Testing hypotheses of Pleistocene population history using coalescent simulations: phylogeography of the pygmy nuthatch (*Sitta pygmaea*)

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In this paper, we use mitochondrial NADH dehydrogenase subunit 2 sequences to test Pleistocene refugial hypotheses for the pygmy nuthatch (*Sitta pygmaea*). Pygmy nuthatches are a common resident of long-needle pine forests in western North America and demonstrate a particular affinity with ponderosa pine (*Pinus ponderosa*). Palaeoecological and genetic data indicate that ponderosa pine was isolated in two Pleistocene refugia corresponding to areas in the southern Sierra Nevada in the west and southern Arizona and New Mexico in the east. We use coalescent simulations to test the hypothesis that pygmy nuthatches tracked the Pleistocene history of their preferred habitat and persisted in two refugia during the periods of glacial maxima. Coalescent simulation of population history does not support the hypothesis of two Pleistocene refugia for the pygmy nuthatch. Instead, our data are consistent with a single refuge model. Nucleotide diversity is greatest in the western populations of southern and coastal California. We suggest that the pygmy nuthatch expanded from a far western glacial refuge into its current distribution since the most recent glacial maximum.

Keywords: coalescent simulations; effective population size (N_e); theta (θ); site-specific mutation rate (μ); pygmy nuthatch (*Sitta pygmaea*); Ponderosa Pine (*Pinus ponderosa*)

1. INTRODUCTION

In the field of phylogeography, the testing of explicit *a priori* hypotheses in a rigorous statistical framework remains in its infancy (Knowles & Maddison 2002). This is due primarily to two formidable hurdles. The first hurdle is a simple lack of knowledge. Often, very little is known concerning the palaeoecology of a region or of an organism's habitat or niche that would allow the formulation of plausible *a priori* historical biogeographic hypotheses. Therefore, phylogeographers are often forced to test the hypotheses that are formed *a posteriori* from the data they would use to test these hypotheses. The few studies that have tested explicit *a priori* hypotheses have done so in organisms, which exhibit restricted ranges and inhabit niches with relatively well-known palaeo-distributions (inhabitants of the Pacific Northwest mesic forests of North America, Carstens *et al.* 2004, 2005a,b; montane grasshoppers (*Melanoplus* spp.), Knowles 2001).

The second hurdle is coalescent stochasticity (Edwards & Beerli 2000; Knowles & Maddison 2002; Hudson & Turelli 2003). Since lineage sorting is random, there is a high probability that large populations with a recent shared history will be genetically similar. In addition, due to coalescent stochasticity, the same population history can result in different genealogical patterns at different loci. Conversely, a single gene tree can also be consistent with

multiple population histories leading to false inferences if the variance in the coalescent process is not evaluated. Therefore, the challenge for researchers is to account for this variability in the coalescent process when testing competing alternative hypotheses of population history. One way to account for coalescent stochasticity is to simulate gene evolution (genealogies or gene matrices) within competing hypotheses of population history (Knowles 2001; Carstens *et al.* 2004). Then, the simulated genealogies can be compared to phylogenies inferred from sequence data to assess which hypothesis is a best fit for the data (Knowles 2001; Carstens *et al.* 2004). In this study, we overcome the two hurdles described earlier by using coalescent simulations to test explicit *a priori* hypotheses concerning the Pleistocene history of the pygmy nuthatch (*Sitta pygmaea*).

The pygmy nuthatch is one of the most common resident avian inhabitants of pine forests in western North America. It has long been considered a specialist on long-needle pines, exhibiting an especially tight association with ponderosa pine (*Pinus ponderosa*; Norris 1958; Kingery & Ghalambor 2001). Pygmy nuthatches require mature open woodland in pine forests for survival and are easily extirpated from long-needle pine forests when stands are harvested (Kingery & Ghalambor 2001).

Ponderosa pine is one of the five most common tree species in western North American forests. It is a major component in 65% of all forest cover south of the boreal forests (Critchfield & Elbert 1966). However, ponderosa pine does have certain soil and moisture requirements and therefore exhibits a patchy distribution. The patchy

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Figure 1. (a) Distribution of the pygmy nuthatch. The colours correspond to major geographic regions within western North America: blue, Californian coastal mountains; yellow, transverse and peninsular ranges of southern California; green, Sierra Nevada and Cascades; pink, rocky mountains; brown, Great Basin; purple, Sierra Madre Occidental, Oriental and Transvolcanic belt of central Mexico. The numbers correspond to populations with sample sizes greater than 10 (table 1). Black dots correspond to populations with sample sizes of less than 10. (b) Median joining network of all haplotypes. The size of the circles is proportional to haplotype frequency. The colours correspond to the colours on the map in (a) to identify the geographic distribution of haplotypes.

Figure 2. Models used to test Pleistocene refugial hypotheses: (a) two refugia hypothesis and (b) an alternative single refugia hypothesis. Hypothesis A1 assumed populations persisted in two Pleistocene refugia for the duration of the glacial episodes ($T_2 = 1\,800\,000$ years BP). Hypothesis A2 assumed populations persisted in two refugia only since the most recent glacial advance ($T_2 = 120\,000$ years BP). Hypotheses B1 and B2 tested fragmentation of a single refugial population with the number of years in the refugium differing (B1, $T_2 = 1\,800\,000$ years BP; B2, $T_2 = 120\,000$ years BP). The effective size of populations was constrained as follows (unless otherwise stated): current populations 50% of total N_e and refugial populations 15% of total N_e (justification for ancestral N_e described in text). Terminal branches (T_1) were all 10 000 years long.

distribution of these pines dictates the patchy distribution of pygmy nuthatches (figure 1a). The distributions of both the pygmy nuthatch and ponderosa pine are nearly an exact match, the only major difference being the absence of ponderosa pine from the transvolcanic belt of central Mexico (figure 1a, no. 14). Here, pygmy nuthatches are instead found in close association with Mexican yellow pines (i.e. *Pinus montezumae* and *Pinus engelmanni*).

The palaeoecology and phylogeography of ponderosa pine are relatively well understood. Palynological and packrat (*Neotoma* spp.) midden data suggest that ponderosa pine was isolated in two refugia towards the southwestern (southern Sierra Nevada) and southeastern (southern Rockies of Arizona and New Mexico) edges of its current distribution during the periods of Pleistocene glacial maxima (Betancourt & VanDevender 1990; Graham 1999).

This historical isolation of ponderosa pine in eastern and western refugia is also supported by genetic data. Latta & Mitton (1999) found fixed differences in mitochondrial and chloroplast DNA and significant F_{st} values in three out of 14 allozyme loci between eastern and western subspecies of ponderosa pine.

The specialization of pygmy nuthatches on long-needle pines and the tight geographic association of ponderosa pine and pygmy nuthatches suggest that they should demonstrate a shared biogeographic history. We use coalescent simulations to test the hypothesis that the Pleistocene history of the pygmy nuthatch matches with that of its preferred habitat, ponderosa pine. Two alternative models are tested: (i) a two refugia model (consistent with the recent evolutionary history of ponderosa pine) and (ii) a single Pleistocene refugium (figure 2).

2. MATERIAL AND METHODS

(a) *Samples, laboratory techniques, analyses*

Tissue samples were obtained for 202 individuals from 20 different localities and represent all recognized subspecies of pygmy nuthatch (figure 1). Since in coalescence theory, variances seldom decrease when sample sizes of over 10 are used (Harding 1996), whenever possible we sampled 10 individuals from each location. Populations with samples sizes of less than 10 (19 individuals from six populations) were excluded from the population level analyses. The brown-headed nuthatch (*Sitta pusilla*), considered part of a super-species complex that includes the pygmy nuthatch (Norris 1958), was used as an outgroup for phylogenetic inference.

Total genomic DNA was extracted from all specimens using a DNeasy tissue extraction kit (Qiagen, Valencia, CA) following the manufacturer's protocol. We amplified the NADH dehydrogenase subunit 2 (ND2) gene using the primers L5215 (Hackett 1996) and H6313 (Johnson & Sorenson 1998). All the 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 and 4°C soak. The 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 and the same primers described earlier following standard ABI protocols. The 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 v. 4.2 (GeneCodes Corporation, Ann Arbor, Michigan). All the sequences were translated and compared to the chicken ND2 sequence (Desjardins & Morais 1990) to confirm the correct reading frame and to check for the presence of stop codons.

Maximum-likelihood (ML) phylogenetic analysis and non-parametric bootstrapping (100 replicates; Felsenstein (1985)) were used to identify major clades and evaluate nodal support for relationships among clades. The hierarchical likelihood ratio test (hLRT) as implemented in MODELTEST v. 3.06 (Posada & Crandall 1998) with the outgroup sequence removed was used to select a model of sequence evolution to be used for the ML phylogeny reconstruction. A median joining network was used to visualize relationships among haplotypes using the program Network (Bandelt *et al.* 1999). Population genetics parameters (i.e. Tajima's *D* and Fu's *F_s*; Tajima 1989a,b; Fu 1997) were calculated for all populations with samples sizes of 10 or more (populations 1–14, hereafter referred to by number) using Dnasp (Rozas & Rozas 1999) and their significance was tested using coalescent simulations. Analysis of molecular variance (AMOVA) was performed with the program ARLEQUIN v. 2.0 (Schneider *et al.* 2000). To explore whether there existed significant genetic variation at multiple geographic levels, nested AMOVA analyses were performed with sequences grouped by region (figure 1) and then by individual population within each region (i.e. sampling locality; figure 1). We conducted two nested AMOVA analyses: the first included the samples from populations with samples sizes of less than 10 individuals, and the second excluded these samples to explore whether these samples biased the analysis.

(b) *Hypothesis testing*

We tested two general hypotheses. The first of the two general hypotheses was a two refugia hypothesis that was consistent with the phylogeography and palaeoecology of ponderosa pine (figure 2a). Under the two-refugia hypothesis, populations of pygmy nuthatches were isolated into two refugia corresponding to the western mountain ranges (e.g. Sierras and Cascades) and the eastern mountain ranges (rocky mountains and great basin ranges). Since pine-adapted species were confined to refugia during periods of glacial maxima, two divergence times for populations in these refugia were tested. The first divergence time corresponded to an early glacial advance (hypothesis A1, $T_2 = 1\,800\,000$ years BP), and the second corresponded to the onset of the most recent glacial advance (hypothesis A2, $T_2 = 120\,000$ years BP; figure 2). The second hypothesis was an alternative single refugium hypothesis. Under this hypothesis, genetic diversity in pygmy nuthatches was the result of recent fragmentation of a single refugial population. The duration of time populations persisted in a single refugium was the same as those tested for the two refugia hypothesis (B1, $T_2 = 1\,800\,000$ years BP; B2, $T_2 = 120\,000$ years BP).

Effective population size (N_e) for all the simulations was estimated using θ values calculated with MIGRATE-N (v. 1.7.3; Beerli 2002) and the following search parameters: 10 short chains of 100 000 steps followed two long chains of 20 000 000 steps, the chains were sampled every 100 steps following a burn-in of 20 000 steps, and default settings used for the initial estimate of theta. The program was run twice to ensure convergence upon similar values with different random seed numbers. Theta was converted to N_e using the equation $\theta = 2 N_e \mu$, and assuming $\mu = 0.027 \times 10^{-7}$ (a site-specific mutation rate calculated for the ND2 gene in Galapagos mockingbirds (*Neosimus parvulus*); Arbogast *et al.* 2006). Population parameter estimates, such as θ , calculated from single locus data have inherently large errors (Edwards & Beerli 2000). Therefore, to ensure that our conclusions were robust to changes in the estimate of θ , coalescent simulations were performed using N_e calculated from the point estimate of θ and from the lower and upper bounds of the 95% CI for the point estimate of θ . Ancestral N_e (N_e for the refugial populations) was estimated using a Bayesian sky-line analysis as described by Drummond *et al.* (2005) in the program BEAST (v. 1.2). The analysis was run for 30 million generations and the first 10% were discarded as burn-in. Ancestral N_e was calculated as a proportion of current N_e .

Hypothesis testing was performed using the program MESQUITE v. 1.05 (Maddison & Maddison 2005). MESQUITE provides the flexibility to build models or hypotheses, simulate trees evolving within the confines of those models and compare these simulated trees with user-defined trees to evaluate model fit. Two types of coalescent simulations were performed. In the first set of simulations, 1000 coalescent genealogies were generated under each historical scenario and the distribution of *S*, the minimum number of sorting events required to explain the population subdivision (Slatkin & Maddison 1989), was recorded. The *S* value of our ML genealogy was compared with the *S* values of the simulated genealogies to evaluate model fit. For this first set of simulations, both the point estimate of N_e and the lower and upper bounds of the 95% CI for N_e were used as model parameters. In the second set of simulations, 100 gene matrices were simulated using the model of DNA substitution selected by MODELTEST and constrained within each historical scenario. PAUP* v. 4.10b was used to reconstruct trees from the simulated gene matrices

Table 1. Intrapopulation statistics for all populations with sample sizes greater than 10 and for all samples combined (total). (The numbers in the first column correspond to the location numbers on figure 1a. All the values were calculated and their significance tested using coalescent simulations under the infinite sites model. Values in italics are significant at $p < 0.05$ level.)

map no.	population	<i>n</i>	no. of haplotypes	<i>s</i>	π	<i>D</i>	<i>F_s</i>	<i>R</i>
1	Pacific Coast	19	8	15	0.00443 ± 0.00047	0.28	0.34	0.05
2	San Diego	13	8	15	0.00309 ± 0.00087	−1.58	−1.99	0.03
3	Riverside	11	5	8	0.00168 ± 0.00065	−1.49	−0.71	0.19
4	Transverse Ranges CA	14	8	10	0.00278 ± 0.00037	−0.65	−2.06	0.18
5	Mono	13	4	6	0.0019 ± 0.00044	0.08	1.03	0.55
6	Siskiyou	12	5	8	0.00293 ± 0.00034	0.59	0.81	0.51
7	Spring Mountains, NV	10	4	6	0.00141 ± 0.0006	−1.28	−0.08	0.08
8	British Columbia	13	5	4	0.00111 ± 0.00026	−0.36	−1.39	0.12
9	Coconino	16	7	11	0.00172 ± 0.00052	−1.74	−2.06	0.09
10	Valencia	11	4	7	0.00199 ± 0.0006	−0.54	0.851	0.4
11	Chiricahua	14	6	7	0.0016 ± 0.00041	−1.27	−1.46	0.04
12	Sacramento	13	4	6	0.00177 ± 0.0005	−0.17	0.87	0.22
13	Nueva Leon	14	2	1	0.00013 ± 0.0001	−1.16	−0.65	0.56
14	Morelos	10	4	6	0.00105 ± 0.00056	−1.85	−0.56	0.16
	total ^a	202	53	60	0.00296 ± 0.00015	−2.17	−49.89	0.09

^a includes 19 individuals from populations with sample sizes less than 10.

and the *S* values for these trees recorded and compared to the *S* value of our ML genealogy to evaluate the model fit. The second set of simulations used only the point estimates of N_e for the model parameters. For all the coalescent simulations, absolute time (years) was converted to coalescent time (generations) assuming a generation time of 2.8 years for pygmy nuthatches (calculated using the equation $T = \alpha + [s/(1-s)]$ from Lande *et al.* (2003), where α is the age at maturity and *s* is the annual adult survival rate).

3. RESULTS

(a) *Phylogeny, population genetics and demography of pygmy nuthatches*

The complete *ND2* gene (1041 bp) was sequenced for all individuals. These sequences yielded 60 variable sites, identifying 53 haplotypes (figure 1b), including 33 that were unique to individuals and 20 shared among several individuals. The three most common haplotypes were found in 29.2, 14.9 and 11.4% of all individuals, respectively. The most common haplotype was found in all except five populations (2, 8, 11, 13 and 14; figure 1a). An hLRT test supported the HKY85 ($\kappa = 4.2$) model as a best fit for our data; however, the resulting phylogeny ($-\ln = 2045.75$; data not shown) was star-like and lacked support for any of the basal nodes. The only two nodes receiving bootstrap support more than 70% were at terminal branches connecting tips. The general lack of support for relationships among haplotypes was supported by the median joining network (figure 1b), which was characterized by a large number of haplotypes nested within loops in the network. The most divergent haplotypes were found mostly in coastal and southern Californian populations (i.e. 1 and 2; figure 1b) with the exception of a single divergent haplotype found in central Mexico (population 14).

AMOVA (Excoffier *et al.* 1992), using HKY85 adjusted distances, indicated that most of the genetic variation was found within populations (63.8%, $p < 0.0001$). However, there were also significant amounts of variation among major geographic regions (all populations included: 24.5%, $p < 0.0001$; populations with 10 or more

individuals included: 24.9%, $p < 0.0001$) and among individual populations (all populations included: 11.67%, $p < 0.0001$; populations more than 10 individuals included: 11.29%, $p < 0.0001$). Nucleotide diversity was highest in the western populations (populations 1–6; table 1), but low overall.

Neutrality statistics (Tajima's *D* and Fu's F_s) were significantly negative in only one population (14, $D = -1.85$, $p < 0.05$; table 1). Otherwise these statistics suggested no deviation from neutral expectations. Raggedness indices (*R*; table 1) were insignificant in 50% of the populations indicating recent population expansions. Pooling all populations resulted in highly significant negative estimates of *D* and F_s ($D = -2.17$, $p < 0.0001$; $F_s = -49.89$, $p < 0.0001$) and a highly insignificant raggedness index ($R = 0.09$; $p < 0.0001$; table 1).

(b) *Hypothesis testing*

Using MIGRATE-N, we estimated an overall θ of 0.02419 (95% CI 0.01406–0.04234) for our data. Using $\mu = 0.027 \times 10^{-7}$ substitutions per site per lineage per million years and a generation time 2.8 years, we calculated a point estimate of $N_e = 160\,000$ and the 95% CI around this estimate ranged from 93 000 to 260 000. Ancestral N_e was estimated to be approximately 15% of current N_e .

We calculated $S = 117$ (Slatkin & Maddison 1989) for our ML genealogy. Figure 3 shows the distribution of *S* values for the simulations and our tree for the point estimate of N_e in the first set of simulations under hypotheses A2 and B2. For the first set of simulations (1000 coalescent genealogies constrained within each model), we were able to reject the two refugia hypotheses for all the values of N_e ($p_{\text{simulationA1}} = 0.0001$ –0.001, $p_{\text{simulationA2}} = 0.0001$ –0.01). We could not reject a hypothesis of a single refugium for our data for the point estimate of N_e or the upper bound of N_e ($p_{\text{simulationB1}} = 0.24$ and 0.14, $p_{\text{simulationB2}} = 0.28$ and 0.18). However, simulations using the lower bound of N_e did reject the single refugium hypothesis ($p_{\text{simulationB1}} = 0.04$, $p_{\text{simulationB2}} = 0.05$). Thus, for most reasonable values of N_e our data support the single refugium hypothesis.

Figure 3. Histograms of S (Slatkin & Maddison 1989) values for simulated coalescent genealogies. (a) Results from simulations within the two refugia hypothesis A2. (b) Results from simulations within the single refuge hypothesis B1. The dashed lines represent the 95% CI for the distribution and the bold black line in (b) represents the S value for our ML genealogy. Results shown here were from the simulation that used the point estimate of $N_e = 160\,000$.

For the second set of simulations (100 gene simulated gene matrices constrained within each model), the HKY85 model described earlier was used to generate the gene matrices. Under these simulations, the two refugia model was rejected ($p_{\text{simulationA1}} < 0.001$, $p_{\text{simulationA2}} < 0.01$). The single refugium model was not rejected ($p_{\text{simulationB1}} = 0.1$, $p_{\text{simulationB2}} = 0.1$).

4. DISCUSSION

Our data support the conclusion of a single Pleistocene refugium for pygmy nuthatches, which is contrary to what we had expected, given the close association between pygmy nuthatches and ponderosa pine. However, the coalescent simulations provide no information regarding the location of this refugium, because under these simulations all populations are in effect, equally likely to be the source population of all current mtDNA diversity in pygmy nuthatches. Traditional measures of genetic diversity (i.e. nucleotide diversity, π) provide an alternative means of inferring the location of a refugium (Hewitt 1996; Avise 2000). Large values of π can be indicative of a larger and stabler historical population size (Avise 2000). Nucleotide diversity is also impacted by other demographic events, such as extreme bottlenecks and gene flow, thus we regard the following conclusion as only our best guess at identifying the location of a glacial refugium for the pygmy nuthatch. In our populations, π is greatest in western and southern California suggesting that these populations may have persisted in a more stable condition for a longer period of time than other pygmy nuthatch populations. In fact, when plotted against degrees west longitude, π appears to be positively correlated with longitude (with π falling as one proceeds further east across the distribution of the pygmy nuthatch; $r^2 = 0.414$, d.f. = 12). Conversely, nucleotide diversity does not appear to be correlated with latitude ($r^2 = 0.089$, d.f. = 12). Thus, we suggest that the pygmy nuthatch Pleistocene refugium was probably located in coastal and/or southern California. This places the refugium in one of the locations, where palaeoecological data indicate that ponderosa pine persisted during Pleistocene glacial maxima (Betancourt & VanDevender 1990; Graham 1999).

A western glacial refugium with subsequent expansion eastward, north-eastward and south-eastward contrasts patterns observed in most other North American birds (Zink 1997; Milá *et al.* 2000) and other north temperate taxa (for review see Hewitt 1996, 2000). In general, nucleotide diversity in temperate taxa declines with increasing latitude due to range expansion following the retreat of glaciers during the Holocene. Thus, this pattern of a negative relationship between nucleotide diversity and latitude is predicated on the assumption that the glacial refugia were all located in the southern most extremes of the current ranges of temperate taxa. If the location of a glacial refugium is found elsewhere, as we hypothesize to be the case for pygmy nuthatches, there is no reason one would not observe a violation of the previously described pattern.

Phylogeography utilizes gene history (genealogy) to draw conclusions concerning population history (Avise 2000). If lineage sorting is incomplete, which is likely in recently isolated populations, the ability to infer population history becomes difficult (Charlesworth *et al.* 2003). The coalescent simulations used in this study provide a means of inferring population history even in the face of incomplete lineage sorting by assessing the stochasticity inherent in the coalescent process. However, these simulations rely heavily upon model parameters (i.e. θ) estimated directly from our data. In coalescent theory, the single most important population parameter that influences the rate of lineage sorting is effective population size (N_e ; Edwards & Beerli 2000; Emerson *et al.* 2001; Charlesworth *et al.* 2003; Hudson & Turelli 2003), which is normally estimated from genetic data using the population parameter, θ . Thus, if coalescent simulations are conducted using poor and/or incorrect estimates of θ , the chance that erroneous inferences will be made is high. Estimates of θ made from single locus data, as in this study, can be unreliable and always have large amounts of error associated with them (Edwards & Beerli 2000; Jennings & Edwards 2005). The error in the estimate of θ must be taken into account for robust conclusions of population history to be made. To overcome this problem, we used both the point estimate of θ and the lower and upper bounds of the 95% CI of θ to calculate N_e when conducting the coalescent simulations.

Thus, the conclusion of a single Pleistocene refugium for all extant pygmy nuthatches is robust across most reasonable values of θ .

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