NOTE

Assessing Clark's nutcracker seed-caching flights using maternally inherited mitochondrial DNA of whitebark pine

Bryce A. Richardson, Ned B. Klopfenstein, and Steven J. Brunsfeld

Abstract: Maternally inherited mitochondrial DNA haplotypes in whitebark pine (*Pinus albicaulis* Engelm.) were used to examine the maternal genetic structure at three hierarchical spatial scales: fine scale, coarse scale, and inter-population. These data were used to draw inferences into Clark's nutcracker (*Nucifraga columbiana* Wilson) seed-caching flight distances. Statistical analyses of fine-scale and coarse-scale distribution of haplotypes showed no apparent signs of deviation from a random pattern. This suggests nutcrackers are effective in dispersal of seed within populations, which is consistent with data gathered on nutcracker seed-caching behavior. However, the lack of homogeneity in haplotype frequencies among populations indicates nutcrackers rarely disperse seeds across large gaps (>20 km) in subalpine habitat.

Résumé : Les auteurs ont utilisé des haplotypes d'ADN mitochondrial transmis maternellement chez le pin à écorce blanche (*Pinus albicaulis* Engelm.) afin d'étudier la structure génétique maternelle à trois échelles spatiales hiérarchiques : à grande et à petite échelles, et parmi les populations. Ces données ont permis d'inférer les distances de vol du casse-noix d'Amérique (*Nucifraga columbiana* Wilson) pour établir ses caches de graines. Les analyses statistiques de la distribution des haplotypes à grande et à petite échelles n'ont pas montré de signes d'une déviation par rapport à une distribution aléatoire. Cette observation suggère que les casse-noix dispersent efficacement les graines à l'intérieur des populations, ce qui est consistant avec les données se rapportant aux habitudes d'entreposage des graines chez cette espèce. Cependant, une hétérogénéité des fréquences d'haplotypes a été remarquée parmi les populations, impliquant que les casse-noix traversent rarement des grandes trouées (>20 km) pour disperser des graines, au sein de l'habitat subalpin.

[Traduit par la Rédaction]

Introduction

The interdependencies of whitebark pine (*Pinus albicaulis* Engelm.) and Clark's nutcracker (*Nucifraga columbiana* Wilson) were not fully recognized until the 1970s (e.g., Tomback 1978). This mutualistic relationship is the predominant force in seed dispersal and regeneration of whitebark pine (Tomback and Linhart 1990; Tomback 2001) and its range-expansion patterns into formerly glaciated regions (Richardson et al. 2002). Thus, Clark's nutcracker seed-caching behavior and flight distances are major factors in determining the biogeography and maternal genetic structure of whitebark pine.

Clark's nutcracker seed-caching behavior has been studied at several whitebark pine sites. Typically, three to five seeds are buried per cache (Hutchins and Lanner 1982; Tomback 1982). Allozyme analyses of whitebark pine have provided evidence that multiple-stemmed clumps, originating from seed caches, are often composed of siblings, and most of the genetic variation in a population is found among clumps (Furnier et al. 1987; Rogers et al. 1999). Observations of nutcracker flights have documented dispersal distances of up to 22 km in piñon pine (*Pinus edulis* Engelm.; Vander Wall and Balda 1977). In whitebark pine, a maximum dispersal distance of 12 km has been observed with most flight distances ranging from 2 to 3 km (Tomback 2001). It is the frequencies of the longer flight distances that can determine dispersal capabilities for colonization and influence gene flow among whitebark pine populations.

This study takes advantage of the fact that mitochondrial DNA (mtDNA) is maternally inherited in the Pinaceae and, thus, can be used to infer seed dissemination patterns of nutcrackers. Therefore, our objectives were to (*i*) assess Clark's

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B.A. Richardson[1] and N.B. Klopfenstein. USDA Forest Service, Rocky Mountain Research Station, 1221 South Main Street, Moscow, ID 83843, U.S.A.
S.J. Brunsfeld. Department of Forest Resources, University of Idaho, Moscow, ID 83843, U.S.A.

[1]Corresponding author (e-mail: brichardson02@fs.fed.us).

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nutcracker seed dispersal within and among populations of whitebark pine, (*ii*) determine potential barriers to nutcracker seed dispersal revealed by mtDNA patterns, and (*iii*) provide information useful for the recovery and establishment of whitebark pine.

Materials and methods

DNA extraction, PCR, and polymorphism detection

Whitebark pine DNA was extracted from 75–100 mg of frozen needle or bud tissue using a rapid cetyltrimethylammonium bromide (CTAB) method (Stewart and Via 1993). This procedure was modified for use with an electric tissue homogenizer. Polymerase chain reaction (PCR) mixtures contained 0.2 mM dNTPs, 4 mM $MgCl_2$, 0.5 μM of primers, and 1 U of AmpliTaq DNA polymerase[2] (Applied Biosystems, Foster City, Calif.) with a final reaction volume of 20 μL. Amplifications were performed using the method of Wu et al. (1998) with a Techne Progene (Princeton, N.J.) thermal cycler.

Five primers (atp6, cox1, cox3, nad5a, and nad5d), developed for California closed-cone pines, were selected that flank mtDNA introns (see Wu et al. 1998). PCR products ranged from 600 to 1500 base pairs and were sequenced with an ABI 377 DNA sequencer (Applied Biosystems) at the Laboratory of Ecological and Conservation Genetics, University of Idaho. The sequences were checked and aligned with Sequencher software (Gene Codes Corp.). Corrected sequences were then compiled for each locus to detect polymorphisms. Out of approximately 5000 sequenced bases, one transversion was detected in the *nad5a* (ubiquinone oxidoreductase subunit 5) intron. The nucleotide basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) determined that this polymorphic sequence was recognized by the restriction enzyme *Mse*I, and the resulting restriction fragment length polymorphism (RFLP) produced two alleles. The remaining samples were screened with *Mse*I to determine the allele type. Haplotype frequencies were compiled for each population, and a *G* test with Williams correction for small sample sizes (Sokal and Rohlf 1995) was performed for populations that contained both haplotypes.

Study area and spatial analysis

The study area comprised seven populations of whitebark pine, located in the Cascade Range of Washington, U.S.A. (Fig. 1). This area was selected because it consisted of a contact zone of two mtDNA haplotypes, which were apparently separated during the Pleistocene by glaciation in the northern Cascades (Richardson et al. 2002). Haplotype 1 is thought to have colonized the northern Cascades from eastern Washington and northern Idaho through a corridor in north-central Washington. Meanwhile, haplotype 2 apparently advanced northward from the Oregon Cascades behind retreating glaciers (Richardson et al. 2002).

The Manastash Ridge population, which was polymorphic for both haplotypes, was selected for an intrapopulation analysis. This is a relatively large population with few multiple-stemmed trunks, and whitebark pine is seral to subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.) and mountain hemlock (*Tsuga mertensiana* (Bong.) Carrière). Whitebark pine was sampled at two scales. First, a coarse-scale transect was installed, which contained randomly selected trees of all age-classes, sampled at an interval of approximately 500 m (Fig. 2*a*). Later, a second, fine-scale transect was sampled, consisting of an additional 19 trees collected at a 100-m interval. This transect was carried out between samples sites within the coarse-scale transect (Fig. 2*b*). Sample locations were determined using a global positioning system (GPS) and mapped according to latitude and longitude coordinates. Statistical spatial analysis was conducted on the coarse- and fine-scale transects to ascertain if there was likely departure from randomness of haplotype distributions. For coarse-scale analyses, a runs test (Daniel 1990) was performed to test the null hypothesis of a random sequence in haplotypes along the transect. In the fine-scale sampling, a nearest-neighbor analysis was performed using the GHAT function in S-PLUS spatial statistics (MathSoft, Inc., Seattle, Wash., version 1.5). Simulations with random data were generated at a similar geographic scale to provide a comparison to detect any nonrandom patterns in the collected data.

Results and discussion

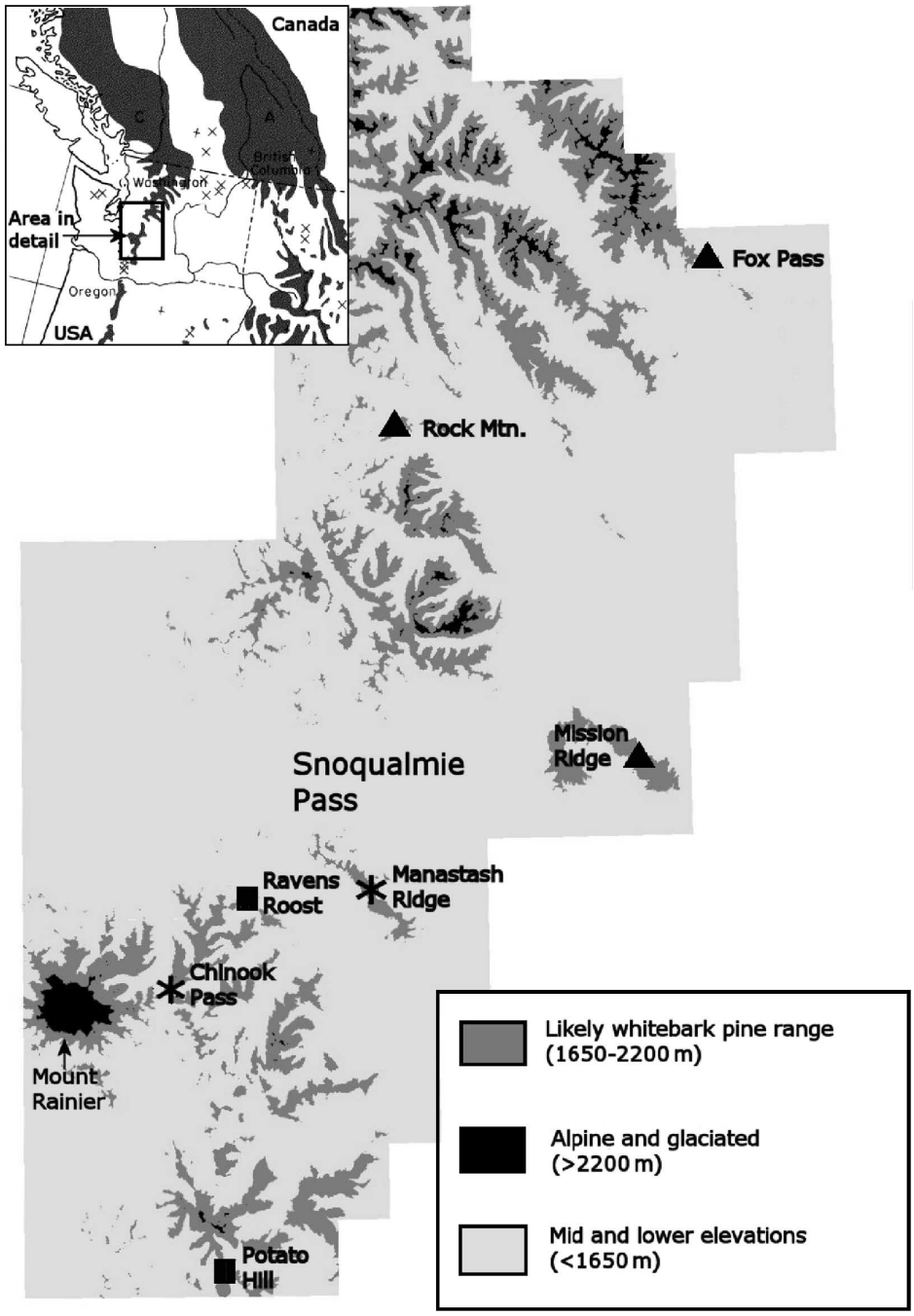
Mitochondrial DNA distribution

Mitochondrial DNA haplotype 1 was the only haplotype found north of Snoqualmie Pass into Canada, and haplotype 2 was exclusive from Potato Hill and southward into the Oregon Cascades (Fig. 1). Both mtDNA haplotypes were detected in the Manastash Ridge and Chinook Pass populations. At Manastash Ridge, haplotype 1 was most frequent, accounting for 54.5% (24 of 44) of the samples, and at Chinook Pass the proportion of haplotype 1 was low (one of nine or 11%). A *G* test (Sokal and Rohlf 1995) conducted between Manastash Ridge and Chinook Pass showed a significant difference in haplotype frequencies ($p = 0.014$). In the Ravens Roost population, 18 km to the southwest of Manastash Ridge, haplotype 1 was absent in a sample size of 27. Haplotype 1 was not detected in 10 samples collected at Potato Hill (41 km to the south) or in sampling from the Oregon Cascades.

To explore the capacity of seed movement within a population, sampling was conducted at two spatial scales within the Manastash Ridge population. At the larger coarse scale (Fig. 2*a*), a runs test did not indicate any significant deviation from a random distribution of the two haplotypes ($p = 0.089$). To test further for a nonrandom pattern of haplotypes, a more intensive fine-scale sampling along a 1-km transect between collection sites 6 and 9 in the coarse scale was conducted to test for spatial clustering of haplotypes (Fig. 2*b*). Among the 19 whitebark pine trees sampled, nearest-neighbor spatial analysis did not detect any departures from a random pattern in the haplotype distribution. At these two scales, intrapopulation analysis of mtDNA haplotypes suggests that nutcracker-mediated seed dispersal provides a locally heterogeneous maternal structure. These

[2] The use of trade names in this publication does not constitute an official endorsement by the USDA Forest Service.

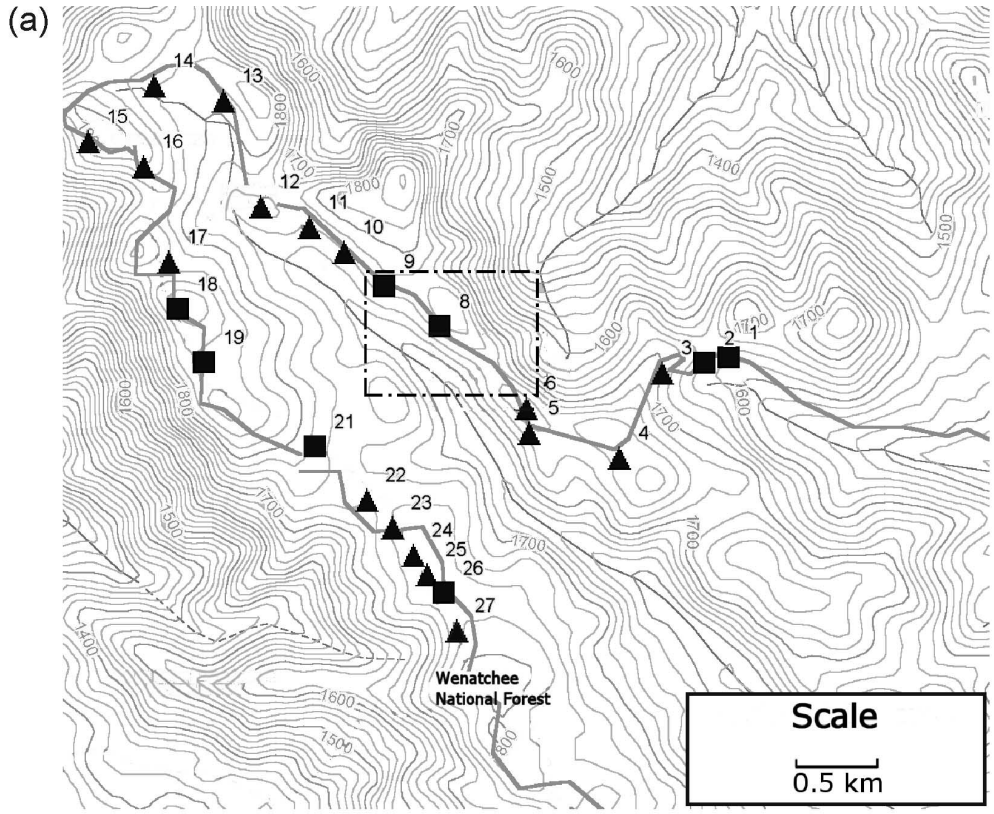
Fig. 1. Inset shows a wider scale of whitebark pine range in Washington, Idaho, and British Columbia and the study area (Little 1971). The study area shows the likely range of whitebark pine based on elevation and sample locations in the Washington Cascades. ▲, haplotype 1; ■, haplotype 2; *, polymorphic populations.

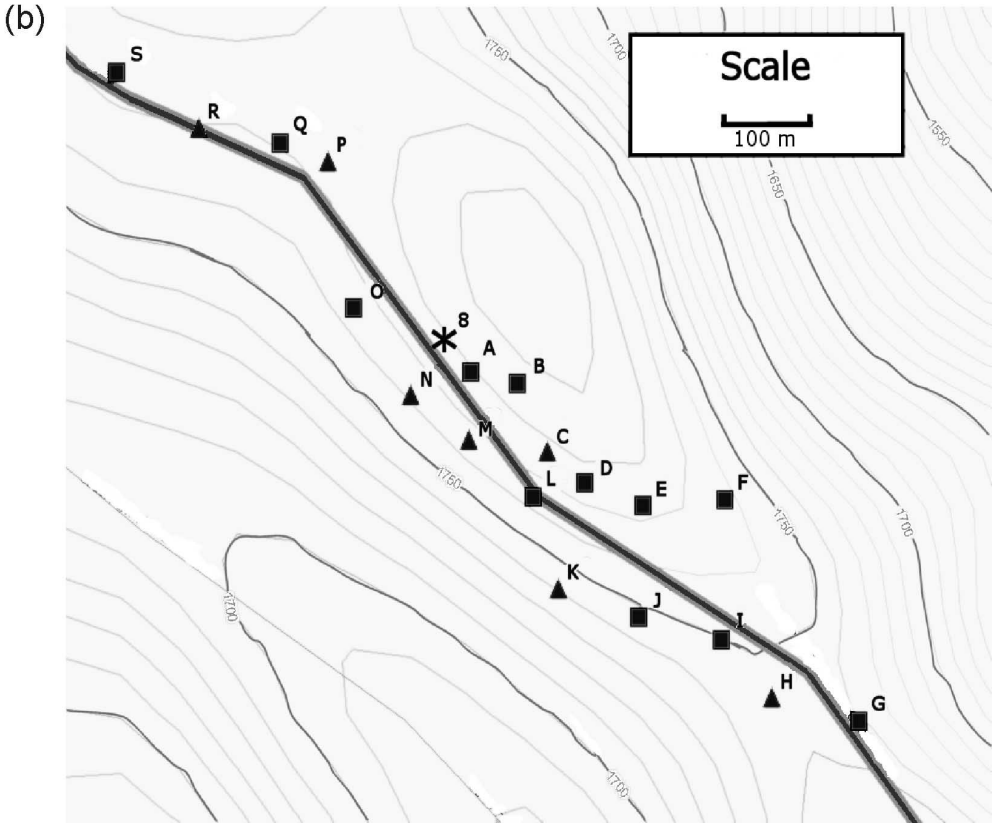


patterns differ from the locally homogeneous fine-scale spatial clustering of mtDNA haplotypes found in wind-dispersed seed of *Pinus ponderosa* Dougl. ex P. & C. Laws. (Latta et al. 1998).

Genetic structuring of mtDNA haplotypes in whitebark pine becomes more apparent as comparisons are made among populations. Because whitebark pine is strictly subalpine (ca. 1650–2200 m elevation in the Cascades), habitats and populations are often isolated on mountain-top islands (Fig. 1). The lack of heterogeneity in the frequencies of haplotypes at Mission Ridge, Manastash, and Ravens Roost suggests reduced seed dispersal among these disjunct populations. These data are consistent with observed seed-dispersal flight distances of Clark's nutcracker rarely exceeding 12 km for whitebark pine (Tomback 2001). Mission and Manastash Ridge are separated by Snoqualmie Pass; one of the largest gaps (ca. 30 km wide) in the distribution of subalpine habitat in the Washington Cascades. This lower elevation pass has apparently acted as a barrier for the northward expansion of haplotype 2 and, perhaps, permitted only

Fig. 2. (*a*) Coarse-scale whitebark pine tree sample locations and haplotypes at Manastash Ridge. The area within the box is shown in detail in Fig. 2*b*. (*b*) Fine-scale sampling of whitebark pine at Manastash Ridge. ▲, haplotype 1; ■, haplotype 2; *, reference point (sample 8 from Fig. 2*a*).





rare southward dispersal events of haplotype 1. Other smaller gaps (18 km) between Manastash Ridge and Ravens Roost have also apparently reduced seed dispersal.

In contrast, pollen dispersal is apparently unrestricted among these populations. Paternally inherited chloroplast microsatellite markers indicated high gene flow via wind-dispersed pollen among Mission Ridge, Manastash Ridge, and Ravens Roost (F_{ST} < 0.006; Richardson et al. 2002). These data are consistent with high pollen dispersal reported in other pine species (Latta and Mitton 1997; Latta et al. 1999). Thus, it is likely that the nutcracker's contemporary role consists more of facilitating regeneration of whitebark pine, while most interpopulation gene flow occurs via wind-dispersed pollen.

The mtDNA distribution associated with seed dispersal by Clark's nutcracker provides some useful information for the management and recovery of whitebark pine populations. It has been argued that the easiest means of promoting whitebark regeneration is the creation of canopy openings by silvicultural treatment, which could be exploited by nutcrackers (Hoff et al. 2001). Our data suggest that canopy openings (e.g., silvicultural treatments) should be in close proximity to seed-producing trees for successful regeneration via nutcrackers. This study also provides additional evidence of the essential ecological role that nutcrackers play in the regeneration and dispersal of whitebark pine.

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