Phylogeography of the white-breasted nuthatch (*Sitta carolinensis*): diversification in North American pine and oak woodlands

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Abstract

Pine and oak woodlands are common North American floral communities with distinct regional species composition. The white-breasted nuthatch (Aves: *Sitta carolinensis*) is a common resident bird of North American pine and oak woodlands, and is distributed continentally across the highly disjunct distribution of these woodlands. We propose three historical hypotheses to explain the evolution of the white-breasted nuthatch in its principal habitat. (i) The species evolved *in situ* in the regional pine–oak communities and the isolation of populations in these regions is captured in cryptic genetic variation. (ii) Migration of individuals between regions is frequent enough to maintain the widespread distributions and prevent regional divergence. (iii) The species have recently expanded to occupy their current distributions and an insufficient amount of time has passed for divergence to occur. Phylogenetic analysis of mitochondrial DNA (ND2 gene) variation (*N* = 216) in the white-breasted nuthatch reveals four reciprocally monophyletic clades concordant with the distribution of the regional North American pine and oak woodlands, and supports hypothesis 1 of *in situ* evolution of populations in the regional pine and oak communities. Within-clade population structure and demographic history are also discussed.

Keywords: continental distribution, divergence times, gene flow, mitochondrial DNA, phylogeography, pine–oak habitats, Sitta

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Introduction

Pine and oak (pine–oak hereafter) woodlands are some of the most common floral communities in North America (Little 1971, 1976, 1977). They are distributed across the continent at midlatitudes, but do exist as far north as British Columbia and as far south as Nicaragua. They are also extremely diverse habitats with distinct regional species assemblages (Eastern North America, Rocky Mountain/ Great Basin, Californian, Sierra Nevada/Cascades, and Mexican/Central American [Madrean] assemblages;

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Remington 1968; Little 1971, 1976, 1977; Graham 1999). In fact, no pine (*Pinus*) or oak (*Quercus*) species is continentally distributed across all of these regions (Little 1971, 1976, 1977). In eastern North America, pine–oak woodland habitats are principle components in three widely distributed biomes: cold-mixed deciduous forests, temperate-mixed deciduous forests, and warm-mixed forests (Jackson *et al.* 2000; Williams *et al.* 2001; see Fig. S1, Supplementary material for a map of these areas). The pine–oak woodlands of the Rocky Mountains, Great Basin, and eastern slope of the Sierra Nevada and Cascade mountains are principally composed of xeric pine forest [primarily Ponderosa pine (*Pinus ponderosa*)]. From the western slope of the Sierra Nevada to the Pacific, the pine–oak habitats of the California floristic province contain many endemics and are more mixed and diverse than the Ponderosa pine-dominated woodlands to the East. Distinct from all other pine–oak

habitats are the Madrean pine–oak communities found at high elevations in Mexico's principal mountain ranges (Sierra Madre Occidental, Sierra Madre Oriental, Sierra Madre del Sur, trans-Mexican volcanic belt, Sierra Madre de Oaxaca, and the Peninsular ranges of Baja California) and the mountains (sky-islands) of the US southwest (West Texas, Southern New Mexico, Southern Arizona, and Southern California). These Madrean pine–oak communities are extremely diverse and exhibit a very high degree of endemism, as they are home to approximately 44% and 30% of the world's pine and oak species, respectively (Little 1971, 1976, 1977), and considered areas of concern by Conservation International and the World Wildlife Fund.

The origination of these distinct regional pine–oak communities is primarily attributed to the fragmentation of the once continentally distributed Tertiary forests in response to Late Miocene and Pliocene (14–2.5 million years ago) orogeny and expansion of the basin and range region in western North America (Graham 1999). However, shifts in the distribution of pine–oak habitats in response to Quaternary glacial cycles also reinforced the isolation and independent evolution of regional communities (Betancourt & VanDevender 1990; Graham 1999). Palaeoecological data suggest that the Quaternary glacial cycles impacted these regional pine–oak communities in different manners. In eastern North America, considerable palynological data exists that the floral communities present during the last glacial maxima retreated into southern refugia; however, these refugial communities have no contemporary analogue and were likely more complex than current communities (Jackson *et al*. 2000; Williams *et al*. 2001). The xeric pine (Ponderosa pine) communities of the Rocky Mountains, Great Basin, and eastern slope of the Sierra Nevada and Cascades mountains were dramatically displaced and confined to localized refugia in the southeastern (southern Arizona and Northern Mexico) and southwestern (southeastern Sierra Nevada) most edges of their current distribution. West of the Sierra Nevada, pine–oak communities retreated south, but displacement was not as extreme as that observed East of the Sierra Nevada because of the moderating effects of the Pacific Ocean (Graham 1999). The climatic cycles of the Quaternary impacted Madrean pine–oak habitats in a radically different fashion than the more northern pine–oak woodlands (McDonald 1993). In the Madrean pine–oak habitats, the cooling associated with glacial advances did not result in the contraction of pine–oak habitats; instead, these habitats remained relatively stable and in some cases may have expanded in association with the cooler temperatures (McDonald 1993).

The division of pine–oak forests into specific regional communities and the separate biogeographic histories of these regional communities should be reflected in patterns of faunal diversity, if the fauna that inhabit these forests evolved *in situ* over time in pine–oak forests. In fact, this is
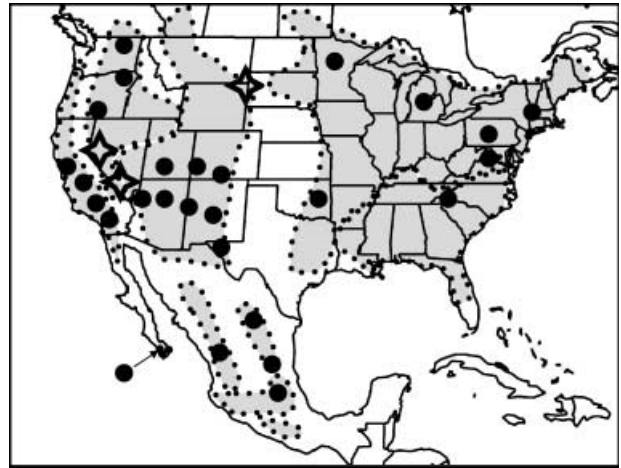

Fig. 1 Geographic distribution of the White-breasted Nuthatch. The dotted lines outline the distributional limits of the described subspecies of White-breasted Nuthatch (AOU 1957), except in Mexico where the distribution is fragmented but only two subspecies are recognized, *S. c. lagunae* (Baja California Sur) and *S. c. mexicana* (all other Mexican populations). Dots and stars mark sampling localities. A star symbolizes a population that contained haplotypes from more than one clade (Fig. 2).

the case for many pine–oak adapted fauna; however, there are a number of bird species that exhibit continental distributions across these disjunct pine–oak habitats [e.g. white-breasted nuthatch (*Sitta carolinensis*)]. The white-breasted nuthatch is a common permanent resident of North American pine–oak forests (Fig. 1). The AOU (1957) officially recognizes eight subspecies of white-breasted nuthatch (Fig. 1): *S. c. carolinensis* (southeastern US), *S. c. cookei* (northeastern US), *S. c. nelsoni* (Rocky Mountains and eastern Great Basin), *S. c. tenuissima* (eastern slope of the Sierra Nevada and Cascades and western Great Basin), *S. c. aculeata* (western slope of the Sierra Nevada, Pacific coast ranges, Transverse and Peninsular ranges of southern California), *S. c. alexandrae* (northern Baja California), *S. c. lagunae* (southern Baja California), *S. c. mexicana* (Sierra Madre Oriental, Occidental, and del Sur and Transvolcanic Belt of Mexico). The described subspecific (geographic) variation in white-breasted nuthatches is based on clinal variation in plumage colouration (darkness of the back, degree of rustiness of the flanks, and pattern on the undertail coverts) and bill shape (Ridgeway 1904; Phillips 1986; Wood 1992; Pravosudov & Grubb 1993). Much of the geographic variation in White-breasted Nuthatches is concordant with the boundaries of the regional pine–oak communities. (For example, *S. c. carolinensis* and *cookei* are found in the eastern North American woodlands and *S. c. nelsoni* and *tenuissima* are associated with the xeric pine forests of the Rocky Mountains, Great Basin, and eastern slope of the Sierra Nevada and Cascade mountains.) Although, exactly how the historical fragmentation of the

principal habitat(s) of the white-breasted nuthatch influenced its evolutionary history remains unknown.

The evolution of the white-breasted nuthatch in its fragmented habitat can be explained by three alternative historical hypotheses. (i) The species evolved *in situ* in the regional pine–oak communities and the isolation of populations in these regions is captured in cryptic genetic variation (deep phylogenetic structure and reciprocal monophyly of regional populations). (ii) Migration (high historical rates of gene flow) of individuals between regions has been frequent enough to maintain the widespread distributions and prevent regional divergence. (iii) The species have recently expanded to occupy their current distributions and an insufficient amount of time has passed for divergence to occur (incomplete lineage sorting). In this study, we explore which of these historical hypotheses best explains the evolutionary history of the white-breasted nuthatch (*Sitta carolinensis*) through a phylogeographic study of genetic variation in this species. Complete mtDNA ND2 sequences are used to examine patterns of genetic structure in the white-breasted nuthatch, and we interpret these patterns within the three historical hypotheses proposed to explain its evolution in the North American pine–oak woodlands.

Methods

Samples and laboratory techniques

Tissue samples were obtained for 216 individuals from 30 different localities and represented all but one (*S. c. alexandrae*; Northern Baja California) of the recognized subspecies of white-breasted nuthatch (see Fig. 1 for sampling localities and Appendix A, Supplementary material, for complete specimen details). Samples of *S. europea* and *S. himalayensis* were obtained from the American Museum of Natural History (AMNH-DOT 5225 and 5598, respectively) and used as outgroup taxa for phylogenetic analyses.

Total genomic DNA was extracted from all specimens using a DNeasy tissue extraction kit (QIAGEN) following the manufacturer's protocol. We amplified and sequenced the NADH dehydrogenase subunit 2 (ND2) gene using the primers L5215 (Hackett 1996) and H6313 (Johnson & Sorenson 1998). The ND2 gene evolves rapidly and has proven useful for uncovering intraspecific genetic structure in Passeriform birds (Drovetski *et al*. 2004; Zink *et al*. 2006). All fragments were amplified in 12.5 μL reactions under the following conditions: denaturation at 94 °C, followed by 40 cycles of 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 1 min. This was followed by a 10-min extension at 72 °C and 4 °C soak. Products were purified using a QIAGEN PCR Purification Kit or Exosap-IT (USB Corporation) purification following the manufacturer's protocols. We performed 20 μL BigDye (ABI) sequencing reactions using 20–40 ng of purified and concentrated PCR product following standard ABI protocols. Sequencing reactions were purified using a magnetic bead clean-up procedure designed by Agencourt Biosciences and run on an ABI 3100-*Avant* automated sequencer. Complementary strands of each gene were unambiguously aligned using SEQUENCHER 4.2 (Gene Codes). All sequences were translated and compared to the chicken ND2 sequence (Desjardins & Morais 1990) to confirm the correct reading frame and to check for the absence of stop codons in the ND2 gene-reading frame.

Phylogenetic methods

Maximum parsimony (MP) and maximum likelihood (ML) phylogenetic analyses were used to identify major clades and evaluate relationships among haplotypes. All MP analyses were performed in PAUP* 4.10b (Swofford 2001) using a heuristic search with 10 random sequence repetitions and tree-bisection–reconnection (TBR) branchswapping. Preliminary MP analysis revealed 63 678 equally parsimonious trees; however, these trees only differed from one another by the relationships among the terminal branches (haplotypes within clades) and the relationships among clades remained stable. Maximum likelihood analyses were performed using TREEFINDER (Jobb *et al*. 2004; version October 2005). First, MODELTEST 3.06 (Posada & Crandall 1998) and the AIC model selection criterion (Posada & Buckley 2004) with the outgroup sequence removed were used to select a model of sequence evolution to be used for ML phylogeny reconstruction. This model was then set as the model of sequence evolution for the TREEFINDER reconstructions. Nonparametric bootstrapping (200 replicates; Felsenstein 1985) performed in the programs PAUP* (MP) and TREEFINDER (ML) was used to evaluate nodal support among clades, with 70% or greater considered to provide strong support (Hillis & Bull 1993).

Divergence times between clades were estimated using the program MDIV (Nielsen & Wakeley 2001). MDIV applies an isolation-with-migration model to the data and uses a Bayesian approach to simultaneously approximate the posterior distribution of three parameters: divergence time between populations ($T = t_{div}/2N_e$), the migration rate between populations since divergence ($M = 2N_e m$), and the population parameter theta ($\theta = 2N_e \mu$, where μ is the per locus mutation rate. The program was first run using default search settings and default priors (for the parameters of interest, θ and *T*). Then, we set our prior value for *T* to equal 10 and *M* to equal 1, because it produced consistent and well-behaved posterior distributions. MDIV analyses were run for 5 million generations following a burn-in period of 500 000 generations, and repeated three times to ensure convergence upon the same posterior distributions for each of the parameter estimates. Estimates of *T* were converted to real time assuming a range of neutral mutation

rate estimates (because a direct rate estimate for the ND2 gene for *Sitta* is not available) for mtDNA in birds: 1.0×10^{-8}–2.5×10^{-8} substitutions/site/year (for reviews see; Arbogast *et al.* 2002; Lovette 2004). These two mutation rates amount to a 2%/Myr (million years) and a 5%/Myr clock, respectively.

Inter- and intra-population analyses

Inter- and intrapopulation level analyses were performed for populations with sample sizes of five or more. Genetic diversity within populations was characterized by the number of unique haplotypes per population and the number of private haplotypes per population. We estimated nucleotide diversity (π; Nei 1987) along with its 95% confidence interval (1000 replicates) using the program ARLEQUIN 2.0 (Schneider *et al.* 2000). ARLEQUIN was also used to compute mismatch distributions (Slatkin & Hudson 1991) and evaluate their significance (1000 replicates). Tajima's *D* (Tajima 1989a, b), which can be used to detect population expansions in geographically structured samples, was calculated using DNASP (Rozas & Rozas 1999) and its significance tested using coalescent simulations. To explore whether there existed significant genetic variation among populations, Analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) was performed with sequences grouped by population within each clade. Only populations with sample sizes ≥ 6 were included in the AMOVAS. All AMOVAS were performed with the program ARLEQUIN. To explore which populations may be responsible for the observed genetic structure in the AMOVAS, pairwise F_{ST} comparisons were performed in ARLEQUIN and their significance evaluated and corrected using a Bonferroni correction for multiple comparisons.

MIGRATE version 1.7.6 (Beerli & Felsenstein 2001, Beerli 2002) was used to estimate gene flow among the isolated populations in the Great Basin with those on the periphery. To reduce the number of parameters and degrees of freedom in our gene flow analyses, we used a stepping-stone model of population structure (Kimura & Weiss 1964). Thus, gene flow was estimated between populations that occurred adjacent to one another. In MIGRATE, we used the maximum-likelihood-based estimation and a Metropolis coupled-Markov chain Monte Carlo (MCMCMC) procedure. Runs consisted of 10 short chains of 100 000 steps followed by one long chain of 10 million steps and each chain had a burn-in period of 100 000 steps. All chains were sampled every 100 steps. Adaptive heating was used with four chains and initial temperatures of 1, 1.3, 1.5, and 2. Starting values of theta and gene flow were estimated using F_{ST}. The F84 model of sequence evolution was used with base frequencies estimated from the data. We ran the program two times with different random seed numbers to examine the robustness of our estimates.

Results

Phylogenetic analyses, geographic distribution of clades, and divergence times

We sequenced the complete ND2 gene (1041 bp) for each individual. These sequences yielded 140 variable sites (111 parsimony informative sites), identifying 90 haplotypes (Fig. 2). Unweighted MP analysis resulted in 63 678 equally parsimonious trees (score = 212). Both MP and ML analyses identified the same well-supported relationships among major clades; thus, we show only the ML phylogeny (Fig. 2). MODELTEST indicated the GTR + G (R matrix 0.306, 10.489, 0.486, 1.509, 3.242, 1.0; $\alpha = 0.0172$) as a best fit for our data. Maximum likelihood analyses using this model of sequence evolution produced a single most likely tree (–ln L = 2770.41755; Fig. 2). The only differences between the two analyses were the arrangement and relationships among haplotypes within clades; however, the branches leading to these haplotypes generally lacked bootstrap support in both MP and ML analyses.

The phylogenetic analyses identified four strongly supported (> 70% nonparametric bootstrap) clades of White-breasted Nuthatch: (i) Eastern Clade, (ii) Pacific Clade, (iii) Eastern Sierra Nevada (ESN hereafter) Clade, and (iv) Rocky Mountain, Great Basin, and Mexico (RGM hereafter) Clade (Fig. 2). These clades exhibited almost complete geographic isolation. The Eastern Clade comprises a monophyletic lineage containing all individuals sampled from East of the Great Plains, including individuals from both described eastern subspecies of white-breasted nuthatch, *S. c. carolinensis* and *S. c. cookei*. The Eastern Clade is sister to the Pacific Clade containing all individuals sampled from West of the Sierra Nevada and the Transverse and Peninsular ranges of southern California, representing the subspecies, *S. c. aculeata*. The uncorrected pairwise sequence divergence between the Eastern and Pacific Clades ranges from 3.6 to 4.7%.

Sister to the Eastern and Pacific Clades is a lineage that includes the ESN and RGM clades. These two clades include all the individuals from the eastern slope of the Sierra Nevada and Cascade mountain ranges, Great Basin, Rocky Mountains, Sierra Madre Oriental, Sierra Madre Occidental, Transvolcanic Belt of Mexico, and the Sierra Laguna of Baja California Sur. Interestingly, the geographic distribution of the haplotypes within the four clades of White-breasted Nuthatch and their phylogenetic relationships results in a leapfrog pattern, where a more distantly related lineage containing the ESN and RGM clades geographically separates two sister clades, Eastern and Western clades. Uncorrected sequence divergences between the Eastern and Pacific Clades and the samples found in the ESN and RGM clades range from 5.5 to 7.2%.

Haplotypes in the ESN Clade are narrowly distributed in populations on the Eastern slope of the Sierra Nevada
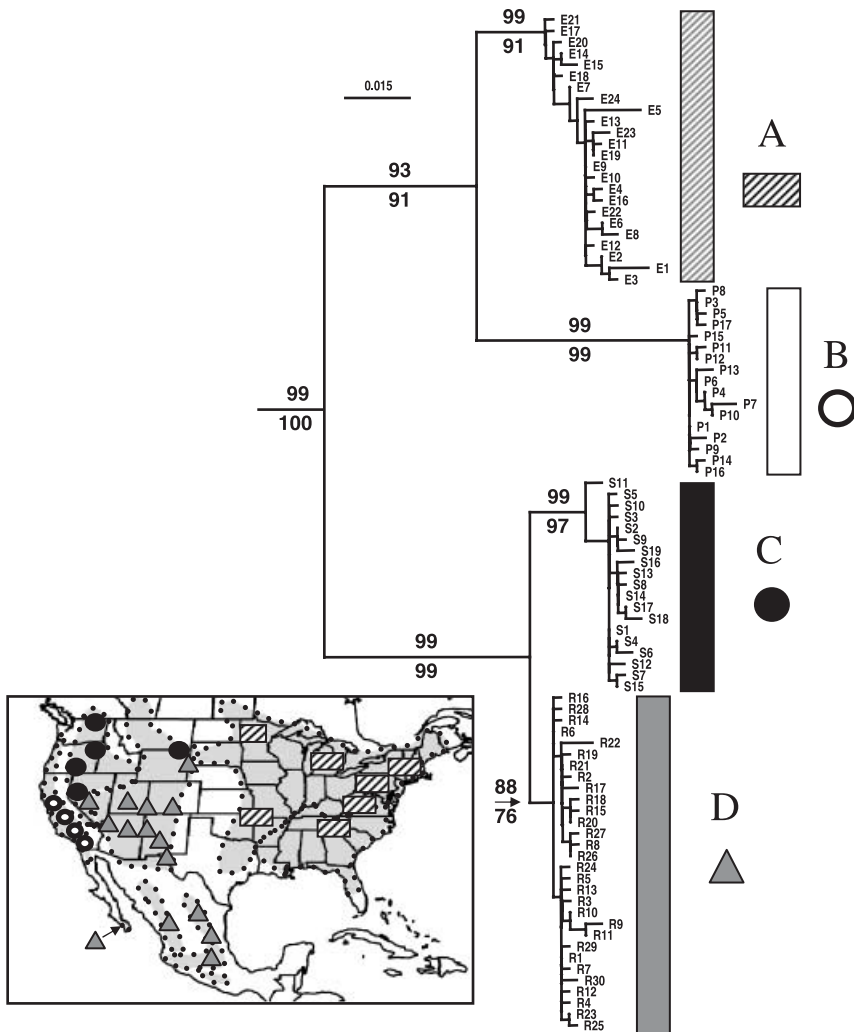
Fig. 2 Maximum likelihood phylogeny. Numbers above and below the branches correspond to maximum parsimony and maximum likelihood bootstrap percentages, percentages on the terminal nodes are not shown. The shaded or patterned shapes below the clade labels are used as identifiers in the inset map and show the geographic distribution of those clades. (A) Eastern Clade (B) Pacific Clade (C) ESN Clade (D) RGM Clade.

and Cascade mountain ranges from southern California to southern British Columbia and come from a single subspecies, *S. c. tenuissima*. Alternatively, haplotypes in the RGM Clade are widely distributed throughout the Rocky Mountains, Great Basin, and Mexico (including the isolated Sierra Laguna of Baja California Sur). The RGM Clade includes samples from three subspecies: *S. c. nelsoni*, *S. c. mexicana*, and *S. c. lagunae*. Uncorrected pairwise sequence divergences between individuals in these two clades range from 0.9% to 1.4%. There is evidence of introgression between the ESN and the RGM clades. Three sampled populations (Mono Crater, Central California, Spring Mountains, NV, and Black Hills, SD) possess haplotypes from both clades (Fig. 1).

MDIV estimates of divergence time (using our two hypothesized rates of molecular evolution) suggest that the basal split between the Eastern/Pacific clades and the ESN/RGM clades occurred 1.4 million or 3.4 million years ago (Table 1). This was followed by the divergence of the Eastern and Pacific clades 630 000 or 1.6 million years ago, and the divergence of the ESN and RGM clades 250 000 or

Table 1 MDIV estimates of divergence times between the four major clades

| Clade 1	Clade 2	t	θ	1.0×10^{-8} 2%/Myr	2.5×10^{-8} 5%/Myr
East/Pacific	RGM/ESN	5.00	14.12	3 400 000	1 400 000
Pacific	East	1.66	19.48	1 600 000	630 000
ESN	RGM	0.76	16.97	640 000	250 000

630 000 years ago. All estimates of migration rates between clades from the MDIV analyses indicated that fewer than 0.2 (range 0.00001–0.02) individuals migrated between clades as they diverged, and many of the posterior distributions for the estimate of *M* included zero.

Population structure and history: within clade patterns

Analyses of molecular variance (AMOVA) suggested that there is a significant amount of genetic variation partitioned

Table 2 Genetic structure within and among populations in each major clade as determined by analysis of molecular variance (AMOVA)

Clade	Category description	% variance	Statistic	P
Eastern				
	Among populations	11.94	$\Phi_{ST} = 0.119$	< 0.027
	Within populations	88.06		
Pacific				
	Among populations in regions	41.30	$\Phi_{ST} = 0.413$	< 0.0001
	Within populations	58.70		
ESN				
	Among populations	14.83	$\Phi_{ST} = 0.148$	< 0.0001
	Within populations	85.17		
RGM				
	Among populations	24.87	$\Phi_{ST} = 0.248$	< 0.0001
	Within populations	75.13		
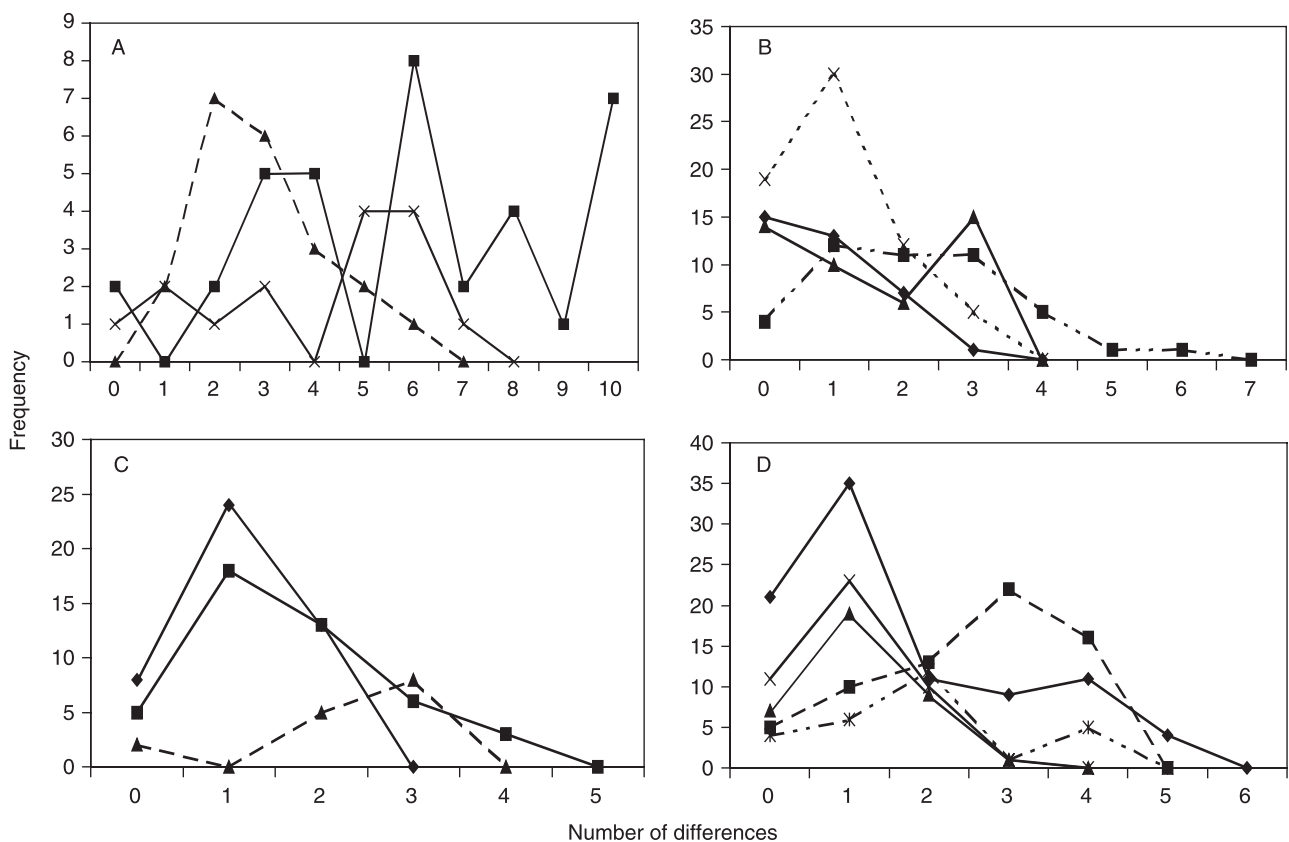
Fig. 3 Mismatch distributions for populations in each clade: (A) Eastern Clade (B) Pacific Clade (C) ESN Clade (D) RGM Clade. In D, the Morelos, Mexico population is represented by the dashed line with a mode of 3.

among populations in every clade of white-breasted nuthatch (Table 2). However, the amount and nature of the genetic partitioning among populations differs greatly by clade. In the Eastern Clade, AMOVA indicated there was significant genetic variation among populations (11.94%), but most of the variation was found within populations (88.06%). Although, the AMOVA of the Eastern Clade

suggested the existence of significant population structure; pairwise F_{ST} comparisons between individual populations provided no significant values following a Bonferroni correction for multiple comparisons (data not shown). Overall, nucleotide diversity was highest in the Eastern Clade, when compared to the other three clades (Table 3). The mismatch distributions (Fig. 3A) for the populations

Table 3 Genetic diversity within populations. The values in the columns correspond to sample size (N), number of unique haplotypes (H), number of private haplotypes (Pri), nucleotide diversity (π) and its 95% confidence interval, and Tajima's D (bold indicates significant values). The category remaining individuals refers to samples from populations with fewer than five sampled individuals. An asterisk marks populations with haplotypes from two clades

	N	H	Pri	π	π C.I.	D
Eastern clade						
Pennsylvania	6	5	4	0.003907	0.002615	0.188
NY, RI	9	7	6	0.005657	0.003384	0.323
Michigan	7	7	5	0.002836	0.001929	−0.503
Remaining inds.	9	8	7	—	—	—
TOTAL	31	24	—	0.004900	0.002717	—
Pacific clade						
Monterrey, CA	10	3	3	0.00143	0.001063	1.45
San Bernardino, CA	9	4	2	0.000801	0.00071	−0.93
San Diego, CA	10	7	4	0.002092	0.001428	−0.508
Northern CA	12	4	3	0.001004	0.000806	−0.741
Remaining individuals	9	5	1	—	—	—
TOTAL	52	17	—	0.0021	0.0012	—
ESN clade						
East Central, CA*	11	6	3	0.006067	0.001058	0.822
Oregon	10	6	3	0.00158	0.001147	−0.279
Washington	5	5	3	0.00317	0.002282	−0.124
British Columbia	6	4	2	0.002177	0.001599	0.197
Remaining individuals	9	6	4	—	—	—
TOTAL	40	19	—	0.0021	0.0013	—
RGM clade						
Coconino, AZ	14	8	5	0.001562	0.001101	**−1.63**
Mohave, AZ	7	4	3	0.001647	0.001245	0.239
Lincoln, NM	9	5	0	0.001067	0.000868	0.025
Valencia, NM	5	3	0	0.001537	0.001267	−1.93
Guadalupe, NM	10	5	0	0.000982	0.000808	−0.129
Spring Mountains, NV*	11	8	2	0.006781	0.001316	0.83
Utah	7	6	1	0.001738	0.001298	−0.561
Colorado	7	1	0	—	—	—
Morelos, Mexico	12	7	7	0.002416	0.001574	−0.633
Remaining individuals	14	9	4	—	—	—
Total	93	30	—	0.00213	0.0009	—

within this clade were generally not unimodal and two differed significantly from the population expansion model suggesting a relatively large or stable population size over time.

In the Pacific Clade, nearly half of the genetic variation was partitioned among populations (41.30%; Table 2), and all pairwise F_{ST} comparisons between populations were significant (data not shown). Nucleotide diversity was generally low, but was greatest in the most southern population (San Diego, CA). The mismatch distributions for Pacific Clade populations were unimodal (Fig. 3B) and did not deviated significantly from that of an expanding population.

A significant amount of genetic variation was partitioned among populations in the remaining two clades, the ESN Clade and RGM Clade, 14.83% and 24.87%, respectively. As in the Eastern Clade, pairwise F_{ST} comparisons failed to

identify a single significant comparison of population structure in the ESN Clade (data not shown). However, in the RGM Clade, pariwise comparisons indicated that a single population, the Morelos population from the Transvolcanic Belt of central Mexico, was responsible for the significant genetic structure among populations in this clade (data not shown). Nucleotide diversity was generally low in both of these clades; however, the two populations that contained haplotypes from both clades [E Central California (Mono Crater) and Spring Mountains, NV] exhibited expectedly high nucleotide diversity (Table 3). The mismatch distributions for populations within these two clades were unimodal and did not differ from that expected of an expanding population. However, the mean number of differences observed in the Morelos, Mexico population from the RGM Clade was larger than those of the other populations in the clade (Fig. 3).

The mountains of the Great Basin (commonly referred to as 'sky-islands') harbour forests (and other montane habitats) that are isolated from neighbouring forests by expanses of sagebrush and other temperate desert habitats. To assess the degree of insularity experienced by White-breasted Nuthatch populations in the Great Basin, we used MIGRATE to estimate rates of gene flow between our one well sampled Great Basin population (Spring Mountains, NV) and its two neighbouring populations from the larger and more contiguous forests of the eastern slope of the Sierra Nevada (Mono Crater) and the forests of northern Arizona (Coconino). The results of the MIGRATE analyses revealed asymmetric rates of gene flow into and out of the Spring Mountains. Gene flow into the Spring Mountains from the Eastern Sierra Nevada and the Rocky Mountains was 1.65 and 5.79 females per year, respectively. However, the rate of gene flow out of the Spring Mountains was less than a single female per year (0.29 and 0.16) to both the Eastern Sierra Nevada and Rocky Mountains.

Discussion

Deep phylogenetic structure reveals regional isolation

The deep phylogenetic structure observed between the four well-supported clades of white-breasted nuthatch is consistent with the long-term regional isolation of populations. To determine if the deep phylogenetic structure in the White-breasted Nuthatch is consistent with historical hypothesis 1 (i.e. the white-breasted nuthatch evolved in concert with the regional pine–oak forests); the divergence times between clades should be concordant with the timing of the fragmentation of North American pine–oak forests (inferred from palaeoecological data). The divergence time for the basal split between the Pacific/Eastern clades and the ESN/RGM clades is 1.4–3.4 million years ago. A 2-million year span makes it difficult to interpret this result; however, a recent study by Ho *et al*. (2005) on the tempo of molecular evolution in avian mitochondrial protein coding genes suggests that the older dates in this range may represent more accurate estimates. Between 3 and 4 million years ago the increased aridification of the intermountain region in western North America and continued uplift of the southern Rocky Mountains led to the isolation of a disjunct patch of pine–oak forest in the southern Rocky Mountains (Graham 1999). The isolation of white-breasted nuthatches from populations in the widespread forests to the North could have resulted in this basal divergence and would provide a mechanism to explain the geographic leapfrog pattern observed among the four clades (Fig. 2).

We estimated the next divergence in the white-breasted nuthatch between the Pacific and Eastern Clades to have occurred between 630 000 years and 1.6 million years ago. The rapid uplift of the Northern Cascades occurred approxi-

mately 2–3 Ma completing the fragmentation of the once widespread North American forests (Graham 1999), coupled with the onset of global cooling and climatic oscillations towards the end of the Pliocene and beginning of the Pleistocene (1.6–2.4 Ma; Jansen *et al*. 1988; Raymo & Ruddiman 1992; Lascoux *et al*. 2004) would have completely isolated eastern and western forests. White-breasted nuthatch populations would also have been fragmented as they tracked their habitat southward, and the presence of populations in the southern Rocky Mountains may have forced the newly fragmented populations to the eastern and pacific forests.

The final divergence between the ESN and RGM clades is estimated to have occurred between 250 000 and 640 000 years ago. This entire time span falls within the Pleistocene; thus, the divergence between these two clades was likely in response to isolation in glacial refugia. White-breasted nuthatches in the Eastern Sierra Nevada Clade and in most of the Rocky Mountain, Great Basin, and Mexico Clade are found in association with mature stands of Ponderosa pine. Palaeoecological (Betancourt *et al*. 1990) and phylogeographic (Latta & Mitton 1999) data indicate that Ponderosa pine populations were isolated in eastern and western refugia (separated by the Great Basin and southwestern deserts) during the Pleistocene. We suggest that white-breasted nuthatches persisted in both the eastern and western Ponderosa pine refugia, which led to the divergence of Eastern Sierra Nevada and Rocky Mountain populations.

The historical biogeographic scenarios described above rely heavily upon the molecular dating of divergence times. Unfortunately, the use of single locus (mtDNA) sequence data to calculate divergence times can be problematic, because of the large standard errors around dates due to coalescent stochasticity (Edwards & Beerli 2000; Jennings & Edwards 2005) and the difficulties of calculating locus specific mutation rates (Ho *et al*. 2005). However, even if the exact times and mechanisms responsible for the divergences in the white-breasted nuthatch are not the ones we propose here; historical hypothesis 1 (independent evolution of white-breasted nuthatches in the regional pine–oak forests) still provides the best explanation for these phylogenetic relationships within the species. There are four distinct and well-supported clades of white-breasted nuthatch. These four clades are geographically associated with the regional pine–oak forests of North America [i.e. California Floristic Province (Pacific Clade), Eastern pine and oak forests (Eastern Clade), Rocky Mountain/Great Basin/Mexican pine forests (RGM Clade), and Eastern Sierra Nevada and Cascade pine forests (ESN Clade)]. Thus, neither hypothesis 2 (frequent gene flow between regional forests) nor hypothesis 3 (recent invasion or expansion across the North America continent) can explain the observed phylogeny. Future phylogeographic research on additional North American bird species that exhibit

continental distributions and occupy pine–oak forests [i.e. brown creeper (*Certhia americana*), chipping sparrow (*Spizella passerina*), dark-eyed junco (*Junco hyemalis*), hairy woodpecker (*Picoides villosus*)] will help determine if the pattern of deep phylogenetic structure observed in the white-breasted nuthatch is common or taxon specific.

Population structure and population history within clades

The estimate of within clade nucleotide diversity (π) was higher in the Eastern Clade (π = 0.0049), than the estimates for the other three clades (π = 0.002). Also, the mismatch distributions for individual populations in the Eastern Clade were generally ragged or multimodal (Fig. 3). High π and a ragged mismatch distribution are normally considered support for a large and stable historical effective population size (Nei 1987; Slatkin & Hudson 1991). However, there is considerable evidence that the pine–oak forests of eastern North America experienced a dramatic contraction of their range during the Late Pleistocene glacial advances (Jackson *et al*. 1997, 2000). If eastern white-breasted nuthatch populations receded with the forests, we would expect π to be low to reflect the population contraction (or at least comparably low to the values observed in the other clades). We propose two mechanisms to explain the high π observed in eastern white-breasted nuthatches. First, there is palaeoecological evidence that forest refugia during Pleistocene glacial maxima were larger in eastern North America than in western North America (Graham 1999). Thus, the higher π in eastern white-breasted nuthatch could be the product of large refugial population size. Alternatively, the fragmentation of eastern pine–oak forests in multiple glacial refugia could also explain the high π and ragged mismatch distributions observed in eastern white-breasted nuthatches. Recent phylogeographic studies of two dominant boreal forest tree species (black spruce, Jaramillo-Correa *et al*. 2004; Jack pine, Godbout *et al*. 2005) found evidence for multiple glacial refugia in eastern North America and the subsequent expansion and current admixture of genotypes from these multiple refugia. Like the boreal forests, it is possible that eastern pine and oak forests and thus white-breasted nuthatches resided in multiple glacial refugia during the Late Pleistocene. Expansion from multiple refugia followed by the interbreeding of these once separate populations could have resulted in the increased π observed in these populations today. Unfortunately, our sampling of eastern white-breasted nuthatches is not sufficient enough to distinguish between these two refugial hypotheses, but this could easily be addressed through future and more thorough phylogeographic study of eastern white-breasted nuthatches.

Low π and unimodal mismatch distributions in the Pacific, ESN, and RGM Clades are consistent with recent population expansions in these three clades (Table 3; Fig. 3).

Shallow genealogies with genetic patterns consistent with recent population expansion northward from southern glacial refugia are found in many North American bird species (Avise *et al*. 1988; Zink 1997; Mila *et al*. 2000; Peters *et al*. 2005). If the recent population expansions in these three clades occurred in a similar fashion, we would expect π to decrease with increasing latitude (if we eliminate the populations with inflated estimates of π due to introgression; Table 3). In fact, this is the case in two of the clades: Pacific Clade and RGM Clade. The most southern populations in these two clades (San Diego, California and Morelos, Mexico, respectively) have the highest estimates of π (Table 3) and mismatch distributions with greater median values than those of other populations in each clade (Fig. 3) suggesting a population expansion in each clade proceeding from south to north. In Mexico, higher π (compared to populations further north) has been observed in montane populations of pines (Ledig *et al*. 1997, 1999, 2006; Ledig 1998, 2000; Delgado *et al*. 1999) and other birds (Mila *et al*. 2000). These patterns of genetic diversity provide additional support for the hypothesis based on palaeoecological data that montane habitats in Mexico were more stable or possibly larger during Quaternary glacial maxima than mountain ranges farther north (McDonald 1993; Graham 1999).

There is significant population structure (supported by both AMOVA and pairwise F_{ST} analyses) in two clades of white-breasted nuthatch: again the Pacific and the RGM clades. In the Pacific Clade, there is significant population structure among all of the sampled populations. The populations of White-breasted Nuthatches sampled from this clade come from the coastal California mountain ranges or the Peninsular and Transverse mountain ranges of southern California (Fig. 1). Recent phylogeographic studies of other bird species occurring in or near these mountain ranges, suggest that species that occupy chaparral, oak, and coniferous forest habitats commonly exhibit a shallow phylogenetic break or population differentiation between Coastal, Northern, and Southern California (Cicero 1996; Barhoum & Burns 2002; Sgariglia & Burns 2003; Spellman *et al*. in press). Within the last 50 000 years, Sonoran and Mohave xeric vegetation has expanded dramatically in southern California (Axelrod 1979; Betancourt & VanDevender 1990). The expansion of desert vegetation into the valleys between southern California mountain ranges resulted in the fragmentation and isolation of chaparral, forest, and woodland habitats. This recent fragmentation of Californian woodlands is also most likely responsible for the shallow population differentiation observed in White-breasted Nuthatches.

The pattern of population differentiation in the RGM clade is driven by the uniqueness of a single population: Morelos, Mexico. This population contains seven private haplotypes (see Appendix A, Supplementary material).

Unfortunately, our population sampling throughout Mexico is not sufficient to address whether the Morelos, Mexico population is truly isolated from the other populations in this clade or whether it just represents the end of a genetic cline (isolation by distance).

Introgression

Since the last glacial maximum, the ESN and RGM clades have expanded and recently came into contact. Introgression between these two clades is evident in three populations: Black Hills, SD; Mono Craters, CA; and Spring Mountains, NV (Fig. 1). In the Black Hills, a single individual possessed the most common haplotype in the ESN Clade and the remaining individuals had haplotypes from the RGM Clade (see Appendix A, Supplementary material). The Black Hills are geographically distant from the core of the ESN Clade, and yet most of the populations sampled between the Black Hills and this clade do not demonstrate evidence of introgression. Thus, the Black Hills introgression may represent an isolated migration event or incomplete lineage sorting between the RGM and ESN clades. Our sampling across the northern distribution of these two clades (Oregon, Washington, Idaho, Montana, Wyoming, and Alberta) is limited, making it difficult to distinguish between these two hypotheses. For example, we cannot rule out the possibility that introgression through this area is extensive and that the sample from the Black Hills represents the southeastern extent of introgression. In fact, the presence of a contact zone and hybridization between eastern and western subspecies of Ponderosa pine in Central Montana (Latta & Mitton 1999) suggests that introgression between these two clades of White-breasted Nuthatch may be common in this area.

Introgression in the Mono Craters is not as surprising as it is in the Black Hills. Remington (1968) proposed the Mono Craters area of east central California as a contact zone between the historically separated biotas of the Sierra Nevada and Rocky Mountains. Support for the Mono Craters areas as a contact zone between the Sierra Nevada and Rocky Mountain avifaunas was also found in a recent phylogeographic study of another pine-associated bird species, the mountain chickadee (*Poecile gambeli*; Spellman *et al.* in press).

Introgession in the Spring Mountains, NV, is more extensive. This population possesses many haplotypes from both the ESN ($N = 3$) and RGM ($N = 8$) clades. Although not hydrologically a part of the Great Basin biogeographic region, the Spring Mountains are functionally equivalent to the mountain ranges of the Great Basin proper. Thus, these mountains (and associated forests) are considered sky-islands separated from neighbouring mountain ranges by vast expanses of desert scrub or sagebrush steppe habitats. Our data indicate that the white-breasted nuthatch population on the Spring Mountains experienced asymmetric rates of historical immigration (high) and emigration (low), as might be expected in an island model. Therefore, it is likely that the genetic diversity and potentially the long-term stability of the white-breasted nuthatch population on the Spring Mountains (and potentially other Great Basin mountain ranges) are greatly dependent upon the frequent influx of migrants from neighbouring populations.

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Supplementary material

The following supplementary material is available for this article:

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