Taxonomy of Douglas fir (*Pseudotsuga menziesii*) infraspecific taxa: vars. *menziesii, glauca* and *oaxacana*: nrDNA, cpDNA sequences and leaf essential oils

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ABSTRACT

Six chloroplast DNA regions of Douglas fir (*Pseudotsuga menziesii*) from trees in 11 populations from Washington southward to Oaxaca, Mexico were sequenced yielding 4857 bp of data as well as sequences for nrDNA from *P. macrocarpa*, *P. menziesii* var. *menziesii*, (3 populations) and var. *glauca* (partial, NM population). *Pseudotsuga macrocarpa* was included as an outgroup. The nrDNA grouped the three var. *menziesii* separate from var. *glauca* (NM), even with only partial sequences. Very little variation was found among the populations of *P. menziesii* but the cpDNA did give support for the recognition of var. *menziesii* and var. *glauca*, with some support for var. *oaxacana*. However, for the cpDNA data, the population of *P. m.* var. *menziesii* on serpentine soil in sw Oregon had its highest affinity to var. *glauca* in Arizona. In contrast, a previous study using terpenoids (Adams et al. 2012) clearly placed the serpentine soil Oregon trees with var. *menziesii*. The Oregon population may be introgressed by the chloroplast from inland (var. *glauca*) germplasm, leading to these results. The var. *oaxacana* differed by 3 MEs (mutational events) from var. *glauca*, and by 2 MEs from a tree in nearby El Chico NP, Hgo. *Pseudotsuga menziesii* var. *glauca* appears very uniform for these cpDNA markers from Wyoming to Arizona, New Mexico, and into northern Mexico. The terpenoid data seem to reflect the status of current evolution and the cpDNA data indicate ancestral relationships.
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KEY WORDS: *Pseudotsuga menziesii,* var. *menziesii,* var. *glauca,* var. *oaxacana,* taxonomy, nrDNA, cpDNA sequences, Douglas fir, *Pseudotsuga macrocarpa.*

Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] is a wide-ranging, common forest tree in North America (Fig. 1). The nomenclatural history of the name is a morass, but seems to have been settled by James Reveal (see http://www.plantsystematics. org/reveal/pbio/LnC/dougfir.html).

In a recent treatment, Eckenwalder (p. 572, 2009) recognizes two varieties: var. *menziesii* and var. *glauca* (Mayr) Franco [cited as (Beissn.) Franco in Eckenwalder, 2009]. Eckenwalder (2009) did not recognize var. *oaxacana* Debreczy & Racz, described from Oaxaca (Debreczy and Racz, 1995).

The leaf essential oils of *P. menziesii* have been exhaustively studied by von Rudloff (1972, 1973, 1984) and von Rudloff and Rehfeldt (1980) who carefully documented the large differences in oil composition between coastal (var. *menziesii*) and inland (var. *glauca*) varieties.

A second team from USDA, Forest Products, Richmond, CA (Snajberk and Zavarin), conducted extensive studies of the terpenoids from the oleoresin of Douglas fir (Snajberk, Lee and Zavarin, 1974; Snajberk and Zavarin, 1976; Zavarin and Snajberk, 1973, 1975). In their most comprehensive study (Snajberk and Zavarin, 1976), they found four chemical races: coastal, northern inland, southern inland and Sierra Nevada. These are shown in Fig. 1, along with populations used in the present study.

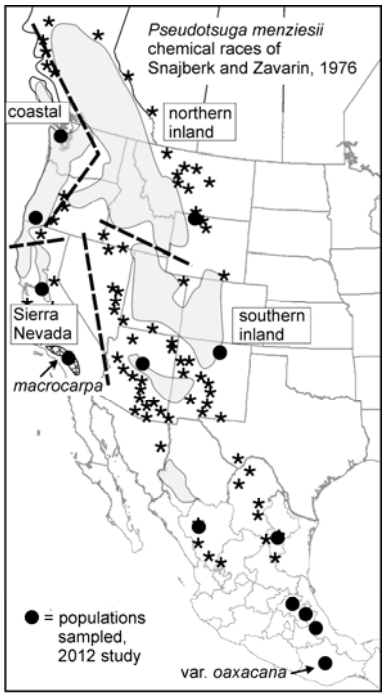

Figure 1. Distribution of *P. menziesii* with chemical races (dashed lines) of Snajberk and Zavarin (1976). Stars denote outlying locations.

There has been previous work at the molecular level on Douglas fir in Mexico. Li and Adams (1989) reported that allozymes divided the Douglas fir into northern coastal (var. *menziesii*) and inland (var. *glauca*) groups, and further divided the inland group into two subgroups (northern and southern inland). They did not find evidence of a subgroup of Sierra Nevada Douglas fir, as Snajberk and Zavarin (1976) found, this based on the oleoresin oils. In addition, Li and Adams (1989) found a distinct pattern in the allozymes from population 103 at General Cepeda, Coah., MX and speculated that it might be *P. flahaultii* Flous (also recognized by Martinez, 1963). However, a nearby collection (104, La Encantada, near Zaragoza, NL) clustered closely with *P. menziesii* from New Mexico, making this an unresolved case.

Gugger et al. (2010) examined mtDNA and cpDNA sequences and found support for coastal (var. *menziesii*) and inland (var. *glauca*) divisions in the United States and Canada. No evidence was found for a Sierra Nevada taxon, but mtDNA suggested the inland (var. *glauca*) taxon might be divided into northern and southern groups. In a subsequent study, Gugger et al. (2011) examined Douglas fir from Mexico. They found considerable divergence in cpDNA from Cerro Potosi, NL and Jamé, Coah. and from other Mexico populations. CpSSRs supported two clades in Mexico (Gugger, Fig. 4c), but that pattern was not recovered with mtDNA (Gugger, Fig. 4a) or cpDNA (Gugger, Fig. 4b) data. In summary, they concluded that "Mexican populations were genetically distinct from USA and Canadian populations, but more closely related to the Rocky Mountain variety than the coastal variety". As Gugger et al. (2010) did not show data from Mexico, and Gugger et al. (2011) showed only data from that country, it is difficult to ascertain the relationship of Mexican populations to those of the USA.

Phenotypic analyses (Reyes Hernández et al. 2006) revealed that *Pseudotsuga* populations of northern Mexico are morphologically similar to *P. menziesii* var. *glauca* from southwestern USA, but the populations from central Mexico differed. They also found a population of NE Mexico (San Francisco) morphologically separated from the rest, even from those of the same geographical region, suggesting an effect of microhabitat selection. This population is just 15 km NW from the one from Cerro Potosi analyzed here.

The leaf terpenes of *Pseudotsuga menziesii* were analyzed by Adams et al. (2012) from throughout its range (Fig. 1). They found (Fig. 2) that terpenes separated Douglas fir into the two classical varieties: var. *menziesii* (coastal) and var. *glauca* (inland). In addition, there appeared to be a slight differentiation between the Yellowstone, AZ-NM and central Mexico populations (Fig. 2). The Cerro Potosi leaf oils also showed some local differentiation (Fig. 2). All three of the populations of *P. menziesii* var. *menziesii* from the coast were very similar in their oils (Fig. 2). In addition, they reported a north-south cline from Wyoming to southern Mexico but found little support for var. *oaxacana* (Fig. 3).

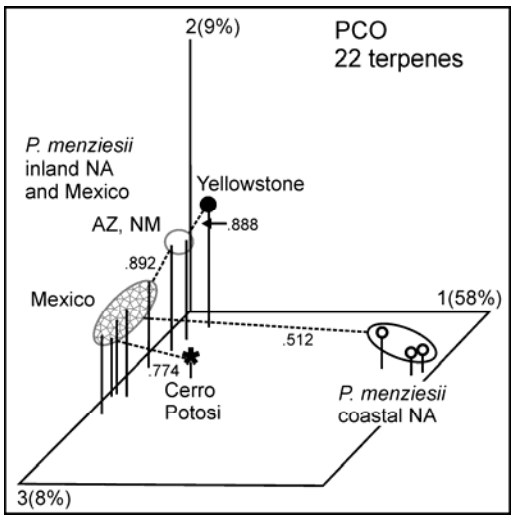

Figure 2. PCO based on 22 terpenes (from Adams et al. 2012).

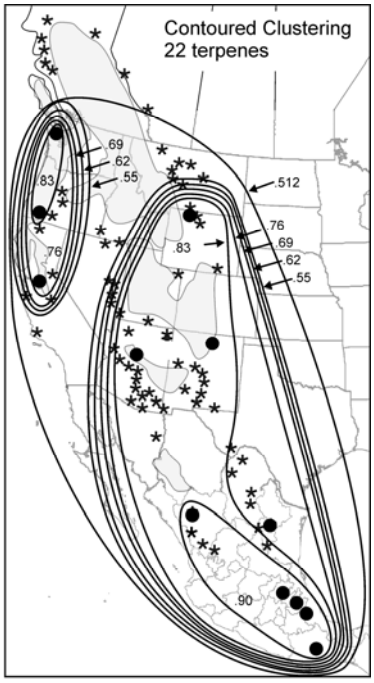

Figure 3. Contoured clustering (from Adams et al., 2012)

The present study was undertaken to complement the terpenoid study of Adams et al. 2012) by sampling the same populations and analyzing six cpDNA regions from the USA and Mexico.

MATERIALS AND METHODS

Plant material (Fig. 4): *P. menziesii* var. *menziesii* (coastal/ Sierra Nevada): *Adams 13239-13240*, Olympic National Park, 48° 02' 48.1"N, 123° 25' 04.08"W. elev. 1720 ft, *Adams 12745-12757*, on serpentine soil, Oregon Mtn., OR, 41º 59' 59.1" N, 123º 47' 10.2" W, 895 m; *Adams 12779-12783*, 6 km e of Buck Meadows, CA, 21 km w of Yosemite NP on US 120, 37º 49.579' N, 119º 58.421' W, 1150 m. var. *glauca*: *Adams 12556-12560*, 13 km w of Cimarron, NM on US 64, 36.54684º N, 105.03321º W, 2125 m; *Adams 12744-12748* (ex *D. Thornburg, 1-5*), 9 km ne of Pine, AZ on Hwy 87, 34º 27.422' N, 111º 24.115' W, 2250 m; *Adams 12818-12822*, 20 km e of Yellowstone NP, on US 14 at the Palisades, 44.45448º N, 109.78182º W, 1910 m; *Adams 13056-13060*, (ex *M. Socorro González Elizondo 7777a-e*), Cerro Potosi, NL, 24º 53' 9" N, 100º 13'14" W, 3141 m.; *Adams 13236-13238* (ex. Marie Deslauiers, Quebec), Cerro Catana, Coah., *Adams 13061-13066*, (ex *Martha González Elizondo 4408-4409, 4413-4416*) Los Altares, Dur., 25º2'56" N, 105º59'48" W, 2310 m; *Adams 13082-13087* (ex *Vargas-Hernandez J1-J6*), El Chico Natl. Park, Mineral del Chico, Hgo., 20º 10' 16" N, 98º 43' 55" W, 2,765 m, var. *oaxacana*: *Adams 13101-13103, 13105-13106* (ex *Vargas-Hernandez I1-I6*, Paraje Peña Prieta, Oaxaca, 17º 09' 38" N, 96º 38' 07" W, 2,700 m.

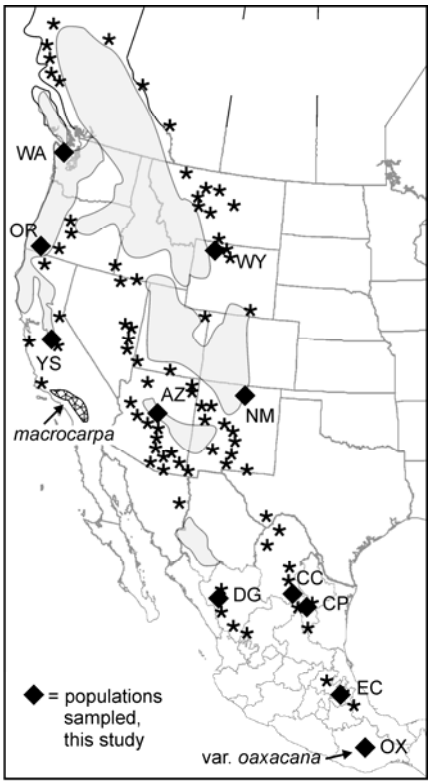

Figure 4. Populations sampled in this study, including *P. macrocarpa*.

P. macrocarpa: *Adams 12776-12778*, USFS Eddy Arboretum, Placerville, CA. Voucher specimens are deposited in the Herbarium, Baylor University (BAYLU, CIIDIR and CHAPA).

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20^{o} C until the DNA was extracted. DNA was extracted from leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions.

PCR amplification: Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. nrDNA was amplified by use of standard ITSA, ITSB, ITS739F, ITS739R, CAA123F primers (Adams et al. 2011).

Chloroplast primers were synthesized based on GenBank sequences for *Pseudotsuga menziesii* and *P. macrocarpa* for the following cp regions (name, forward and reverse primer, Tm):

psbA-matK:	psbA57f	GTTTTCGGTGCTAGTAATC	matK16r	CAGGATCTGAAAGTAGAAAA, 50°C
trnM-trnS:	trnM30f	AAGGCTCATAACCTTGAG	trnS1006r	TACTATACCGGTTTTCAAGA, 50°C
psaJ-petG:	psaJ8f	TGGAAAGATAGGTCTTTAGAT	petG27r	AACTGCATATTCACAATACC, 50°C
rbcL-atpB:	rbcL20f	AATCCGACACTAGCTTTAG	atpB724r	AATACGTCCCACATTTTT 50°C
petN-rpoB:	petN25f	AAAAACTACCATTAAAGCAG	rpoB6r	CTCCCTCATTTTCATCTAAT 50°C
trnS-clpP:	trnS32f	CGTACTACGGATTAGCAA	clpP11r	AGGAACTTTTGGGACAC 50°C

The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.). Minimum spanning networks were constructed from mutational events (ME) data using PCODNA software (Adams et al., 2009; Adams, 1975; Veldman, 1967).

RESULTS AND DISCUSSION

The nrDNA proved recalcitrant in sequencing for var. *glauca* and all the materials from Mexico. Repeated efforts and the synthesis of gene-walking primers failed to generate complete sequences. Complete sequences for all the var. *menziesii* and *P. macrocarpa* plants were obtained, but only var. *glauca* from New Mexico (NM) yielded partial sequences. Analysis revealed one (1) substitution and two (2) indel differences between var. *menziesii* (WA, OR, YS) and var. *glauca* (NM). A Neighbor Joining tree (NJ) showed high support for the vars. *menziesii* and *glauca*, even with partial sequences from the NM plants.

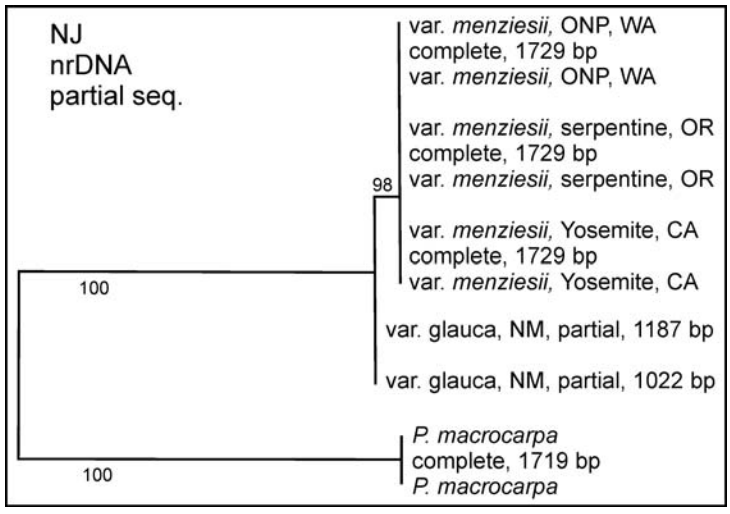

Figure 5. NJ analysis based on nrDNA. Note the partial sequences for the NM plants (1187, 1022 bp).

Sequencing the six cpDNA regions resulted in few mutations (Table 1). Most substitutions were either between *P. macrocarpa* and *P. menziesii,* or mutational events that were only found once.

Table 1 Chloroplast DNA regions sequenced in Douglas fir. subs = substitution event.

cp region	length(bp)	# subs.	# indels	# mut. events (ME)	comments
psbA-matK	814	6	1	7	6 subs. in *P. macrocarpa*
trnM-trnG-psbZ-trnS	932	11	3	14	3 subs. in *P. macrocarpa*
psaJ-petG	781	3	1	4	
rbcL-atpB	731	3	1	4	
petN-rpoB	795	3	3	6	2 subs. in *P. macrocarpa*
trnS-clpP	804	6	2	8	2 subs. in *P. macrocarpa*
totals	4857	32	11	43	

The most variable region was trnM-trnG-psbZ-trnS with 14 MEs (Table 1) and psaJ-petG and rbcL-atpB were the least variable regions with only 4 MEs. Of the 32 substitutions, only 13 were found multiple times in *P. menziesii*. Of the 11 indels, only 2 were present multiple times in *P. menziesii.*

A Neighbor-Joining (NJ) analysis resulted in a tree with very low boot strap values for most branches (Fig. 6). There is support for var. *menziesii* (59) and var. *oaxacana* (56) and limited support for var. *glauca*. This is in strong contrast to the terpenoid data (Fig. 2). The population on serpentine soil in Oregon (OR, Fig. 3) is not in the clade with var. *menziesii* from Olympic NP and Yosemite NP (Fig. 6). The two plants from Yellowstone, WY are somewhat separated (Fig. 6).

However, it should be noted that several studies have shown that var. *menziesii* and var. *glauca* hybridize and form introgressants inland from the coast to the rocky mountains (von Rudloff 1972; 1973; 1984; von Rudloff and Rehfeldt 1980). It is very likely that our samples may contain some introgressed individuals. If so, then a phylogenetic algorithm is not the best choice for analysis of this data set.

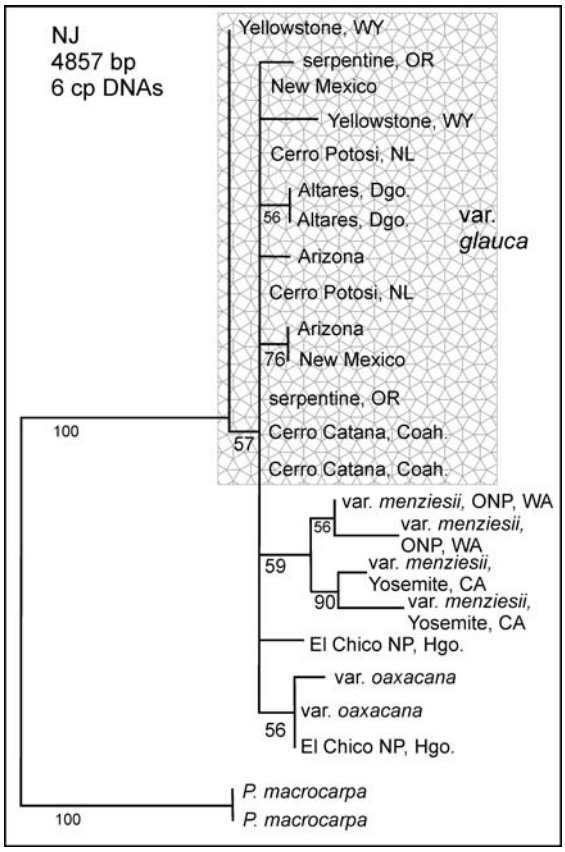

Figure 6. NJ based on 4857 bp of cpDNA data.

A UPGMA analysis of the data revealed a slightly different pattern (Fig. 7) in which vars. *menziesii*, *glauca* and *oaxacana* are resolved into groups. Again, the serpentine soil, OR plants are grouped with var. *glauca*. Interestingly, var. *oaxacana* is now grouped with the other Mexican populations, except for the Cerro Catana plants (CC, Fig. 3) that are grouped with the inland plants of AZ, NM and WY (Fig. 7).

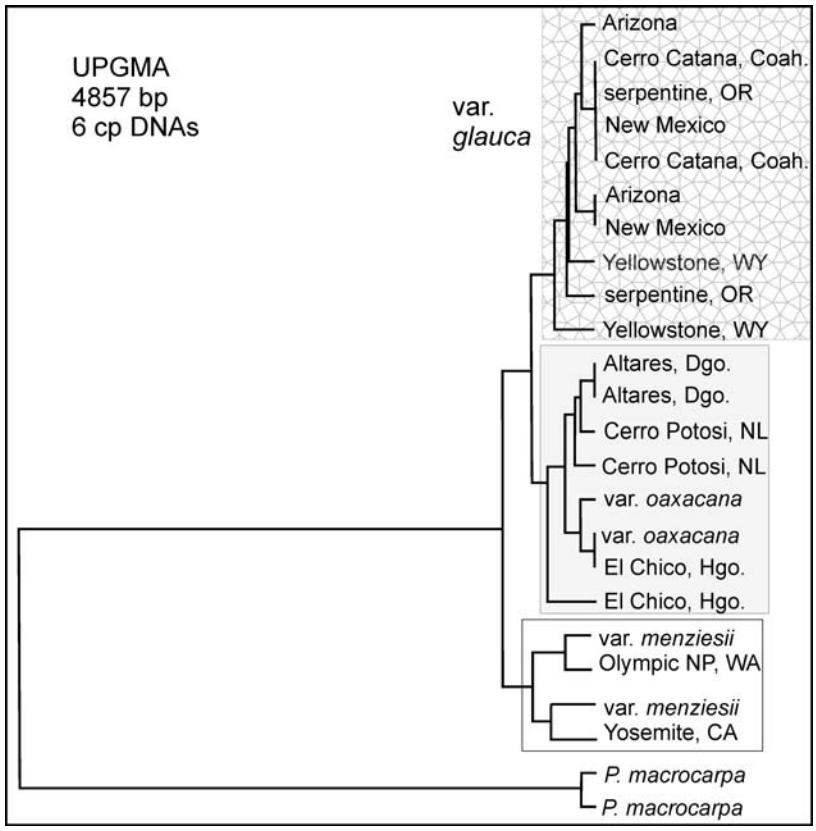

Figure 7. UPGMA based on 4857 bp from 6 cpDNAs

Additional insight can be obtained by examination of the mutations in a minimum spanning network (MSN). This analysis involved only the 15 mutations that occurred more than once in the individuals of *P. menziesii*. Of these 15 MEs, 13 were substitutions and 2 were indels. The MSN shows that by DNA data var. *menziesii* consists of only the Olympic NP and Yosemite, CA plants (Fig. 8) and within the Olympic NP plants (WA), there are 3 MEs. That group is separated by only 4 MEs from var. *glauca* / var. *oaxacana*. In short, it is clearly not well defined by cpDNA data. The entire var. *glauca* group from the Rocky Mountains to northern Mexico differs among populations by only 0, 1 or 2 MEs (Fig. 8). There is a hint of divergence in the central Mexico populations of Durango and Cerro Catana, as they differ by 2 MEs, and the group differs from other var. *glauca* by only 2 MEs (Fig. 8). The var. *oaxacana* group is separated by 3 MEs, but in itself, is diverse, having 2 MEs differences compared to a plant from El Chico, which differs by 3 MEs from another El Chico plant (Fig. 8).

Mapping the MSN onto a map gives a geographic perspective (Fig. 9). Very few mutations separate the populations of Douglas fir from OR, WY, AZ, NM, CC and CP. The Douglas fir on serpentine soil (southern Oregon, OR) differs by only 1 ME from Arizona (var. *glauca*, Fig. 8), however, its terpenes were like those of coastal Douglas fir (var. *menziesii*, Fig. 2 above). It may be that the Oregon population has been introgressed by inland Douglas fir (var. *glauca*).

There were no differences between Arizona (AZ) Douglas fir and Cerro Potosi (CP, Fig. 9); and only one ME difference was found between AZ and CC (Cerro Catana, Fig. 9). Clearly, the cpDNA data show very few changes in sequences in these intergenic regions from Wyoming southward to northern Mexico.

The Altares, Dgo. (DG) trees differ by 2 MEs from Cerro Potosi populations (CP, Fig. 9), indicating they are part of the var. *glauca* complex (at least in these cpDNA data).

The results from 6 cpDNAs are similar to those of Wei et al. (Fig. 1, 2011) who, using only trnfM-trnS data, found haplotypes characteristic of var. *menziesii* (coastal, Pacific Northwest), var. *glauca* (inland, northern Rockies to southern New Mexico) and var. *oaxacana* (central Mexico to Oaxaca). Using data from the first intron of nad7 (mt DNA), they found haplotypes supporting var. *menziesii* (coastal, Pacific Northwest) and var. *glauca* (inland, northern Rockies to southern Mexico), with no support for var. *oaxacana*. It is of interest that they date the split of var. *menziesii* from *glauca* as 8.5 Ma

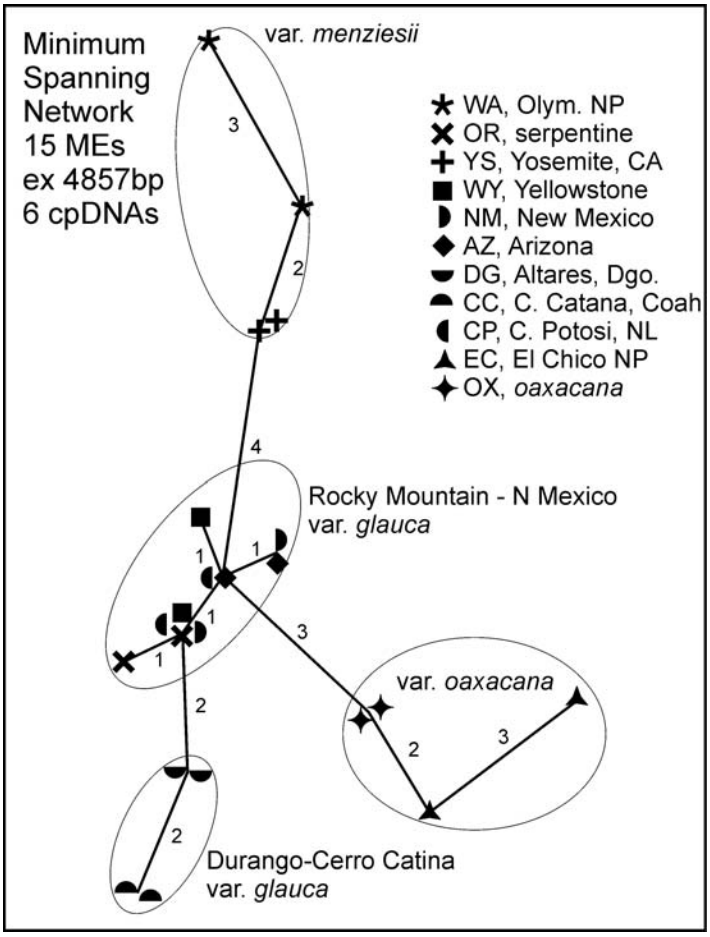

Figure 8. Minimum spanning network based on 15 MEs.

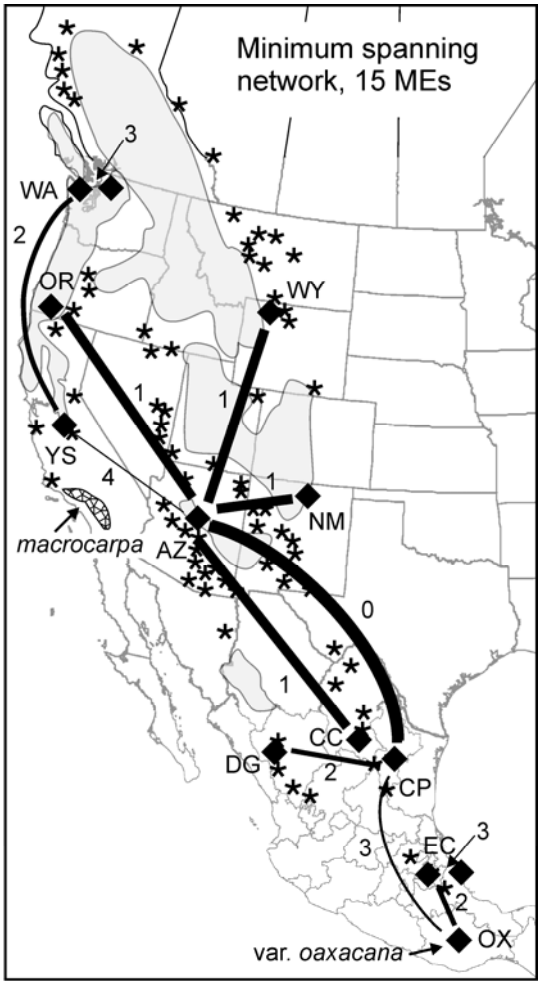

Figure 9. MSN on geographic map. Numbers next to lines are # MEs. Line width is inversely proportional to # ME differences.

and divergence of the Mexican populations as 3.2 - 4.8 Ma. Syring et al. (2007) concluded that the presence of shared haplotypes among *Pinus* species was due to incomplete lineage sorting. They estimated that reciprocal monophyly will be more likely than paraphyly in 1.7 to 2.4 Ma with complete genome-wide coalescence in species in up to 76 Ma. If Wei et al. (2011) are correct about the ages of the divergence of *Pseudotsuga* varieties (3.2 to 8.5 Ma), and Syring et al. (2007) are correct in their dates, then it is not unexpected that one would find ancestral haplotypes from one variety in the genome of another variety. This could explain our finding of var. *glauca* haplotypes in the southern Oregon population. Wei et al. (2011) did not find var. *glauca* haplotypes in their southern Oregon population, but they did find var. *glauca* haplotypes in their central Oregon population (Wei et al. (Fig. 1, 2011). Their trnfM-trnS data showed a similar result.

The difference in the terpene data, the nrDNA and the cpDNA data for the southern Oregon, serpentine population is a major difference in the two studies. The terpenes from the Oregon population are most similar to var. *menziesii* from Olympic NP, WA (Fig. 2 above and Adams et al. 2012). Adams and Stoehr (2012) showed that the terpenes from hybrids between var. *menziesii* (coastal) and var. *glauca* (inland) are more like var. *glauca* (inland) than var. *menziesii*, due to dominant inheritance towards var.

glauca. If the southern Oregon population is of hybrid origin (var. *menziesii* x var. *glauca*), then one might expect the terpenes to be like var. *glauca*. But this is not the case. The terpenes from the Oregon population are most similar to var. *menziesii* from Olympic NP, WA. Additional research is needed to resolve this problem.

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