RE-EXAMINATION OF THE TAXONOMY OF *JUNIPERUS FLACCIDA* VAR. *MARTINEZII*, AND VAR. *POBLANA* (*CUPRESSACEAE*)

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ABSTRACT

Recent DNA sequencing data have shown that *J. flaccida* var. *flaccida, J. f.* var. *martinezii* and *J. f.* var. *poblana* are polyphyletic taxa. Additional analysis using Random Amplified Polymorphic DNAs (RAPDs) analyses for *J. durangensis*, *J. flaccida* var. *flaccida*, var. *martinezii*, and var. *poblana*, *J. jaliscana*, *J. monticola* and *J. standleyi* revealed exactly the same pattern of relationships as seen with the sequence data. Specifically, that *J. flaccida* and its varieties are polyphyletic. Upon re-examination of the morphology, *J. f.* var. *martinezii* and var. *poblana* are recognized as distinct species: *J. martinezii* Perez de la Rosa and *J. poblana* (Mart.) R. P. Adams, **stat. nov.**

KEY WORDS: *Juniperus flaccida, J. f.* var. *martinezii, J. f.* var. *poblana, J. martinezii, J. poblana, Cupressaceae*, taxonomy, RAPDs.

The taxonomy of *Juniperus flaccida* Schltdl. has been somewhat unsettled. Generally, flaccid (weeping) foliaged junipers in Mexico (and the Chisos Mtns., Texas) have been referred to as *J. flaccida*. The first systematic treatment of these junipers was by Martinez (1963) who recognized two varieties: *J. f.* var. *typica* and *J. f.* var. *poblana* Mart. *Juniperus f.* var. *flaccida* has large seed cones (9-20 mm), with 6-10

seeds, and pendulous (flaccid) foliage and branchlets (Fig. 1). *Juniperus f.* var. *poblana* also has large seed cones (9-12 mm), with 6-10 seeds, but the foliage is distichous and in vertical planes like *Thuja*, and not very flaccid (Zanoni and Adams, 1976, 1979; Adams, 2004). In fact, the earliest name for this taxon was *Cupressus thurifera* Kunth which is indicative of the planate nature of the foliage (shown in Fig. 1). Perez de la Rosa (1985) discovered a population of trees that had small seed cones (5-7 mm), with 1-2 seeds per cone and with foliage somewhat drooping but branchlets erect (Fig. 1). He described this taxon as a new species,

J. f. var. *flaccida* *J. f.* var. *martinezii* *J. f.* var. *poblana*

Figure 1. Comparison of leaf foliage among J. f. var. flaccida, J. f. var. martinezii and J. f. var. poblana.

J. martinezii Perez de la Rosa. Except for the seed cones, the taxon looks similar to *J. flaccida*; indeed Silba (1985) treated it as *J. flaccida* var. *martinezii* (Perez de la Rosa) Silba. Each of these varieties has leaf margins that are hyaline and nearly entire, with either a few small teeth or merely a wavy margin (Adams, 2004). However, they are considered part of the serrate leaf margined *Juniperus* species of the western hemisphere (Adams, 2004).

Adams et al. (1990) compared the leaf essential oils and found considerable differences among the *J. flaccida* varieties. However, they decided to accept *J. flaccida* var. *martinezii* until "...additional data, such as from DNA analysis, are available." (Adams et al. 1990).

Recently, DNA sequencing of nrDNA (ITS) and trnC-trnD (Schwarzbach, et al. 2007) has revealed that *J. flaccida* varieties are not monophyletic (Fig. 2). It will be noticed that *J. f.* var. *poblana* is not closely related to any juniper and that *Juniperus f.* var. *martinezii* is more closely related to *J. durangensis* than to *J. f.* var. *flaccida* (Fig. 2).

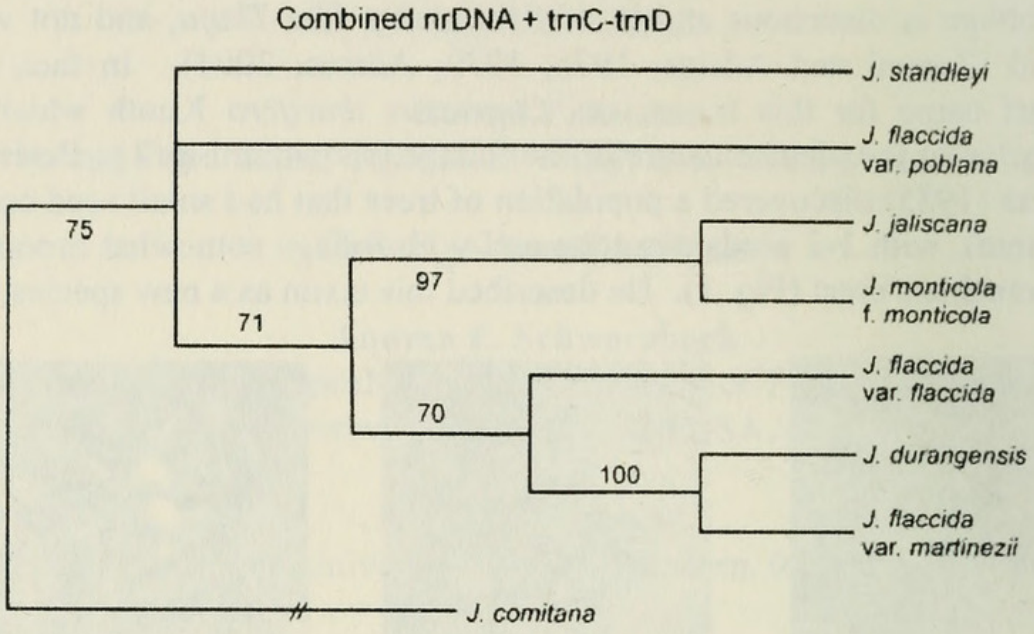

Figure 2. Partial phylogenetic tree derived from nrDNA + trnC-trnD sequence data (adapted from Schwarzbach et al., 2007). Values above branches are posterior probabilities. Note that *J. f.* var. *flaccida*, *J. f.* var. *martinezii* and *J. f.* var. *poblana* are in separate clades that are well supported.

To investigate this pattern more closely, DNA fingerprinting analyses were performed for the same taxa (Schwarzbach et al., 2007). RAPDs (Random Amplified Polymorphic DNAs) is a form of DNA fingerprinting that has been used in several *Juniperus* studies and has proved useful in systematics (Adams, 1999, 2000a-d; Adams and Demeke, 1993; Adams and Nguyen, 2005), when stringent laboratory procedures are followed (Adams, Flournoy and Pandey, 1998). In this study, we report on RAPDs analysis and combine these results with the DNA sequence data and morphology to evaluate the taxonomic status of *J. flaccida* and its varieties.

MATERIALS AND METHODS

Specimens collected: *J. comitana*, Adams 6858-62, 14 km s of Comitan, thence 14 km e on road to Montebello, Chiapas, Mexico; *J. durangensis*, Adams 6832-35, at km 152 on Mex. 40, 52 km w of El Salto, Durango, Mexico; *J. flaccida* var. *flaccida*, Adams 6892-96, 23 km

e of San Roberto Junction on Mex. 60, Nuevo Leon, Mexico; *J. flaccida* var. *martinezii*, Adams 5950-52, 8709, 40 km n of Lago de Moreno on Mex. 85 to Amarillo, thence 10 km e to La Quebrada Ranch, 21° 33.08' N, 101° 32.57' W, Jalisco, Mexico; *J. flaccida* var. *poblana*, Adams 6868-70, 62 km s of Oaxaca, Mexico on Mex. 190; *J. jaliscana*, Adams 6846-49, 19 km e of Mex. 200 on dirt road to Cuale, Jalisco, Mexico; *J. monticola* f. *monticola*, Adams 6874-78, El Chico Nat. Park, 8 km ne of Pachuca, Hidalgo, Mexico; *J. standleyi*, Adams 6852-56, 24 km nw of Huehuetango on road to San Juan Ixcoy, Huehuetango, Guatemala. Voucher specimens are deposited at BAYLU.

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° C until the DNA was extracted. DNA was extracted using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia, CA). The RAPD analyses follow that of Adams and Demeke (1993). Ten-mer primers were purchased from the University of British Colombia (5'-3'): 134, AAC ACA CGA G; 153, GAG TCA CGA G; 184, CAA ACG GAC C; 212, GCT GCG TGA C; 218, CTC AGC CCA G; 239, CTG AAG CGG A; 249, GCA TCT ACC G; 250, CGA CAG TCC C; 338, CTG TGG CGG T; 346, TAG GCG AAC G; 347, TTG CTT GGC G; 375, CCG GAC ACG A; 431, CTG CGG GTC A; 478, CGA GCT GGT C.

PCR stock solutions (Taq, primer, and buffer) were made in bulk so that all the PCR reaction tubes for a primer were prepared using the same bulk stock. This is a critical factor for minimizing variation in band intensities from sample to sample (see Adams, Flournoy and Pandey, 1998, for protocols to minimize PCR band variation). PCR was performed in a volume of 15 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 2.0 mM $MgCl_2$, and 0.1% Triton X-100, 0.2 mM of each dNTPs, 0.36 µM primers, 0.3 ng genomic DNA, 15 ng BSA and 0.6 unit of Taq DNA polymerase (Promega). A negative control PCR tube containing all components, but no genomic DNA, was run with each primer to check for contamination. DNA amplification was performed in an MJ Programmable Thermal Cycler (MJ Research, Inc.). Samples were run in duplicate to insure reproducibility (Adams, Flournoy and Pandey, 1998). A temperature profile was obtained for each well of the thermocycler to be sure that no variation existed among wells in the heating/ cooling block. The thermal cycle used was: 94° C (1.5 min) for initial strand separation, then 40 cycles of 40° C (2 min),

72° C (2 min), 91° C (1 min). Two additional steps were used: 40° C (2 min) and 72° C (5 min) for final extension. The temperature inside a PCR tube containing 15 µl buffer was monitored with a temperature probe, quantitated and printed for each step for each of the 40 cycles for every PCR run (Adams, Flournoy and Pandey, 1998) to insure that each cycle met temperature specifications and that each PCR run was exactly the same. Amplification products were analyzed by electrophoresis on 1.5% agarose gels, 75V, 55 min, and detected by staining with ethidium bromide. The gels were photographed over UV light using Polaroid film 667 and scanned to digital images. The digital images were size normalized in reference to pGem® DNA size markers before band scoring. Bands were scored as present (1) and absent (0). Bands that were inconsistent in replicate analyses were not scored.

Associational measures were computed using absolute character state differences (Manhattan metric), divided by the maximum observed value for that character over all taxa (= Gower metric, Gower, 1971; Adams, 1975). Principal coordinate analysis (PCO) was performed by factoring the associational matrix using the formulation of Gower (1966) and Veldman (1967). It should be noted that problems of homology of RAPD DNA bands on agarose gels can be significant (Rieseberg, 1996), but these errors can be accounted for using multivariate statistical methods (PCO) (see Adams and Rieseberg, 1998). A minimum spanning diagram was constructed by selecting the nearest neighbor for each taxon from the pair-wise similarity matrix, then connecting those nearest neighbors as nodes in a network (Adams, et al. 2003).

RESULTS AND DISCUSSION

The RAPDs data (Fig. 3) are essentially identical to the sequence data (Fig. 2). As with the sequence data (Fig. 2), the RAPDs show (Fig. 3) the *J. flaccida* varieties do not form a monophyletic group. *Juniperus flaccida* var. *poblana* is very distinct. *Juniperus flaccida* var. *flaccida* and *J. f.* var. *martinezii* are in the same group that includes *J. durangensis* (Fig. 3). *Juniperus durangensis* does share some seed cone characteristics with *J. f.* var. *martinezii* (Table 1). However, *J. durangensis* and *J. f.* var. *martinezii* are quite distinct in their morphology.

Zanoni and Adams (1976) have shown that *J. f.* var. *flaccida* and *J. f.* var. *poblana* are about as different in morphology as other serrate

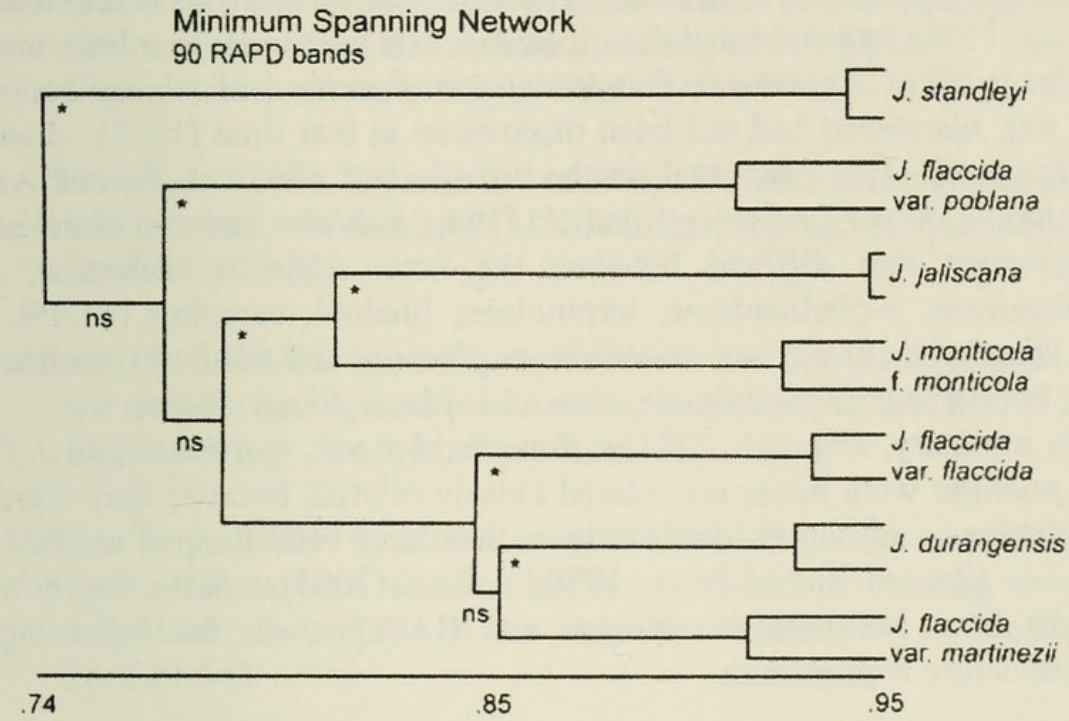

Figure 3. Minimum spanning network based on 90 RAPD bands.
* = significant at p = 0.05. ns = not significant.

Table 1. Morphological comparison between *J. f.* var. *flaccida*, *J. f.* var. *poblana*, *J. f.* var. *martinezii*, and *J. durangensis*.

	flaccida	*poblana*	*martinezii*	*durangensis*
seeds/cone	(4-)6-10(-13)	(4-)6-10(-13)	1-2(-3)	1-3(-4)
cone size	9-20 mm	9-15 mm	(5-)6(-9) mm	6-7 mm
cone color	tan to brownish/ purple	bluish-brown	brown	bluish-red
cone shape	spherical	spherical	ovoid, gibbous	ovoid, gibbous
terminal branch tips	hanging, but straight	hanging, but, straight	erect and straight	curved on tips
branching	radially	planate	radially	radially

leafed juniper species in Mexico. Essential leaf oil analysis (Zanoni and Adams, 1976) indicated that the oil of *J. f.* var. *poblana* was a little more similar to oil of *J. comitana* than to *J. f.* var. *flaccida* leaf oil. Of course, *J. f.* var. *martinezii* had not been discovered at that time (1976). Later, Adams et al. (1990) reported on the volatile leaf oils of *J. flaccida* var. *flaccida*, *J. f.* var. *martinezii* and *J. f.* var. *poblana* and found several components that differed between the taxa: α-pinene, sabinene, α-phellandrene, β-phellandrene, terpinolene, linalool, camphor (11.4% in var. *martinezii* vs. 0.5 and trace in vars. *flaccida* and *poblana*), terpinen-4-ol, bornyl acetate, γ-cadinene, manoyl oxide, and kaur-16-ene.

In summary, although *J. f.* var. *flaccida*, *J. f.* var. *martinezii* and *J. f.* var. *poblana* were never considered closely related, because they were not similar to any other Mexican taxa, they have been lumped under *J. flaccida* (Zanoni and Adams, 1979; Adams, 2004). With the new insight from both DNA sequence and RAPDs data, the following nomenclature is proposed:

Juniperus poblana (Martinez) R. P. Adams, **stat.. nov.,**

Basionym: *Juniperus flaccida* var. *poblana* Martinez., Anales Inst. Biol. Univ. Nac. Mexico 17:31 (1946).

Cedro, poblana juniper, Pueblo juniper. Type: Mexico: Puebla: Amozoc at 2300 m, *Martinez 507* (Holotype: MEXU!)

Distribution: from Jalisco, east to Oaxaca, Mexico. See map, p. 112, Adams (2004).

Both the DNA sequence and RAPD data support the morphology for the recognition of *Juniperus f.* var. *martinezii* at the specific rank (*Juniperus martinezii* Perez de la Rosa). Additional research (in progress) on geographic variation in *J. flaccida* (sensu stricto) is needed to understand the variation within this species.

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