing, 1976; Gillespie & Turelli, 1989). Positive associations between MHC diversity and individual fitness resulting from greater resistance to infections and disease have been documented in several species (Bernatchez & Landry, 2003; Kloch *et al*., 2013; Lenz *et al*., 2013; Sepil, Lachish & Sheldon, 2013). As a result, loss of MHC variation due to bottlenecks may compromise population viability (Altizer, Harvell & Friedle, 2003). Variation in MHC among populations is thought to be maintained by fluctuating selection, where pathogen selection pressures vary over space and time (Hedrick *et al*., 1976; Gillespie & Turelli, 1989). Thus, regional differences in pathogen communities could generate greater population structure at MHC genes than expected with neutral markers (Hedrick, 2002; Spurgin & Richardson, 2010).

Here, we characterized variation in the MHC class II B gene in marbled murrelets sampled from five regions spanning the range of the species. We assessed the suitability of this marker for inferring adaptive genetic variation in murrelets and used it to characterize population differentiation among four of the sampled regions. We also determined if population structure based on MHC correlated with patterns of neutral genetic variation and evaluated previous delineations of conservation units for this species.

Material and methods

All marbled murrelet samples in this study were used in previous genetic studies based on neutral markers (Friesen *et al*., 2005; Peery *et al*., 2008, 2010; Hall *et al*., 2009). Most were collected at sea using the 'night-lighting/dip-netting' technique (Whitworth *et al*., 1997) from an inflatable vessel during the breeding season from 1992 to 2007, but some were shot at sea for dietary analysis (Supporting Information Table S1). Only samples of adults were included in the analysis to avoid genotyping non-resident juveniles that may have recently dispersed from other populations (Hall *et al*., 2009). A total of 60 samples from five populations spanning the species' range were screened for variation at the MHC (Fig. 1, Supporting Information Table S1) including Aleutian Islands ($n = 8$), southeast Alaska ($n = 17$), Oregon ($n = 17$), northern California ($n = 2$) and central California ($n = 16$). Samples from the Aleutian Islands were collected 4–14 years earlier than samples from other populations, but we doubt this result affected comparisons of genetic variation among populations given that the difference in sampling was equivalent to only ~1 murrelet generation and effective population sizes remain large (Peery *et al*., 2010). Samples consisted of 50 μL of blood collected from the medial metatarsal vein using capillary tubes and stored in 95% ethanol, or in the case of the Aleutian Islands samples, solid tissue collected from the heart, liver or kidney kept frozen.

DNA extraction and amplification

Genomic DNA was extracted from blood and tissue samples using DNeasy Tissue Extraction Kits (Qiagen, Valencia, CA, USA). A 576 bp fragment of two MHC class II B genes, encompassing 110 bp of the 5′ end of intron 1, the entirety of exon 2 that codes the PBR (270 bp), and the entirety of intron 2 (196 bp), was amplified by polymerase chain reaction (PCR) using the primers *MamuF7* (5′ GGTGCAGGAGGATGCTGTG 3′) and *MamuR7-1* (5′ GTAGGTCCAGTCTCCGTTCTG 3′). These primers were designed based on sequences used to amplify the same MHC genes for auklets (Walsh & Friesen, 2003) and modified after several rounds of PCR, cell-cloning and sequencing to obtain primers specific for marbled murrelets. PCR conditions were set as follows: Initial denaturation at 95°C for 5 minutes; 30 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 60 s; and a final extension at 72°C for 5 min. For each 20 μL PCR, we added 100 ng of genomic DNA, 1 unit of Hotstart HiFidelity® DNA polymerase (Qiagen), 0.5 μM of each primer, 3.0 mM $MgCl_2$, 100 mM of each deoxynucleotide triphosphate, and 150 μg mL^{-1} bovine serum albumin.

Cloning and sequencing

Previous studies with MHC class II B genes in alcids suggested the presence of two loci, yielding up to four alleles

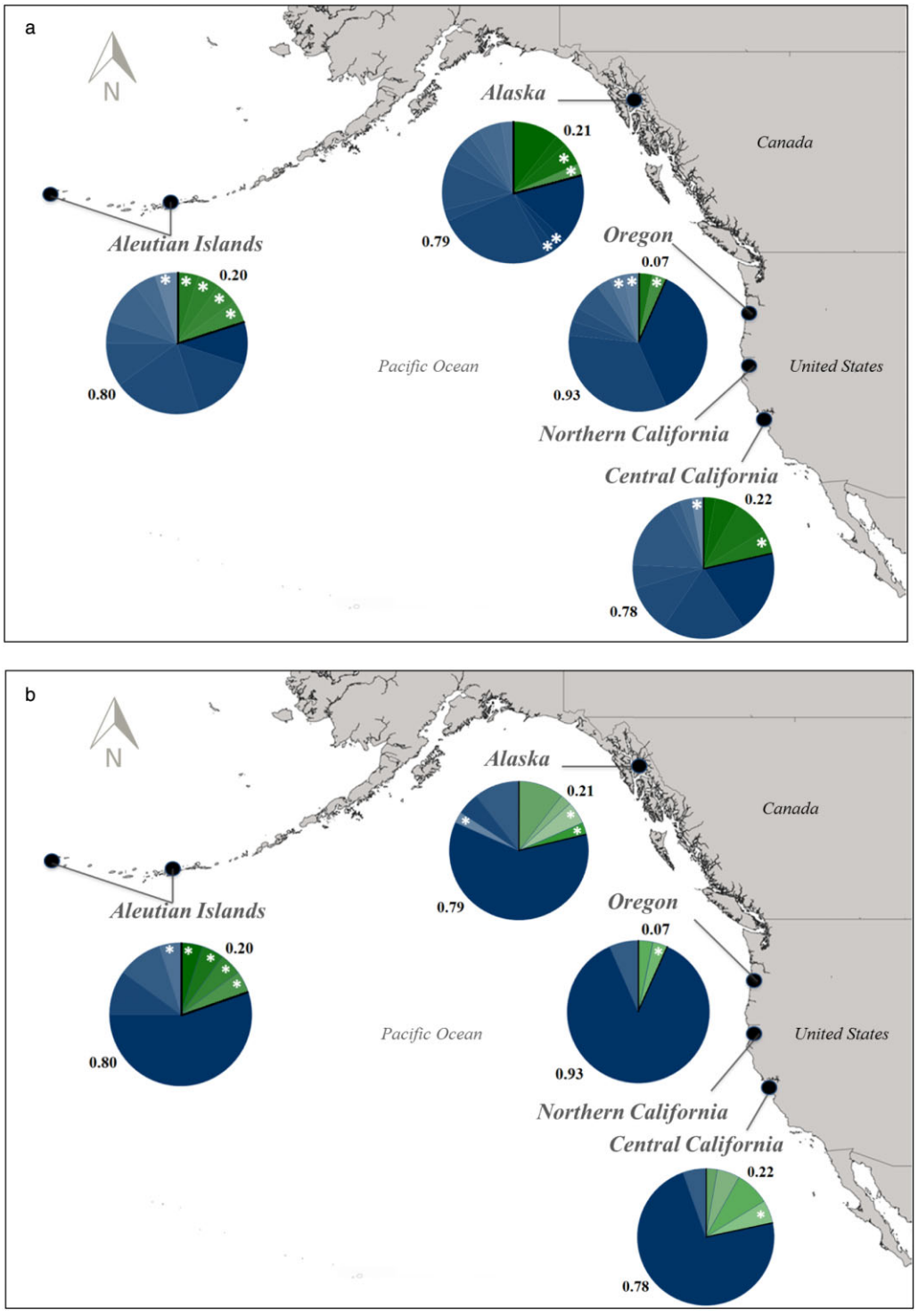

Figure 1 Sampling locations for marbled murrelets *Brachyramphus marmoratus* in the present study. Individuals sampled at the two locations in the Aleutian Islands were pooled into a single population sample. Pie charts show the frequencies of alleles (a) and inferred peptides (b) from exon 2 of major histocompatibility complex class II B, where each 'pie slice' represents an allele or peptide. Green and blue pie slices represent allele/peptide lineages 1 and 2, respectively. White asterisks indicate private alleles and peptides. Data for northern California are not presented because of small sample sizes.

per individual (Walsh & Friesen, 2003). However, the tendency for gene duplication in this marker made it necessary to verify the number of loci present in marbled murrelets. Therefore, cell cloning was performed to sequence all possible alleles. PCR products were ligated to linearized plasmid vectors using the Topo® TA Cloning® Kit (Invitrogen®, Grand Island, NY, USA) and *Escherichia coli*-competent cells were transformed with these recombinant vectors. Sixteen transformant clones were selected per individual for sequencing based on Monte Carlo simulations, which indicated that doing so resulted in a 95% probability of sampling all four alleles. To obtain the inserts (MHC class II B genes) from transformant clones, a second round of PCR was run under the same conditions as mentioned earlier, and product sizes were verified by electrophoresis using 1% agarose gels. Correctly sized PCR products were purified with Exonuclease/Shrimp Alkaline Phosphatase enzyme (ExoSAP-IT, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Once cleaned, products were cycle-sequenced using BigDye® Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) labels in both forward and reverse directions. Labeled products were cleaned from unincorporated dye using Agencourt® paramagnetic beads (Beckman Coulter, Inc., Fullerton, CA, USA) and visualized using an ABI 3730xl DNA analyzer (Applied Biosystems) at the Biotechnology Center of University of Wisconsin-Madison.

All MHC class II B sequences obtained from each clone were edited by comparing forward and reverse sequences, then, sequence identity (as MHC class II DAB-like) was verified using nucleotide BLAST (Basic Local Alignment Search Tool) searches on GenBank (http://www.ncbi.nlm.nih.gov). All 16 sequences from each sampled murrelet were aligned to obtain genotypes (list of different MHC sequences an individual carries) using a Clustal-W multiple alignment application in Geneious v.5.6 (Drummond *et al.*, 2012). Each individual's alignment was checked for PCR recombination events; sequences that were intermediate between two others (i.e. the first segment of the sequence was identical to one sequence and the last part was identical to a second sequence in the alignment) were considered PCR recombination errors and discarded from the analysis. Only three such sequences were detected and the three individuals containing these sequences were discarded from the analysis. Moreover, we conducted independent PCR reactions for five individuals without detecting recombination events. Replication errors and polymorphic sites were identified in an *ad hoc* manner. All alleles from all individuals were aligned in a Clustal-W multiple alignment and a polymorphic site was identified as a position that had two or more different nucleotides in at least two different individuals. Sites where all but one nucleotide were identical were treated as PCR/sequencing errors. Gene functionality was assessed by searching for frame-shift mutations and stop codons, and by looking for highly conserved residues within the putative amino acid sequences (Schaschl, Goodman & Suchentrunk, 2004).

Data analysis

Because assignment of alleles to one of the two presumed loci found was not possible, the frequency of an allele was estimated as the number of genotyped individuals with that allele divided by the total number of alleles in the population. Genetic diversity was characterized in the entire sequenced gene, exon 2 and intron 2 based on the number of alleles (defined as the number of different sequences detected), the number of segregating sites, nucleotide diversity, and haplotype diversity estimated using DNAsp 7.0.1 (Rozas & Rozas, 1999). Peptides were inferred from MHC exon 2 sequences via translation. Population-specific frequencies of inferred peptides were calculated as the number of individuals having a given peptide sequence divided by the total number of peptide sequences detected in the population.

Selection on MHC exon 2 sequences was tested using two methods. First, we calculated ω, the ratio of non-synonymous to synonymous substitutions. This calculation was conducted for both peptide binding site (PBS) codons and non-PBS codons (Nei & Gojobori, 1986), where PBS and non-PBS codons were identified according to Brown *et al.* (1993). Under the influence of positive selection, the number of non-synonymous mutations is expected to be greater than the number of synonymous mutations and ω is therefore expected to be greater than 1 (Bamshad & Wooding, 2003). The significance of ω was assessed with a *Z*-test under the null hypothesis of neutrality (i.e. $\omega = 1$) using the methods of Nei & Gojobori (1986) and a Jukes–Cantor correction. Second, Tajima's *D*-test statistic was used to test for balancing selection by comparing the number of segregating sites and the number of pairwise nucleotide differences within a sliding window of 12 nucleotides (step size = 3 nucleotides). Values > 1.5 were considered to be statistically significant (Tajima, 1989). The statistics ω and Tajima's *D* were estimated using software DNAsp v7.0.1, and the *Z*-test for ω was conducted using 1000 permutation in Mega v5 (Tamura *et al.*, 2011).

To characterize relationships among exon 2 alleles, a phylogenetic network was constructed using a neighbor–net algorithm based on Kimura's two-parameter genetic distance in the software SplitsTree v4.0 (Bryant & Moulton, 2004). This approach was more likely to accurately reflect relationships among alleles than a phylogenetic tree because it accounts for recombination and gene conversion, which are known to be important processes shaping variability in MHC sequences (Huson & Bryant, 2006).

The level of genetic differentiation between pairs of populations was assessed using F_{st} (Wright, 1984) based on both exon 2 allele frequencies and the frequencies of peptides inferred from exon 2. Northern California samples were excluded from analyses of population structure given that only two samples from this region were sequenced. To account for multiple comparisons, critical values were adjusted using the approach of Narum (2006), which resulted in a significance level of 0.020 given that six

Table 1 Genetic diversity statistics for the MHC class II B gene in marbled murrelets *Brachyramphus marmoratus*, presented for the entire gene fragment (MHC) as well as for exon 2 and intron 2 separately

Diversity statistic	MHC (576 bp)	Exon 2 (270 bp)	Intron 2 (196 bp)
Number of alleles	74	27	29
Haplotype diversity	0.97	0.86	0.92
Nucleotide diversity	0.038	0.079	0.040
Number of segregating sites	79	49	26
With two variants	73	44	25
With three variants	6	5	1

MHC, major histocompatibility complex.

pairwise tests of H_o ($F_{st} > 0$) were conducted per marker type. Also, a hierarchical analysis of molecular variance (AMOVA; Weir & Cockerham, 1984) was performed in Arlequin (Excoffier *et al.*, 2005) to determine how much genetic variation was distributed among individuals, populations and regions. In this analysis, central California and Oregon were grouped into a southern region because they are considered Threatened under the ESA; Aleutian Islands and southeastern Alaska samples were grouped into a northern region because neither of these populations is protected under the ESA. Allele and peptide richness (corrected for differences in sample size among populations) were estimated by randomly re-sampling 20 alleles or peptides from each population (20 being the observed number of gene copies in the Aleutian Islands; see later). This process was repeated 1000 times, and both the mean and the fifth and 95th percentiles were estimated for the number of alleles and peptides in the re-sampled datasets for each population.

To test for differences in patterns of putatively adaptive and neutral genetic variation, we compared levels of genetic population structure at both MHC exon 2 alleles and inferred peptides to the level of genetic population structure based on 12 microsatellite loci, the latter of which was obtained from a reanalysis of data presented in Friesen *et al.* (2005). To make sample sizes comparable, we randomly selected samples from Friesen *et al.* (2005) such that microsatellite and MHC sample sizes were equal for each population. The same murrelets were screened for MHC (this study) and microsatellite (Friesen *et al.*, 2005) variation in the Aleutian Islands population, but samples differed for the other three populations. Nevertheless, the pattern of genetic population structure observed with microsatellites in Friesen *et al.* (2005) was very similar to the microsatellite population structure detected with the samples used to screen for MHC variation (Hall *et al.*, 2009; Peery *et al.*, 2010). Pairwise F_{st} for microsatellites was estimated using the program Arlequin v3.5. Concordance in genetic population structure between marker types was assessed by comparing genetic distance (F_{st}) matrices with a Mantel test. Correlations between F_{st} and geographic distance (km) were also explored for both marker types using Mantel tests. All tests were performed using the Ecodist v1.2.7 package (Goslee & Urban, 2007) in R v3.0 (R Development Core Team, 2007) and were one-way given that relationships were expected to be positive.

Results

Polymorphism at MHC class II B genes

High levels of polymorphism were observed at MHC class II B genes with 74 different alleles being detected for the entire 560 bp amplified gene fragment (Table 1). Nucleotide diversity and the number of segregating sites were approximately two times greater in exon 2 than intron 2, although the number of alleles and haplotype diversity in the exon and intron were similar. Almost all segregating sites were characterized by two variants (Table 1).

A maximum of four alleles were detected within an individual at exon 2, suggesting that two copies of this gene occur within the marbled murrelet genome. Thirteen individuals (22%) had only one allele, 27 (45%) had two alleles, 17 (28%) had three alleles, and 3 (5%) had four alleles at exon 2. Twenty-seven unique alleles were detected for exon 2 (*GenBank Accession Nos. KC533147 to KC533173*; Table 1, Supporting Information Fig. S1) and two clearly distinct allele lineages differing by 22 mutations (8% of sites) were visible in the phylogenetic network (Fig. 2). Lineage 1 had slightly fewer alleles (12) than lineage 2 (15) and lineage 1 occurred at a relatively low frequency in all populations (0.07–0.22; Fig. 1a). Forty of the 60 sampled individuals possessed alleles from only one lineage, indicating that each lineage was present on both MHC class II B loci.

Functionality and selection of exon 2

Frame-shift mutations or stop codons were not detected in the nucleotide sequences of any of the 27 exon 2 alleles, suggesting that exon 2 of both loci is functional. Moreover, after translating nucleotide sequences into amino acid sequences, conserved amino acid residues thought to be important for protein-folding in human leukocyte antigen such as cysteine residues and NGT (Asparagine-Glycine-Threonine) glycosilation sites (Schaschl *et al.*, 2004), were also conserved in murrelets across all 17 different peptides derived from exon 2 alleles (C residues 10 and 74 and NGT residues 14–16 in murrelets; Fig. 3). Inferred peptides were arranged in two distinct groups that differed from each other by nine amino acids and corresponded to the two allele lineages described earlier (Fig. 3). Peptide group 1 was composed of 12 different peptides compared with only five

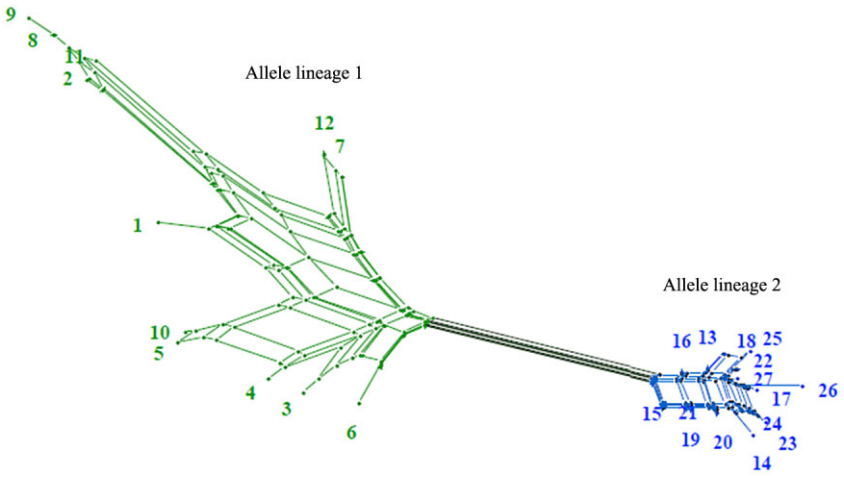

Figure 2 Neighbor–net phylogenetic network for exon 2 of major histocompatibility complex class II B genes in marbled murrelets (*Brachyramphus marmoratus*). Lines represent mutation events and nodes represent alleles. Alleles were grouped in two highly distinct allele lineages 1 (in green) and 2 (in blue) separated by 22 mutations.

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Peptide1   YFQVMFKANCYFTNGTERVRFVETYIYNRQALLHFDSDVGRYVADTPLGKPSAEYWNSQPEILEQKRAAVDTFCRHNYRVWTPFTVDRR
Peptide2   ...E.....................................................P...F.........R.............V........
Peptide3   ............................PYV.....................D.................E.....................
Peptide4   ...D...................V......EPNV.......H...........D.................E.....................
Peptide5   ...D.....................EPNV.......H...........D.............R.......................
Peptide6   ............................PFV.................T...................E.....................
Peptide7   ...D.....................PYV.......................C............E...........V........
Peptide8   ...E.....................................................P...F.........R.............GV........
Peptide9   ...E.....................................................P...F.........R..P..........GV........
Peptide10  ...D.....................EPNV.......H.........T.D.............R.......................
Peptide11  ...E.....................................................P...F.......................GV........
Peptide12  ...D.....................PYV.......................C............E...........GV........
Peptide13  ...D...SD...........L.TRH....EQYA.................T.....F.........R...E...V..N..K.....A.E..
Peptide14  ...E...SD...........L.TRH....EQYA.................T.....F.........R...E...V..N..K.....A.E..
Peptide21  ...D...SD...........L.TRH....EQYA.................T.....F.........R...E...V..N..K.......E..
Peptide25  ...D...SD...........L.TRH....EQYA.......H........T.....F.........R...E...V..N..K.....A.E..
Peptide26  ...D...SD...........L.TRH....EQYA.............I...T.....F.........R...E...V..N..M.....A.E..
           1        10        20        30        40        50        60        70        80
                    PBS1      PBS2      PBS3                          α-Helix
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Figure 3 Alignment of inferred peptides from exon 2 of major histocompatibility complex class II B genes. Peptide1 is the reference amino acid sequence and dots represent identical sites. Peptide-binding sites (PBS) are residues highlighted in grey in the reference sequence and identified at the bottom of the figure. Peptides of group 1 are highlighted in light grey and were obtained from alleles in lineage 1. Peptides of group 2 are highlighted in dark grey and were obtained from alleles in lineage 2.

different peptides in group 2. However, group 1 was the least common across all four populations (0.07–0.22; Fig. 1b).

The rate of non-synonymous to synonymous substitutions was significantly greater than 1 in the PBSs, but not the non-PBSs of exon 2, suggesting that PBSs have historically been under positive selection (Table 2). Also, evidence of balancing selection acting on exon 2 of the MHC class II B genes surveyed here was found with an excess of segregating sites reflected in positive, significant Tajima's *D*-test values at PBS sites, but not at non-PBS sites (Table 2).

Population genetic structure

Ten to 15 exon 2 alleles were detected in the four populations considered in analyses of genetic population structure (Table 3). The fewest number of alleles and inferred peptides were detected in Oregon. After rarefaction, conducted to correct for differences in sample size among populations, peptide and allelic richness was significantly lower in Oregon than the other three populations (i.e. the mean in Oregon was below the lower 95% confidence interval of the other populations). Similarly, the mean number of alleles per individual was lower in Oregon [1.76; 95% confidence interval (CI): 1.86–3.14] than in the other three populations (Aleutian Islands: 2.50, 95% CI: 1.86–3.14; southeast Alaska: 2.24, 95% CI: 1.92–2.55; central California: 2.31, 95% CL: 1.82–2.81). All four populations harbored private alleles (range: 2–5) and 59% (15 of 27) of all exon 2 alleles were private (i.e. detected in a single population; Table 3). Similarly, all four populations harbored private peptides (range: 1–5), and 63% of peptides (10 of 16) were unique to a single population. Finally, alleles from both lineages were present in all populations and their frequencies per population were very similar among populations;

Neither regional ($F_{ct} = 0.001$, $P = 0.664$) or population ($F_{sc} = 0.013$, $P = 0.128$) structure was detected at exon 2, and 98% of adaptive genetic variation occurred within popula-

tions ($F_{st} = 0.014$, $P = 0.081$) using AMOVA. Oregon and the Aleutian Islands were very nearly significantly differentiated based on F_{st} calculated for exon 2 allele frequencies given the 'protected' significance level of 0.020 ($F_{st} = 0.054$, $P = 0.025$; Table 4). Oregon was significantly differentiated from the Aleutian Islands ($F_{st} = 0.095$, $P = 0.016$) and very nearly significantly differentiated from southeast Alaska ($F_{st} = 0.065$, $P = 0.023$) based on inferred MHC exon 2 peptides (Table 4).

Generally, patterns of population structure differed between adaptive genetic markers and neutral markers for marbled murrelet. No significant correlation was detected between the level of structure (based on F_{st}) obtained with microsatellite markers and the level of structure obtained with either MHC exon 2 allele frequencies ($r_M = -0.23$, $P = 0.282$) or inferred peptides from MHC exon 2 alleles ($r_M = -0.25$, $P = 0.705$; Fig. 4). Furthermore, genetic differentiation was nearly positively correlated with geographic distance between pairs of populations for microsatellites ($r_M = 0.68$, $P = 0.085$), but not MHC alleles ($r_M = 0.09$, $P = 0.560$) or inferred peptides ($r_M = 0.249$, $P = 0.37$; Fig. 4).

Table 2 Tests of selection for exon 2 of the MHC class 2 B genes in marbled murrelets *Brachyramphus marmoratus*, presented separately for codons in PBS and non-PBS codons

Statistic	PBS codons ($n = 72$)	Non-PBS codons ($n = 15$)
Mean number of synonymous differences between sequence pairs	5.7	4.5
Mean number of non-synonymous differences between sequence pairs	15.1	6.0
Substitutions per synonymous site (*dS*)	0.073	0.112
Substitutions per non-synonymous site (*dN*)	0.204	0.042
ω (*dN*/*dS*)	3.242	0.378
Z-test of selection, $\omega = 1$	($Z = 2.08$, $P = 0.040$)	($Z = -1.50$, $P = 0.137$)

Codons were assigned as PBS or non-PBS based on Brown et al. (1993). The Z-test at the bottom of the table provides a test of the null hypothesis of neutrality ($\omega = 1$). MHC, major histocompatibility complex; PBS, peptide-binding sites.

Discussion

Adaptive genetic variation at MHC in marbled murrelets

The likely presence of two MHC class II B genes in marbled murrelets is consistent with studies of other alcid species (Walsh & Friesen, 2003) as well as other birds (Ekblom *et al.*, 2007), although greater numbers of gene copies have been detected in several avian species (Sato *et al.*, 2000; Alcaide, Edwards & Negro, 2007). Levels of sequence and haplotype diversity observed in murrelets were also consistent with levels estimated in other bird species (Briles *et al.*, 1993; Edwards, Gasper & March, 1998; Ekblom *et al.*, 2004;

Table 3 Allele and peptide number and richness for exon 2 of MHC class II B genes in four marbled murrelet *Brachyramphus marmoratus* populations

Population	*n*	Alleles		Peptides		Private allele number	Private peptide number
		N	*R* (5–95%)	*N*	*R* (5–95%)		
Aleutian Islands	20	12	9.0 (6.7–11.3)	8	6.0 (4.0–8.0)	5	5
Southeast Alaska	38	15	10.4 (7.8–13.1)	8	6.6 (4.9–8.3)	5	3
Oregon	30	10	6.9 (4.4–9.3)	4	3.2 (1.8–4.6)	2	1
Central California	37	12	8.8 (6.5–11.2)	6	5.2 (3.8–6.6)	3	1

Allele and peptide richness were calculated using rarefaction. Also presented are private allele and peptide number, where private alleles and peptides were only detected in a single population. *n*, total number of DNA or peptide sequences detected per population; *N*, the number of alleles or peptides detected per population; *R*, the rarefied number of alleles or peptides per population; MHC, major histocompatibility complex; PBS, peptide-binding sites.

Table 4 Pairwise F_{st} estimates (*P*-values) for four marbled murrelet *Brachyramphus marmoratus* populations based on the frequencies of exon 2 alleles in MHC Class II B genes, the frequencies of peptides inferred from exon 2, and microsatellites

Population pair	Marker type		
	MHC alleles	MHC peptides	Microsatellites
Aleutian Islands – southeast Alaska	0.007 (0.249)	–0.012 (0.615)	0.033 (< 0.001)
Aleutian Islands – Oregon	0.054 (0.025)	0.095 (0.016)	0.036 (< 0.001)
Aleutian Islands – central California	0.001 (0.414)	0.025 (0.110)	0.063 (< 0.001)
Southeast Alaska – Oregon	0.016 (0.146)	0.065 (0.021)	0.002 (0.240)
Southeast Alaska – central California	0.002 (0.373)	0.013 (0.184)	0.039 (< 0.001)
Oregon – central California	0.018 (0.107)	0.009 (0.242)	0.024 (< 0.001)

MHC, major histocompatibility complex.

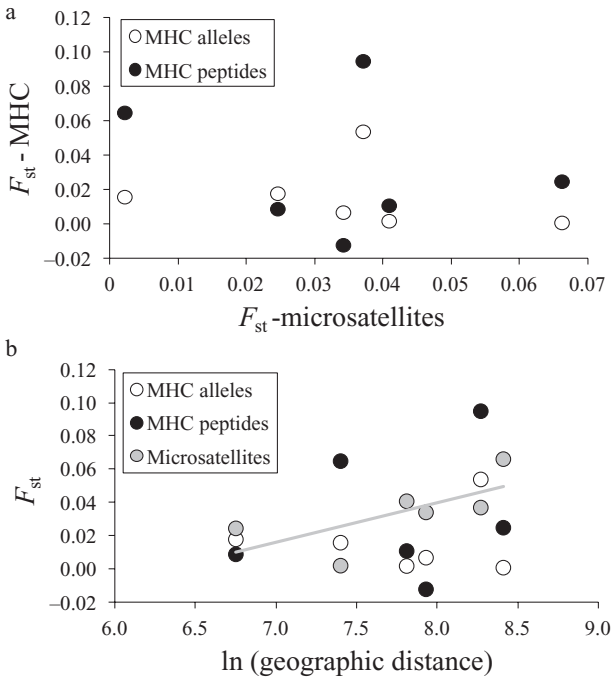

Figure 4 Correlations between the level of genetic differentiation and the geographic distance separating pairs of marbled murrelet populations using microsatellites and both major histocompatibility complex (MHC) class II B exon 2 alleles and inferred peptides. (a) Correlations in genetic differentiation between microsatellites and MHC markers; and (b) correlation between genetic differentiation and geographic distance.

Jarvi *et al*., 2004). For example, nucleotide diversity in exon 2 for murrelets (0.079) fell within the range estimated in other bird species (0.03 and 0.10; Hess, 2000). High polymorphism at MHC class II B genes is thought to be the result of balancing selection that maintains multiple gene variants in the population gene pool (Takahata, Satta & Klein, 1992; Hughes & Yeager, 1998). Our results support balancing selection as a factor influencing MHC variability in marbled murrelets given significant, positive values of both Tajima's D and ω in the PBS of exon 2 (Hughes & Nei, 1989).

While reduced MHC diversity does not necessarily compromise population viability or the potential for adaptation (Radwan, Biedrzycka & Babik, 2010; Gangoso *et al*., 2012), MHC diversity has been linked to disease resistance and fitness in several bird species (Bonneaud *et al*., 2006; Kloch *et al*., 2013; Sepil *et al*., 2013). In fact, recent work suggests that presence of divergent MHC alleles within individuals can reduce susceptibility to disease and increase fitness (Lenz *et al*., 2013). In marbled murrelets, exon 2 alleles were distributed in two lineages that were highly distinctive in nucleotide composition and inferred peptides, and individual murrelets often possessed alleles from both lineages. While we did not link MHC variability to fitness in marbled murrelets, results from Lenz *et al*. (2013) suggest that the presence of both lineages might confer important disease resistance capability to individuals carrying both lineages. Moreover, while allele lineage 2 occurred at a relatively high frequency in all populations and may currently be at a selective advantage, lineage 1 might constitute a reservoir of genetic variation in the context of frequency-dependent selection when rare alleles become advantageous.

Adaptive versus neutral population genetic structure

Some notable differences in patterns of population genetic structure were detected between MHC and microsatellite markers in marbled murrelets, a finding that may reflect different aspects of the demographic and evolutionary history of this species (Holderegger *et al*., 2006). In broad terms, the level of differentiation between MHC and microsatellites was uncorrelated, recognizing the limitation of conducting Mantel tests using only four sampled populations for inferences in this regard. Differences in patterns between neutral and adaptive markers occurred largely because the Aleutian Islands and central California were differentiated from southeast Alaska and based on microsatellites, whereas only Oregon was divergent from the Aleutians and southeast Alaska based on peptides inferred from exon 2 of MHC class II B genes. This result suggests that regional variation in pathogen-mediated selection pressures may have favored different MHC variants in Oregon and southeast Alaska. Weak genetic drift associated with large effective population sizes has likely limited divergence between Oregon and southeast Alaska at microsatellites, but natural selection is more efficient and can be the most important microevolutionary process shaping patterns of adaptive genetic variation in large populations. In contrast to MHC peptides, we did not detect a difference in exon 2 allele frequencies between Oregon and southeast Alaska. However, the distribution of putative peptide frequencies between populations could be a more appropriate statistic to infer regional variation in adaptive traits because natural selection acts directly on the phenotype. In addition, peptide frequencies might be a more appropriate measure of differences in adaptive traits among populations because individual peptides were often coded by several different alleles (e.g. one peptide was coded by six different alleles).

The Aleutian Islands were differentiated from continental populations based on microsatellites, but only differed from Oregon based on MHC exon 2 alleles and inferred peptides. Thus, genetic drift may have played an important role in shaping patterns of neutral variation between the Aleutian Islands and some mainland populations, but less so in shaping patterns of MHC variation. Similarly, central California, the southernmost murrelet population, was divergent from all three northern populations based on microsatellites, but not based on MHC alleles or peptides. The central California population appears to have diverged from northern populations at neutral markers because of the enhanced effects of genetic drift and reduced gene flow associated with its peripheral location and fragmentation of

the species' old-growth nesting habitat (Peery *et al.*, 2010). In contrast, MHC allele frequencies in central California and Oregon/southeast Alaska could have been similar because similarities in pathogen-mediated selection overrode the effects of genetic drift and isolation in this small population (Spurgin & Richardson, 2010).

Implication for the conservation of marbled murrelets

The intensive laboratory work associated with cloning prevented characterizing MHC variation in all of the populations included in previous studies of neutral variation and necessitated modest sample sizes of individuals per population. While these limitations may have prevented a comprehensive screening of MHC variation in marbled murrelets, this assessment provides novel and meaningful insight into the delineation of conservation units and other aspects of murrelet conservation for at least three reasons. First, (1) the four sampled sites span the range of the species; (2) exon 2 of MHC appeared to be under selection; (3) 'ecological non-exchangeability' based on genes with known functionality such as MHC is considered by some to be a key criterion for delineating conservation units (Crandall *et al.*, 2000).

In contrast to previous characterizations of neutral genetic variation in marbled murrelets, we detected significant population differentiation within the large central portion of the species range, particularly in the form of differences in MHC-derived peptide frequencies between southeast Alaska and Oregon. In addition, low allele and peptide richness at both the individual and population level in Oregon suggests that individuals in this population may be the most susceptible to novel diseases or pathogens. These findings suggest that the Oregon population could be considered of special conservation concern.

Both the Aleutian Islands and southeast Alaska harbored a large number of private alleles and peptides; population declines in these areas could result in the loss of MHC variability that is important to the evolutionary potential of the species. Moreover, private alleles and peptides typically occurred at low frequencies and will be the first to be lost with further population declines in these regions. In contrast to microsatellites, we did not detect differentiation between central California and other populations based on MHC exon 2 alleles or peptides. Nevertheless, previous studies of neutral genetic variation indicate that permanent immigration into this population is very low and that the population may not be re-colonized if it becomes extirpated (Peery *et al.*, 2010). As such, and similar to Friesen *et al.* (2005), we recommend that central California be considered a separate conservation unit despite the lack of MHC differentiation.

While exon 2 of MHC class II B genes appears to be a reasonable proxy for pathogen and disease adaptation when identifying conservation units in murrelets, these genes are just a few of several involved in the immune system responses to pathogenic environment responses (Acevedo-Whitehouse & Cunningham, 2006). Indeed, patterns of population structure may differ considerably for other genes involved in the immune response such as toll-like receptors (Roach *et al.*, 2005) or other ecologically important functions such as physical tolerance. In addition, patterns might change if signatures of selection were averaged across a range of markers at the genomic level (Peterson *et al.*, 2012). As genes under selection become more accessible in non-model species because of the development of next-generation sequencing methods, delineations of conservation units based on both adaptive and neutral genetic variation will become increasingly feasible.

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Data accessibility

DNA sequences: GenBank accession numbers: KC533147 to KC533173.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Multiple alignment of MHC Class II B exon 2 sequences. Nucleotides are shown at sites that differ from the corresponding site in the first sequence. Sites identical to the corresponding site in the first sequence are shown as dots.

Table S1. Sampling locations and genotypes for exon 2 of the MHC Class II B gene in marbled murrelets. Location and year of sampling for marbled murrelets screened for variation at exon 2 of MHC Class II B genes. Genotypes are based on the identity of the one to four sequences detected per individual.