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RESEARCH ARTICLE

Genetics of divergence in the Northern Saw-whet Owl (*Aegolius acadicus*)

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ABSTRACT

Differentiation, often leading to speciation, is common among island populations of birds. However, migratory species tend to differentiate less because increased movements often preclude the extended periods of allopatry thought to be required for speciation. This interaction of isolation and migration–gene flow is a complex issue in evolutionary biology. We examined the genetics of divergence in Northern Saw-whet Owls (*Aegolius acadicus*), in which a migratory form (*A. a. acadicus*) occurs during fall migration and winter, but not at other times of the year, in the range of a sedentary, island form (*A. a. brooksi*) on Haida Gwaii, British Columbia. We used 2,018 base pairs of mtDNA and genomic data from 405 amplified fragment-length polymorphisms to assess the population genetics and evolutionary history of these two subspecies. Both mtDNA and nuclear genomic markers showed significant divergence between these subspecies, and mtDNA gene flow between them was very low (<1 individual generation^{-1}). These subspecies likely diverged in association with the Wisconsin glacial maximum, ~16,000 yr BP. The refugial history of the region, life-history traits, and the maintenance of lineage integrity despite current contact suggest that this divergence occurred or is maintained through heteropatric differentiation, probably driven by a loss of migratory behavior in *brooksi* accompanied by local adaptations.

Keywords: AFLPs, migration, mtDNA, population genetics, speciation

Genética de divergencia en *Aegolius acadicus*

RESUMEN

La diferenciación, que frecuentemente resulta en especiación, es común entre poblaciones isleñas de aves. Sin embargo, las species migratorias tienden a diferenciarse menos porque sus movimientos excluyen periodos prolongados de alopatría, los cuales se consideran necesarios para la especiación. Esta interacción entre aislamiento y migración/flujo genético es un tema complejo en la biología evolutiva. Examinamos la genética de divergencia en *Aegolius acadicus*, en el cual ocurre una forma migratoria (*A. a. acadicus*) durante el otoño e invierno, pero no durante otras épocas del año, dentro del rango de distribución de una forma sedentaria isleña (*A. a. brooksi*) en Haida Gwaii, British Columbia. Usamos 2018 pb de ADN mitocondrial (ADNmt) y datos genómicos de 405 polimorfismos de longitud de fragmentos amplificados (AFLPs) para evaluar la genética poblacional y la historia evolutiva de estas dos subespecies. Tanto el ADNmt como los marcadores genómicos nucleares mostraron divergencia significativa entre las subspecies, y el flujo de ADNmt entre ellas fue muy bajo (<1 individuo por generación). Estas subespecies probablemente divergieron con el máximo glacial de Wisconsin, hace ~16,000 años. La historia refugial de la región, características de historia de vida, y el mantenimiento de integridad de linaje pese a contacto actual sugieren que esta divergencia occurió y es mantenida por diferenciación heteropátrica, probablemente causada por una pérdida de comportamiento migratorio en brooksi acompañado por adaptaciones locales.

Palabras claves: ADNmt, AFLPs, especiación, genetica de poblaciones, migración

INTRODUCTION

The repeated cycles of glaciation at high latitudes during the Quaternary are thought to have caused diversification in many taxa (Hewitt 1996, 2000). The genetic effects of this history have been studied in diverse life forms, using several molecular markers (e.g., Taberlet et al. 1998, Shafer et al. 2010). Many of these studies have found evidence of isolation during Pleistocene glacial cycles, and allopatric diversification is often thought to be the main route of divergence and speciation in birds (Mayr 1963, Coyne and Orr 2004, Price 2008). Recently, some speciation research has focused on the importance of attributes other than geography in the process of differentiation (Gavrilets 2003, Butlin et al. 2008, Crispo 2008, Li et al. 2010). Behavioral, ecological, and environmental differences between populations are increasingly seen as important drivers of divergence (Schluter 2001, McKinnon et al. 2004, Ruegg

et al. 2012, Verzijden et al. 2012), and all of them can operate within each category of the geographic models of speciation (allopatric, parapatric, and sympatric; Gavrilets 2003). A recent refinement of geographic context is heteropatric speciation (Winker 2010), a type of parapatric speciation (Gavrilets 2003) in which diverging migratory organisms occur in allopatry and sympatry cyclically through an annual cycle yet undergo divergence through a type of ecological speciation, often with some gene flow. This type of speciation requires relatively strong divergent selection to cause and maintain lineage divergence in the presence of gene flow (Nosil et al. 2009). Populations that occur in heteropatry often have an opportunity for gene flow (e.g., stop and mate), yet many diverge nonetheless; we can thus infer that divergent selection is operating, probably through ecological adaptations for exploitation of resources that are available cyclically and that are heterogeneously distributed in space and time (Winker 2010, Winker et al. 2013). Accurately assessing the main drivers of historical (and contemporary) divergence is a very complex task (Coyne and Orr 2004), stemming both from an inability to know that strict allopatry did not occur at some critical historical point (Mayr 1982) and from difficulties in reconstructing temporal aspects of gene flow (e.g., Becquet and Przeworski 2009, Strasburg and Rieseberg 2011). The most productive avenues of research will use a holistic approach, incorporating all available evidence to shed light on the mechanisms that promote and maintain divergence. Here, we use such an approach to better understand divergence between a pair of lineages with a heteropatric distribution.

Throughout most of their range, Northern Saw-whet Owls (*Aegolius acadicus*) do not vary significantly in size or coloration (Rasmussen et al. 2008). *Aegolius acadicus brooksi*, the population on Haida Gwaii (Queen Charlotte Islands), British Columbia, Canada, is an example of a resident endemic subspecies that has undergone differentiation from its mainland counterpart (Fleming 1916; Figure 1). The other subspecies, *A. a. acadicus*, occupies the mainland range. Like other taxa that are distributed heteropatrically, these two subspecies do not exhibit multigenerational spatial isolation, which is a key component of the allopatric speciation model. The nominate subspecies (*A. a. acadicus*) occurs on Haida Gwaii in small numbers during fall migration and winter (e.g., 7 of 115+ specimens from Haida Gwaii, spanning dates October 12 to January 11; Sealy 1998, University of Alaska Museum specimens), but these birds are not known to breed there. The breeding seasons of Haida Gwaii and mainland owls do not appear to differ significantly (Campbell et al. 1990, Cannings 1993, COSEWIC 2006). Egg dates are known from late March to early June in British Columbia, and the scant data from southeastern Alaska, USA, suggest similar timing (Campbell et al. 1990, Heinl and Piston 2009). The resident subspecies *brooksi* is darker in color, exhibits unique feeding habits, and is nonmigratory (Hobson and Sealy 1991, Sealy 1998, 1999); initial data using limited sampling showed genetic differentiation in mtDNA from mainland *acadicus* (Topp and Winker 2008).

The genetic and morphological differentiation seen between these subspecies of *A. acadicus* are likely related to glacial cycles during the Pleistocene, as seen in many avian and other taxa (e.g., Avise and Walker 1998, Johnson and Cicero 2004, Weir and Schluter 2004, Shafer et al. 2010). The Haida Gwaii area, in particular, is thought to have been a refugium during the last glacial maximum (Warner et al. 1982, Heusser 1989, Pielou 1991). Endemic species and subspecies are described from Haida Gwaii for many classes of organisms, including plants (Ogilvie 1989), insects (Kavanaugh 1989, Clarke et al. 2001), fish (Moodie and Reimchen 1976, O'Reilly et al. 1993), and mammals (Osgood 1901, Fleming and Cook 2002). Birds, in particular, have been shown to exhibit genetic differentiation consistent with a refugial history there (e.g., Burg et al. 2005, 2006, Pruett and Winker 2005, Topp and Winker 2008, Pruett et al. 2013), although some differentiation may have occurred after glaciers receded (e.g., Bull et al. 2010).

Currently, Hecate Strait separates Haida Gwaii from the nearest mainland by ~70 km. Historical isolation distances between these islands and the mainland are unknown, but the migratory capacity of many bird species and the glacial history of northwestern North America suggest that a Haida Gwaii refugium was probably no more removed from other habitable areas than is seen today. We cannot definitively reconstruct the historical biogeography between these two lineages (i.e. were they at one time entirely allopatric and now exhibit secondary contact, or have they existed in heteropatry since they began to split?). However, we can study them as a contemporarily heteropatric pair of lineages and ask a series of questions to determine the evolutionary history of *brooksi* in relation to nominate *acadicus*. (1) These taxa (subspecies) are morphologically distinct, but is there significant genetic differentiation? (2) How does their divergence date correspond with the past climatic history of the area? (3) Is there evidence of gene flow and, if so, at what level? (4) Is there evidence of selection among sampled loci? And (5) although we cannot explicitly test models of speciation in this system, we synthesize the available evidence and consider the possible modes of divergence that likely drove this case of differentiation.

METHODS

Tissue samples from 51 individuals were obtained from the collections of the University of Alaska Museum and the University of Washington Burke Museum. Study-skin

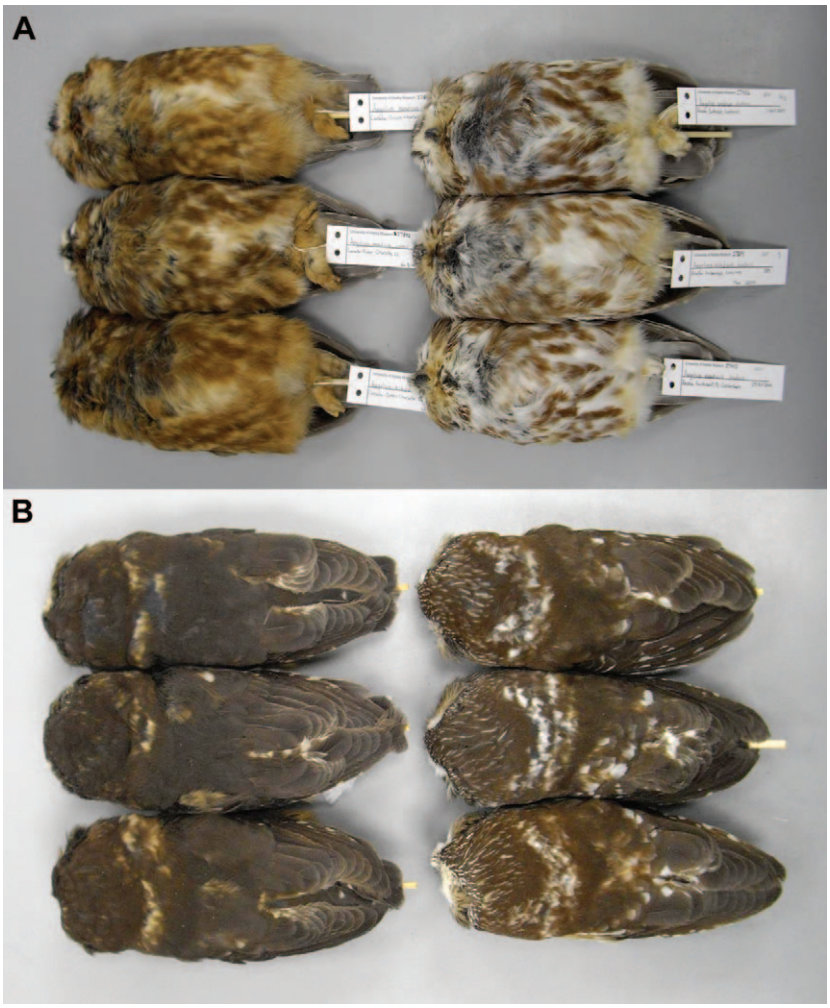

FIGURE 1. Ventral (**A**) and dorsal (**B**) views of *Aegolius acadicus brooksi* (left) and *A. a. acadicus* (right). Photo credit: J. J. Withrow

vouchers are at these institutions and at the Queen Charlotte Islands Museum and University of Manitoba Zoology Museum (Table 1).

Mitochondrial Sequence Data and Sampling

We amplified 1,047 base pairs (bp) of the mitochondrial NADH dehydrogenase subunit 2 (ND2) gene for *acadicus* ($n = 19$ individuals) and *brooksi* ($n = 24$); one additional *acadicus* sequence was obtained from GenBank (EU601051; Table 1). We amplified 971 bp of the cytochrome *b* (cyt *b*) gene for 1 individual *acadicus* and 14 *brooksi* and supplemented these with 20 *acadicus* and 10 *brooksi* from Topp and Winker (2008; Table 1). Most *acadicus* were from Alaska, but we also included individuals from New Mexico, Oregon, and Washington, USA, to encompass broader geographic variation (sample sizes varied by marker; see Figure 2 and Table 1). ND2 is a well-known marker in birds and has been shown to be particularly informative and approximately neutrally evolving (Zink et al. 2005); similarly, cyt *b* is a well-studied gene with a fairly constant rate of evolution (Moore and DeFilippis 1997, Avise 2000), allowing population parameters to be estimated with reasonable confidence (Lovette 2004).

DNA was extracted from frozen tissues using a DNeasy Tissue Kit following the manufacturer's protocol (Qiagen, Valencia, California, USA). Initial amplifications were performed using cyt *b* primers L0-25 and H1117 (Topp and Winker 2008) and ND2 primers H6313 and L5219 (Sorenson et al. 1999). Polymerase chain reaction (PCR) amplification was conducted using 2.5 μL of each primer at 10 μM, 3 μL of a 10-μM solution of dNTPs, 0.2 μL (1 unit) of *Taq* DNA polymerase, 6μL of 25 mM MgCl2, 5 μL of 5× *Taq* Buffer (Promega, Madison, Wisconsin, USA), 2.5 μL of 1 mg mL^{-1} BSA, and 23.3 μL water, for a total reaction volume of 50 μL. The PCR thermal regime started with 2 min at 94°C, followed by 29 cycles of 94°C for 1 min, 48°C

TABLE 1. Subspecies, voucher numbers, and GenBank accession numbers for cytochrome *b*, ND2, and AFLP (amplified fragment-length polymorphism) data from *Aegolius acadicus acadicus* and *A. a. brooksi*. All voucher numbers are University of Alaska Museum (UAM) unless otherwise noted. University of Washington Burke Museum is abbreviated UWBM. Study skin vouchers for several birds are at Queen Charlotte Islands Museum (QCIM) and University of Manitoba Zoology Museum (UMZM), and numbers for these are given in parentheses after the UAM number; EU601051 was obtained from GenBank for this study.

	GenBank accession numbers	
Museum voucher numbers	Cytochrome *b*	ND2
A. a. acadicus		
5488§, 5851§, 6500, 6501, 6901, 6904, 8990, 8989, 9180, 9181*, 9277‡, 13949, 13995, 13996, 14940, 15184‡, 17882, 17883, 17953‡, 17954‡, 17955‡, 17957, UWBM68205, UWBM79081, UWBM67190, UWBM67021*, MVZ181707†.	EU075383–EU075387, EU075398–EU075412, KC620183	KC620150–KC620168 EU601051
A. a. brooksi		
10153, 10154, 19042 (QCIM A12), 19472, 19473‡, 19474 (QCIM 94–199), 19479 (QCIM B110), 19481 (UMZM 2872), 19482 (UMZM 2869), 19483, 19484 (UMZM 2870)*, 19485 (UMZM 2874), 26388–26390, 27886–2789, 27891–27896	EU075388–EU075397, KC620169–KC620182	KC620126–KC620149

§ Individuals for which only cytochrome *b* data were used.
* Individuals for which no AFLP data were generated.
‡ Individuals for which only AFLP data were generated.
† Individuals for which only ND2 data were used.

for 2 min, 72°C for 2 min, and with a final elongation step at 72°C for 5 min.

The PCR cleanup and sequencing were done at the High-Throughput Genomics Unit (University of Washington, Seattle, Washington, USA), using an ExoSAP cleaning process, cycle-sequenced using BigDye chemistry on an ABI 3730KL high-throughput capillary sequencer (Applied Biosystems, Foster City, California, USA). Cycle-sequencing amplifications were done using the initial sequencing primers and internal primers for some individuals. These internal primers were as follows: cyt *b* internal forward primer (5′-TTCTCAGCCGTACCATACATTGGC-3′), cyt *b* internal reverse primer (5′-ATCACAGCTGGATGGGATTCCT-3′), ND2 internal forward primer (5′-TCTTGCCTCCTCCTAACAACAGCA-3′), and ND2 internal reverse primer (5′-TGTTGATAGGATGGCCATGGAGGT-3′). Sequences were aligned and edited using Sequencher version 4.7 (Gene Codes, Ann Arbor, Michigan, USA).

Amplified Fragment-Length Polymorphism Data and Sampling

Amplified fragment-length polymorphisms (AFLPs) were generated using a protocol modified from Vos et al. (1995). These were generated for 24 *brooksi* and 22 *acadicus* from throughout the species' range (Figure 2 and Table 1). Initial sample DNA concentration was quantified on a spectrophotometer; all samples had concentrations of 30–70 ng μL^{-1}. The restriction digestion and ligation of adapter pairs were performed in one step at 37°C for ~12 hr. This reaction mixture was subsequently diluted with 94.5 μL of 0.1M TE buffer and frozen until preselective amplification. Preselective amplification was done using standard protocols with an annealing temperature of 56°C (Withrow 2013). After preselective amplification, reactions were diluted with 80 μL of 0.1× TE buffer and frozen until selective amplification. Selective amplifications used the same PCR regime as before, but nine primer–pair combinations of dye-labeled *MseI* and *EcoRI* primers (Table 2) were used to selectively amplify a subset of DNA. Thermal-cycler regime consisted of 2 min at 94°C followed by 11 cycles of 94°C for 20 s, 66°C for 30 s, and 72°C for 2 min. During each cycle the annealing temperature was dropped by 1°C; at 56°C, 19 additional cycles were run at that annealing temperature followed by 30 min at 60°C. Samples were run on an ABI 3100 automated sequencer (Applied Biosystems).

GeneMapper version 3.7 (Applied Biosystems) was used to score the chromatograms. Only unambiguous loci with clean, well-defined peaks were scored. We used a minimum peak width of 1.5 bp and a minimum peak height of 75 as a starting point, but then examined each peak individually to maximize the useful phylogenetic signal (Holland et al. 2008). In a similar study, a genotyping error rate of <1% was estimated (Withrow 2013), a rate generally considered not to result in inaccurate inferences of phylogeographic pattern (Zhang and Hare 2012). We discarded two *brooksi* samples that did not amplify larger fragments, likely because of tissue degradation related to the salvaged nature of these specimens. Data were transformed into a binary state (presence–absence) matrix using a Microsoft Excel macro, which also transformed the matrix into nexus format (Rinehart 2004). All bands were considered independent, homologous loci.

Genetic Differentiation and Population Structure

A median joining network illustrating haplotype frequencies of mtDNA was generated using Network version

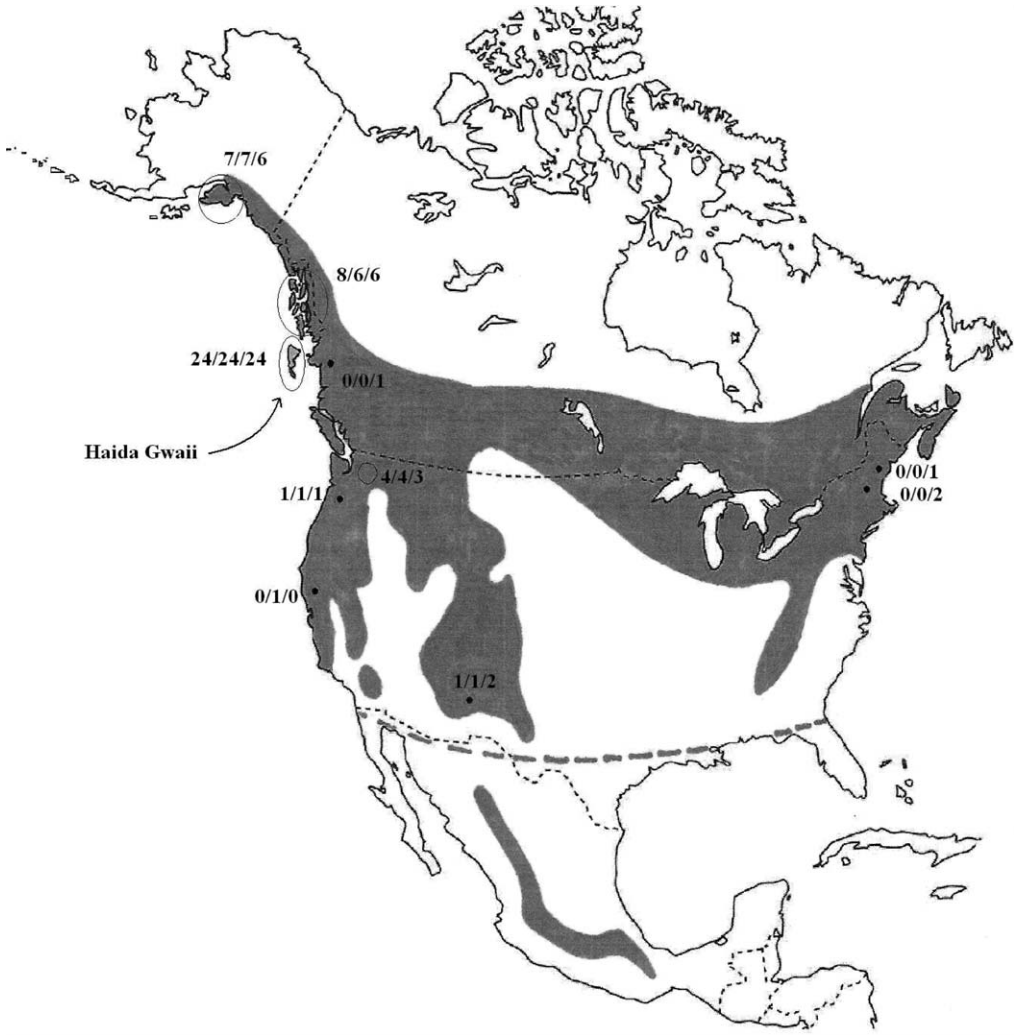

FIGURE 2. Breeding range (gray) and sampling locations of *Aegolius acadicus acadicus* and *A. a. brooksi*. Numbers indicate sample sizes for a given sampling location for three markers: cytochrome *b*, NADH dehydrogenase subunit 2, and amplified fragment-length polymorphisms, respectively. Dashed line marks the southern extent of wintering *A. a. acadicus*; the Mexican population south of this line is nonmigratory.

TABLE 2. Amplification and scoring results, for each primer pair (*EcoR1* and *MseI*) and dye (and totals), from amplified fragment-length polymorphisms generated for *Aegolius acadicus acadicus* and *A. a. brooksi*: total peaks (T), number of polymorphic peaks (P), and percentage of peaks that were polymorphic (%P).

			Both subspecies			Within *acadicus*			Within *brooksi*		
EcoR1	*MseI*	Dye	T	P	%P	T	P	%P	T	P	%P
–ACT	–CAG	FAM	34	13	38.2	34	8	23.5	34	10	29.4
–ACT	–CAT	FAM	22	4	18.2	22	2	9.1	22	1	4.5
–ACT	–CTA	FAM	55	11	20.0	55	9	16.4	53	8	15.1
–ACA	–CAA	FAM	62	18	29.0	60	15	25.0	59	11	18.6
–ACA	–CAC	FAM	51	16	31.4	50	10	20.0	51	13	25.3
–ACA	–CAG	FAM	67	11	16.4	63	6	9.5	66	8	12.1
–ACA	–CAT	FAM	27	5	18.5	27	2	7.4	26	4	15.4
–ACA	–CTA	FAM	51	18	35.3	51	14	27.5	51	12	23.5
–AAG	–CAT	JOE	36	6	16.7	36	5	13.9	36	5	13.9
Totals			403	102	25.2	398	71	17.8	398	72	18.1

4.6.0.0 (Bandelt et al. 1999). We used Arlequin (Excoffier et al. 1992) to calculate pairwise Φ_{ST} values between subspecies for cyt *b* and ND2 sequences separately and for a combined mtDNA dataset. Genotypes were permuted 1,000× to obtain *P* values to determine whether Φ_{ST} values were significant. We calculated F_{ST} and *P* values following Lynch and Milligan (1994) for the AFLP data, using AFLP-SURV version 1.0 (Vekemans et al. 2002); we assumed Hardy-Weinberg equilibrium and used the Bayesian method with non-uniform priors, 10,000 random permutations, and 1,000 bootstraps for genetic distances.

We also analyzed AFLP data in STRUCTURE version 2.2 (Pritchard et al. 2000, Falush et al. 2007) to determine the most likely number of populations and to identify any admixed individuals. STRUCTURE uses MCMC simulations in a Bayesian framework to assign individuals to populations and determine the most likely number of populations (Pritchard et al. 2000). The program's model-based clustering method effectively deals with the genotypic ambiguity present in dominant markers such as AFLPs (Falush et al. 2007). Preliminary runs indicated that a burn-in of 20,000 iterations was sufficient. We then ran three independent simulations, using the admixture model, for 100,000 iterations for values of (*K*) from 1 to 5. The likelihood of *K* given the data was calculated as P(*K*|*X*). To avoid biasing the inference of population structure, we did not use subspecies origin information, although all individuals were phenotypically identifiable. We used the program Distruct (Rosenberg et al. 2002) to transform and apply information from a representative run of the most likely number of *K* into a postscript file that was viewed and manipulated in Adobe Illustrator. Although Pritchard et al. (2000) demonstrated STRUCTURE's ability to correctly identify *K* in such datasets, we also evaluated the log-likelihood values for Δ*K* (Evanno et al. 2005) using STRUCTURE HARVESTER version 6.93 (Earl and vonHoldt 2012).

To independently assess STRUCTURE results, we also performed an admixture analysis in BAPS version 5.3 (Corander and Marttinen 2006, Corander et al. 2008), using 1,000 simulations for each value of *K* (1–5). Like STRUCTURE, BAPS assesses population structure and individual admixture using Bayesian clustering algorithms.

Divergence Time, Effective Population Size, and Gene Flow

The program IM (Nielsen and Wakeley 2001) was used on the concatenated mtDNA data to estimate seven parameters, most scaled to the neutral mutation rate, μ: *t* (*T*/μ, where *T* is time since divergence in years before present [yr BP]), θ_2 ($4N_e\mu$ for *A. a. acadicus*), θ_1 ($4N_e\mu$ for *A. a. brooksi*), θ_a ($4N_e\mu$ for the ancestral population at time of divergence), m_1 ($2M/\theta_1$, where *M* is the effective number of migrants moving into Haida Gwaii per generation), m_2 ($2M/\theta_2$, migration rate from Haida Gwaii to the rest of North America), and *s* (a splitting parameter, the proportion of nominate *acadicus* that founded the *brooksi* population; 1 – *s* is the proportion of *brooksi* contributing to *acadicus*; Hey 2005). We ran three independent runs using the initial parameter starting maxima of $\theta = 100$, $m = 3$, and $t = 3$, with a burn-in of 500,000 steps, parameters that worked well in preliminary runs, and a different random number seed for each run. We calculated generation time (*G*) using the equation $G = \alpha + [s/(1 + s)]$, where α is the age of maturity and *s* is the expected adult survival rate (Sæther et al. 2005). Most *A. acadicus* probably begin breeding when 1 yr old ($\alpha = 1$; Rasmussen et al. 2008), and we assumed a survivorship percentage of 0.66% based on Rasmussen et al. (2008) and Marks and Doremus (2000), giving an estimated generation time of 3 yr. Because the timing of divergence was unknown, the HKY model of molecular evolution (Hasegawa et al. 1985) was used in all runs to account for multiple nucleotide substitutions at the same position, nucleotide frequency differences, and any transition–transversion bias as recommended in IM documentation. ModelTest (Posada and Crandall 1998) did not rule out the HKY model as appropriate in this dataset. Each run proceeded for >10 million updates to achieve a minimum effective sample size of 100 for any given parameter estimate; most were several orders of magnitude higher than this. Results from the three runs were similar, and we report here only the parameters estimated in the longest run of 30,002,974 updates after burn-in.

The parameters estimated by IM are dependent on the mutation rate, which is an uncertain quantity (Lovette 2004, Ho et al. 2005, 2011). A mutation rate of 2% divergence Ma^{-1} (0.00002018/yr/2,018 bp) was used (following Weir and Schluter 2008). We also set a range of mutation rates at 1–4% divergence Ma^{-1} (0.00001–0.00004/yr/2,018 bp) to set priors on the mutation rate. This range was also used to calculate a range of demographic parameter values because mutation rates are uncertain (Weir and Schluter 2008, Ho et al. 2011, Pacheco et al. 2011).

Genetic Diversity and Selection

We used DNAsp (Rozas et al. 2003) on mtDNA data to calculate haplotype diversity (H_d; Nei and Chesser 1983) and nucleotide diversity (π). We conducted chi-square tests of genetic differentiation between subspecies based on haplotype frequencies and nucleotide diversity indices. The chi-square tests with Yates continuity corrections were conducted in PopTools version 2.6.9 (Hood 2005), an add-in for Microsoft Excel.

Because heteropatric speciation requires divergent selection to occur, we sought evidence for selection in the AFLP data (though given the size of the genome in relation to our dataset, an absence of evidence for selection would not be surprising). To determine whether the genes sampled

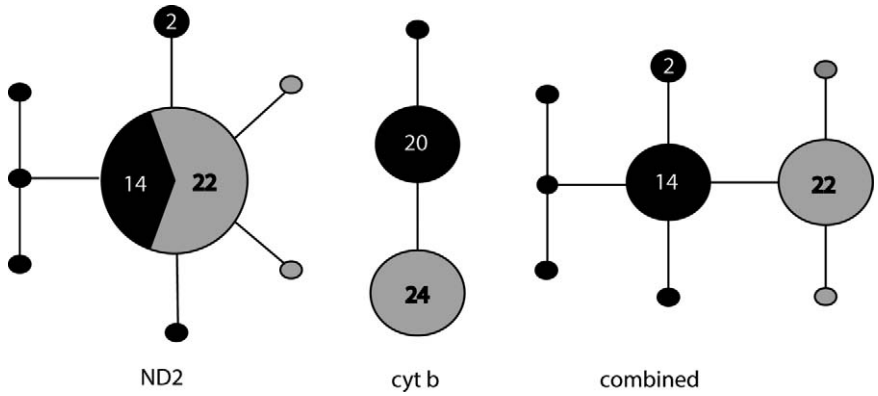

FIGURE 3. Haplotype networks showing ND2 sequences, cytochrome *b* (cyt b) sequences, and concatenated sequences (combined) from 24 *Aegolius acadicus brooksi* (gray) and 21 *A. a. acadicus* (black). Numbers of sampled individuals are given; small circles represent one individual.

through AFLPs diverged via genetic drift or selection, we compared F_{ST} and heterozygosity estimates for each locus in our dataset with a simulated dataset acting under drift alone, using an infinite-alleles model. To produce these simulated data, we used the program fdist2 (Beaumont and Nichols 1996), which uses an average divergence of F_{ST} and expected heterozygosity (H_S) to simulate the expected distribution of differentiation across loci (Campbell and Bernatchez 2004, Bonin et al. 2006). It uses an F_{ST} calculated by the method of Nei (1977) as modified by Nei and Chesser (1983) and generates a uniform distribution of heterozygosities in place of a specified mutation rate. This distribution was then used to calculate quantiles of the median and upper and lower 99% confidence intervals of the distribution of loci for the population diverging under drift alone. The program also calculates F_{ST} and H_S for all polymorphic loci in the dataset, which were then plotted against the confidence intervals. Loci falling outside this distribution in excess of expected false positives are assumed to be under selection or closely linked to loci under selection (Beaumont and Nichols 1996, Nosil et al. 2009, Flaxman et al. 2012). The data were analyzed to obtain an estimate of the average F_{ST} across all loci. The model was then fit to this F_{ST} for simulation. We ran the simulation for 20,000 realizations, with two demes total, sampling the two subspecies of *A. acadicus* with an expected $F_{ST} = 0.11$ and an average sample size per subspecies of 22 individuals.

RESULTS

Genetic Differentiation and Population Structure

Haplotype networks of both ND2 and cyt *b* showed some structuring between subspecies. Interestingly, with only three haplotypes, cyt *b* still exhibited a fixed difference between the subspecies, with two haplotypes occurring in the larger *acadicus* (Figure 3). ND2 was less structured, with most individuals sharing a single common haplotype. Of the eight ND2 haplotypes, three occurred in *brooksi* and six in *acadicus* (Figure 3).

Population structuring in mtDNA was apparent in both genes separately and together, with Φ_{ST} for cyt $b = 0.96$ ($P < 0.001$), Φ_{ST} for ND2 $= 0.67$ ($P < 0.001$), and a combined $\Phi_{ST} = 0.67$ ($P < 0.001$). These are consistent with, and reinforce, the significant structure seen visually in the haplotype networks (Figure 3). The AFLP data also showed significant structure between the subspecies, with $F_{ST} = 0.074$ ($P < 0.0001$).

The STRUCTURE analysis estimated that the most likely number of populations involved was two ($\ln P(X|K) = -1{,}438.4$; $P(K|X) = \sim 1$; Table 3). Most individuals (35 of 45; 78%) had estimated membership coefficients (Q) suggesting >90% posterior probability that their genomic alleles originated from their putative (phenotypic) population of origin (Figure 4). Three (of 24; 12.5%) Haida Gwaii birds had >50% of their membership coefficients estimated to originate from *acadicus*, and two (of 22; 9.0%) mainland birds had more than half of their membership coefficients estimated to come from *brooksi*. One of these birds was from the Kenai Peninsula, Alaska, and the other from mainland British Columbia. Six (25%) Haida Gwaii birds had >10% of membership coefficients estimated to come from *acadicus*, whereas four (18%) mainland birds had similar estimates of membership coefficients matching *brooksi*.

The BAPS analysis also estimated the most likely number of populations to be two ($\ln \Pr(K|X) = -1{,}631.1$; Table 3), and identified the same individuals as being genomically admixed in the same proportions as STRUCTURE (Table 3; individual data not shown).

Divergence Time, Effective Population Size, and Gene Flow

Each of three replicate but independent coalescent analyses in IM produced similar results. We report here only a representative 30-million-step run. Coalescent

TABLE 3. Estimates from the programs STRUCTURE (Pritchard et al. 2000) and BAPS (Corander and Marttinen 2006) of the number of clusters or likely populations involved (*K*) in our analysis. We used STRUCTURE and BAPS without using prior population information. Δ*K* (Evanno et al. 2005) values calculated in STRUCTURE HARVESTER (Earl and vonHoldt 2012) are also shown.

	STRUCTURE			BAPS	
K	mean Ln Pr(*X*|*K*)	Pr(*K*|*X*)	Δ*K*	Ln Pr(*X*|*K*)	Pr(*K*|*X*)
1	−1,534.7	∼0	–	–	∼0
2	−1,439.0	∼1	110.2	−1,631.1	∼1
3	−1,463.9	∼0	7.5	−1,662.4	∼0
4	−1,610.8	∼0	0.9	−1,694.6	∼0
5	−1,823.0	∼0	–	−1,739.2	∼0

analysis in IM estimated the high point (and 95% HPD) of the probability distribution for the following model parameters (Table 4): effective female population size of *brooksi* (θ_1) = 0.35 (0.65–96.15), effective female population size of *acadicus* (θ_2) = 60.75 (8.45–97.75), effective ancestral female population size (θ_a) = 0.05 (0.05–38.85), migrants from *acadicus* into *brooksi* (m_1) = 0.0015 (0.017–2.520), migrants from *brooksi* into *acadicus* (m_2) = 0.0045 (0.017–2.630), divergence time (t) = 0.33 (0.14–3.29), and the proportion of the ancestral population that gave rise to *brooksi* (s) = 0.0005 (0.0025–0.9700). We converted these values into demographic units using a divergence rate of 2% Ma^{-1} (Table 4). The posterior distribution of t did not include zero and, using this mutation rate and a generation time of 3 yr, the analysis estimated a divergence date of ∼16,228 yr BP (Table 4). At a 4% divergence rate, t = 8,187 yr BP, and at 1% t = 32,750 yr BP. The effective population size of *acadicus* (θ_2; 250,867 females) did not converge well, likely because of its extremely large size in relation to *brooksi* and inadequate signal in the dataset for an accurate estimate. The effective population size of *brooksi* (θ_1) was estimated as 1,445 females (Table 4); θ_1 converted to 7,243 individuals at a 4% divergence rate, and to 2,891 individuals at 1%. Estimates of mtDNA migration rates in both directions peaked at zero (these are not affected by mutation rate; Table 4), substantially lower than would be predicted by random mating in a population on Haida Gwaii comprising ∼6.5% nominate *acadicus* (given that 7 of ∼115 modern specimens from the island are by plumage *acadicus*; Sealy 1998, University of Alaska Museum specimens). The proportion of the ancestral population founding *brooksi* was estimated to be very small (s = 0.05%).

Genetic Diversity and Selection

Nucleotide diversity was lower in *brooksi* than in *acadicus* (π = 0.00008 and 0.00039, respectively; χ^2 = 2342.8, df = 1, $P < 0.001$), but haplotype diversity was not significantly different between the two taxa (*brooksi*, H_d = 0.163; *acadicus*, H_d = 0.538; χ^2 = 2.1, df = 9, P = 0.144).

The nine primer pairs produced 405 AFLP loci. Of these, 102 (25.2%) were polymorphic (Table 2) when both subspecies were included. There were 19 AFLP loci that were fixed in *brooksi* but polymorphic in *acadicus*, and there were 23 loci fixed in *acadicus* but polymorphic in *brooksi*; these differences were not significant (χ^2 = 0.0477, df = 2, P = 0.977). There were six AFLP loci present in *brooksi* that were absent in *acadicus*, and seven loci present in *acadicus* that were absent in *brooksi*, and these differences also were not significant (χ^2 = 0.844, df = 2, P = 0.981).

One of 405 loci (0.25%) fell outside the simulated dataset's 99% quantile (data not shown). This result includes fewer loci than would be expected by chance (expected n = 4; 1%), which suggests that these two populations are probably not experiencing strong divergent selection at the loci examined.

DISCUSSION

These owl subspecies exhibit significant genetic differentiation and low gene flow despite their occurrence today in heteropatry. This reinforces the validity of the phenotyp-

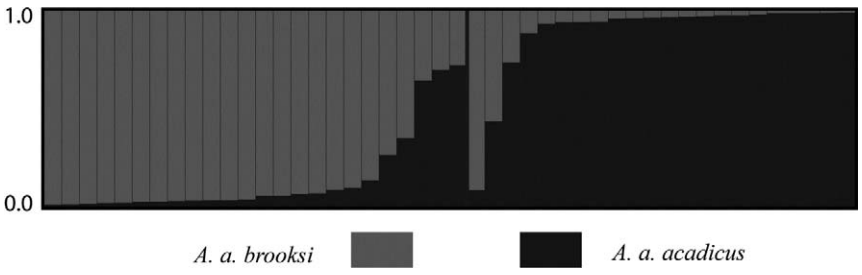

FIGURE 4. Genotypic makeup of 22 *Aegolius acadicus acadicus* and 24 *A. a. brooksi* inferred using the program STRUCTURE (Pritchard et al. 2000). Each bar represents a phenotypically identified individual. Haida Gwaii birds are on the left (gray), mainland birds on the right (black), separated by dark vertical line. The three most admixed individuals in the mainland sample are, from left to right: Homer, Alaska; Bulkley River, British Columbia; and Anchorage, Alaska.

TABLE 4. Demographic parameter estimates for *Aegolius acadicus acadicus* and *A. a. brooksi* from a representative 30-million-step run in the program IM (Nielsen and Wakeley 2001).

Demographic description	Model parameter	Demographic value at 2% divergence	95% lowest and highest densities
Aegolius acadicus brooksi effective population size	θ_1	1,445	34,894 and 403,658
A. a. acadicus effective population size	θ_2	250,867	2,684 and 397,051
Ancestral effective population size	θ_a	206	206 and 160,431
Migrants from *acadicus* into *brooksi*	m_1	0.00026	0.0165 and 2.51
Migrants from *brooksi* into *acadicus*	m_2	0.136	0.0165 and 2.63
Time since divergence	t	16,228	6,813 and 163,156

The estimate of effective population size is in units of individuals. The estimate of time since divergence is in years. Migrants are individuals per generation. For estimates of other parameters and estimates using other mutation rates, see text.

ically well-defined *brooksi* subspecies (Figure 1). Our analyses of mtDNA data suggest that there is effectively no gene flow between populations (Table 4). By contrast, Bayesian clustering analyses of AFLP data suggested nuclear allele sharing between populations in some individuals (Figure 4). The nature of AFLP data precludes us from determining whether these alleles are a result of incomplete lineage sorting or ongoing gene flow. (No test for gene flow in AFLP data exists.) Most birds, including owls, have female-biased dispersal (König et al. 2009), and *A. acadicus*, in particular, appears to fit this pattern (Beckett and Proudfoot 2012). Nominate *acadicus* may also be nomadic to some extent (Marks and Doremus 2000, Bowman et al. 2010). We therefore expect mtDNA to be a leading indicator of gene flow in this species. Thus, the very low estimates of gene flow seen in maternally inherited mtDNA (effectively zero), coupled with the shallow level of divergence observed, suggest that the shared nuclear alleles are due to incomplete lineage sorting rather than gene flow. This is a pattern similar to that seen between Snow and McKay's buntings (*Plectrophenax nivalis* and *P. hyperboreus*), another case of island differentiation, in which mtDNA suggested that no gene flow was occurring, but in which AFLP markers showed a sharing of some nuclear alleles (Maley and Winker 2010).

The IM simulations estimated a divergence date of ~16,000 yr BP (though confidence intervals are broad; Table 4), which was about the time of the oldest known evidence for a terrestrial environment on Haida Gwaii (Warner et al. 1982), at or just after the maximum extent of late Wisconsin glacial extent in the area (Clague and James 2002, Clark et al. 2009). However, other genetic data suggest a deeper forested refugial history (Pruett et al. 2013). Uncertainties about substitution rates make precise estimates of divergence dates from a single molecular marker difficult (Ho et al. 2005, 2011, Price 2008, Tinn and Oakley 2008). Nevertheless, our estimate of their divergence peaked sharply at a time corresponding reasonably well with the last glacial maximum of the Pleistocene, and mutation rates of 1–4% Ma^{-1} did not change this association, which makes *brooksi* a relatively recently derived taxon. Whether these owls were isolated on the current Haida Gwaii archipelago as glaciers retreated after the last glacial maximum or occupied a nearby refugium is uncertain.

Evidence also exists for a small effective population size and a possible founder effect in *brooksi*. Population size estimates for *brooksi* (1,445 females) and *acadicus* (250,867 females) correspond well with the current census size estimates of 1,900 individual *brooksi* (COSEWIC 2006) and 100,000–300,000 individual *acadicus* (Rasmussen et al. 2008).

We did not find evidence of selection in our sample of nuclear loci, so our dataset suggests that drift has been important. Our sampling of the genome was small, however (405 loci, compared with ~20,000 genes in the chicken genome; Hillier et al. 2004), so selection may not be absent. In fact, given differences in plumage, migration, and diet, the existence of some divergent selection may be inferred, though whether drift may also have affected these phenotypic traits is unknown.

Biogeography and Models of Speciation

Glaciation, tectonic rebound, and fluctuating sea levels make geological inferences of historical refugia complex, but much of the continental shelf of south coastal Alaska and coastal British Columbia north and south of Haida Gwaii remained ice free during the last glacial maximum (Hetherington et al. 2003, Kaufman and Manley 2004, Carrara et al. 2007). Haida Gwaii may even have been connected to the mainland briefly, 10,000 to 11,000 yr BP (Hetherington et al. 2003). Thus, a string of refugia along northwestern North America (Shafer et al. 2010) likely existed, with Haida Gwaii only one of several ice-free areas. Therefore, we do not suggest that *brooksi* necessarily arose on Haida Gwaii, but rather that it arose in an unglaciated area in the region and likely shifted its range as sea levels rose and suitable habitats shifted.

Today, why do migrant mainland *acadicus* apparently not stop, overwinter, and mate with the *brooksi* population in a part of the *acadicus* range that is suitable, at least at the species level, for both wintering and breeding? Given this contemporary situation, our results showing genetic differentiation and low gene flow suggest that key aspects

of the heteropatric speciation model are at least maintaining the distinctiveness and low gene flow between these two lineages. The heteropatric speciation model, like most speciation models, makes two key predictions when comparing two diverged or diverging lineages: Significant genetic differentiation will be present, and gene flow will be low (Winker 2010). The allopatric speciation process predicts the former, but not necessarily the latter—secondary contact often results in considerable gene flow. Clearly determining the historical condition between these owl lineages (i.e. allopatry followed by secondary contact versus long-term heteropatry) is not possible at present (e.g., Becquet and Przeworski 2009, Strasburg and Rieseberg 2011). However, classic allopatric divergence, although possible, may not be the most parsimonious model responsible for the existence of the current differentiation. Instead, the heteropatric model may apply historically as well.

A migratory *Aegolius* ancestor could well have used this coastal refugial region just as nominate *acadicus* does today. The ability of *Aegolius* to colonize areas much more remote than a Haida Gwaii refugium is highlighted by the extinct *A. gradyi* from Bermuda, a close relative of *A. acadicus*, which likely arose by peripatric speciation (a type of allopatric speciation) after an ancestral *A. acadicus* reached that island (Olson 2012). A species with the high dispersal capability of *acadicus* likely would not have been absent from a Haida Gwaii refugium long enough for extended periods of multigenerational allopatry to occur. Current geographic and life-history characteristics thus suggest a process of heteropatric divergence. Alternatively, as an island form, *brooksi* may have arisen through allopatric isolation (or through peripatric speciation) and retains its distinctive genetic and phenotypic attributes despite secondary contact with nominate *acadicus*. However, secondary contact zones of taxa that diverged via allopatry often exhibit more pronounced hybridization than seen here (Coyne and Orr 2004), a common phenomenon among birds in the Pacific Northwest, including *Setophaga townsendi* and *S. occidentalis* (Krosby and Rohwer 2010), *Larus occidentalis* and *L. glaucescens* (Hoffman et al. 1978), *Trogolodytes pacificus* and *T. hiemalis* (Toews and Irwin 2008), *Setophaga coronata* subspecies (Milá et al. 2007), *Picoides villosus* subspecies (Klicka et al. 2011), *Catharus ustulatus* subspecies (Ruegg et al. 2012), and *C. guttatus* subspecies (Topp et al. 2013). Even if allopatry was the main driver of divergence in these owls, mechanisms of heteropatric differentiation could still have acted to maintain differences acquired largely in allopatry and may explain why we apparently do not see reticulation of these lineages despite opportunities for *acadicus* to stop and mate. However, we cannot distinguish between historical scenarios of allopatric or heteropatric divergence using only our genetic data.

We consider it likely that the Haida Gwaii refugium was accessible and accessed by ancestral continental *acadicus* while a sedentary population was established and began diverging. It is possible, however, that continental ice sheets contributed to lowering the number of continental immigrants while strong divergent selection occurred, creating sufficient isolating mechanisms that when the degree of heteropatry increased to the level observed today (there is, after all, a large continental population in the region that could have been present on Haida Gwaii during the last glacial maximum), lineage integrity was maintained. Further study of nuclear gene flow is warranted.

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