Evolutionary relationships of the 'sky island' pines (*Pinus* subsection *Ponderosae*) based on nuclear and plastid microsatellite loci

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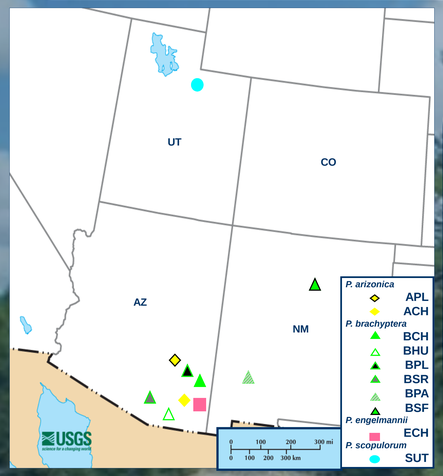
Introduction

The taxonomy of *Pinus* subsection *Ponderosae* remains largely unsettled. Recent evidence suggests that *P. scopulorum* Lemmon 1897 from the northern Rockies is distinct from *P. ponderosa* Douglas ex. P. & C. Lawson 1836 [1] and that the ponderosa pines on a high-altitude 'sky island' (Mt. Lemmon, AZ) may not belong to either *P. ponderosa* or *P. scopulorum*. Rather, the Mt. Lemmon pines resolve as sister to several *Ponderosae* from south central Mexico on low-copy nuclear gene trees [2].

Pinus scopulorum has predominantly 2 needles per bundle. Despite no obvious discontinuity, the pines of the southern Rockies, as well as the sky island pines, have predominantly 3 needles per bundle. If these 3-needle pines are distinct from *P. scopulorum*, then *P. brachyptera* Engelmann 1848, collected near Santa Fe, NM may be their appropriate classification.



Objectives

We consider four alternative hypotheses:

H_1: The sky island, southern, and northern Rockies pines are homogeneous.

H_2: The sky island pines fit within an extensive southern Rockies group, distinct from the northern Rockies.

H_3: The sky island pines of Arizona are distinct from the southern Rockies pines of New Mexico.

H_4: Introgressive hybridization between sky island pines and sympatric *P. arizonica* Engelmann and/or *P. engelmannii* Carriere has occurred.

Methods & Results

			13 Polymorphic cpSSR Loci				5 Polymorphic nSSR Loci			
OTU	Pop	Locale	N	N_h	N_p	H_e	N	N_a	N_p	H_e
P. arizonica					**11**				**2**	
	APL	AZ: Pinalenos	14	12	10	0.978	23	23	2	0.535
	ACH	AZ: Chiricahuas	12	5	1	0.742	10	16	0	0.446
P. brachyptera					**77**				**10**	
Arizona Sky Islands:										
	BCH	AZ: Chiricahuas	24	18	12	0.967	26	23	0	0.532
	BHU	AZ: Huachucas	28	21	10	0.976	30	23	1	0.477
	BPL	AZ: Pinalenos	29	18	6	0.948	25	21	1	0.512
	BSR	AZ: Santa Ritas	22	16	7	0.948	30	21	3	0.509
New Mexico:										
	BPA	NM: Pinos Altos	19	18	7	0.994	20	20	2	0.499
	BSF	NM: Santa Fe	28	22	18	0.974	30	25	1	0.455
P. engelmannii					**16**				**0**	
	ECH	AZ: Chiricahuas	25	16	16	0.930	21	19	0	0.433
P. scopulorum					**12**				**4**	
	SUT	UT: Salt Lake City	18	14	12	0.967	26	21	4	0.466
	Total (no missing data)		219				241			
	Mean		22	16	10	0.943	24	21	2	0.486

OTU, operational taxonomic unit; N, no. of individuals; N_h/N_a, no. of haplotypes/alleles; N_p, no. of private haplotypes/alleles; H_e, expected heterozygosity.

Methods: DNA was isolated (Dneasy; Qiagen) from 258 samples representing 10 populations. Multiplexed PCR (Qiagen) used a 3-primer protocol with fluorescently labeled 3rd primers. Primers for cpSSRs and nSSRs were developed for other pine species [3-8]. We performed PCRs in 4 batches: a) 15 pooled cpSSRs at 58° annealing; b) 2 pooled nSSRs (PtTX2128, PtTX3025) at 53° annealing; c) 4 pooled nSSRs (PtTX2123, PtTX3030, PtTX3098, PpWD40) at 60° annealing; d) LOP5 at 53° annealing. Fragments were genotyped (1 pool for 15 cpSSRs and 1 pool for 7 nSSRs) using a 4-color dye set (ABI3130xl; University of Arkansas, Fayetteville).

Results: One cpSSR locus was excluded (nonspecific amplification) and 1 was monomorphic, yielding 13 polymorphic cpSSR loci that revealed 122 haplotypes for 219 individuals with no missing data; 116 cpSSR haplotypes were private to an OTU. Two nSSR loci are incomplete; results for 5 polymorphic loci are reported here. We used 45 alleles in 241 individuals with no missing data for these preliminary analyses; 16 nSSR alleles were private to an OTU.

Plastid Results

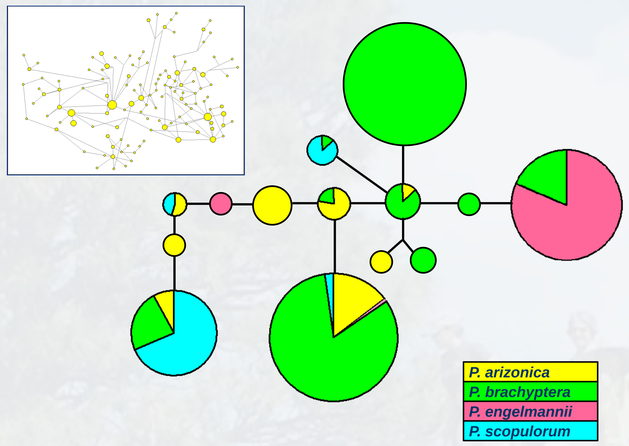


Fig. 1. Plastid SSR haplotype network. Circle sizes are proportional to number of individuals. Based on a Reduced Median network (inset; NETWORK software; Fluxus Engineering) of 122 haplotypes, simplified by collapsing related nodes.

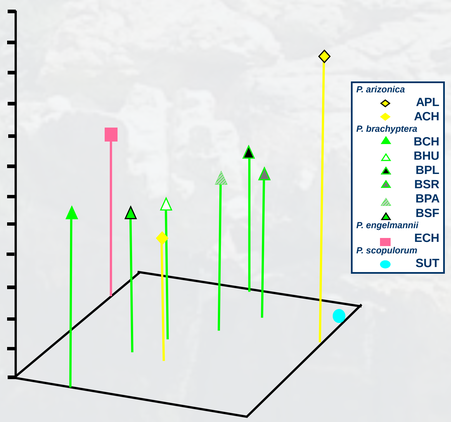


Fig. 2. Principal Components (PCA) based on VARCOV matrix of multi-locus cpSSR haplotypes (NtSys).

Nuclear Results

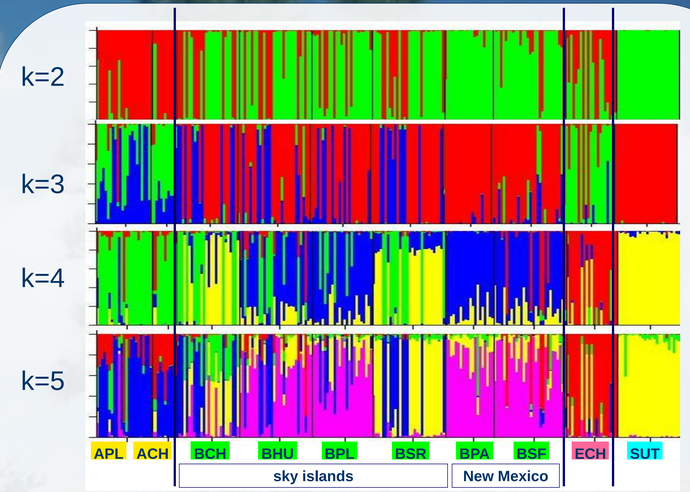


Fig. 3. Assignment of individuals to 2, 3, 4, or 5 groups based on multi-locus nuclear alleles (STRUCTURE) . Colors are arbitrary and do not reflect assignment to OTU.

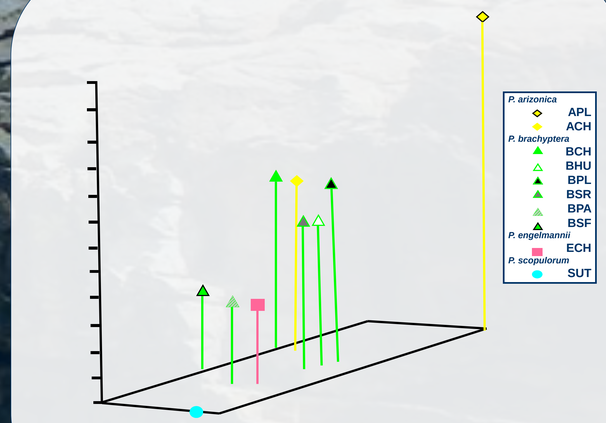


Fig. 4. Principal Components (PCA) based on VARCOV matrix of multi-locus nSSR alleles (NtSys).

Conclusions

H_1: *Pinus scopulorum*, represented here by one population from Utah, is distinct (SUT; Figs. 2, 3, 4), despite sharing some related haplotypes (Fig. 1). Therefore, we reject the hypothesis that these pines are homogeneous. The relationship between the northern and southern Rockies populations of ponderosa is an interesting question that we will pursue with collaborators.

H_2: We cannot reject the hypothesis that the sky island pines of Arizona fit within the wide-ranging southern Rockies ponderosa pines (Fig. 2).

H_3: We have weak signal supporting the hypothesis that the sky island pines may be distinct from the southern Rockies. The nSSR assignment test at k=4 (Fig. 3) fits our 4 putative OTUs quite well if the 2 populations from New Mexico (BPA, BSF) are taken to represent *P. brachyptera*. In this scenario, individuals from the 4 sky islands have unclear group assignments. They are also somewhat separate on the nSSR PCA (Fig. 4). We will reexamine this alternative after we genotype a population from Mt Lemmon, AZ.

H_4: Based on PCA (Figs. 2, 4), the Chiricahua population of *P. arizonica* (ACH) appears more closely related to *P. brachyptera* than to the Pinalenos (APL). However, individual assignment (Fig. 3) groups these 2 *P. arizonica* populations together. Because of sympatry, our results could be explained by introgression or by sample misidentification of immature trees in the field.

The high number of polymorphic loci and haplotypes reported here are typical of multi-locus cpSSRs in pines [9]. Despite an abundance of private haplotypes, the simplest network implies lineages that are shared among OTUs (Fig. 1). Homoplasy, as well as shared ancestral polymorphism, likely contribute to this pattern. Nevertheless, multivariate analyses of cpSSR frequencies are revealing (Fig. 2) and a comparison of results from plastid (paternally inherited in pines) and nuclear genomes for the same samples is useful.

We are in the process of completing genotypes for 2 additional polymorphic nSSR loci. We found that multiplexed PCRs were extremely efficient for cpSSRs, but more challenging for nSSRs. We have also found length variation in mtDNA (maternally inherited) that may enhance our data set.

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References: [1] Gernandt et al. 2009. Sys. Bot. 34:481; [2] Epperson et al. 2009. Am. J. Bot. 96:707; [3] Vendramin et al. 1996. Mol. Ecol. 5:595; [4] Stoehr & Newton. 2002. Can. J. For. Res. 32:469; [5] Liewlaksaneeyanawin et al. 2004. Theor. Appl. Genet. 109:361; [6] Elsik et al. 2000. Genome 43:550; [7] Kutil & Williams. 2001. J. Hered. 92:327; [8] Willyard et al. 2009. Mol. Phylo. Evol. 52:498.; [9] Marshall et al. 2009. Theor. Appl. Genet. 104:367.