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The *Juniperus flaccida*-*J. poblana* complex revisited: insights from molecular and oil analysis**Robert P. Adams**Biology Department, Baylor University, Baylor-Utah Lab, 201 N 5500 W, Hurricane, UT 84737,
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Andrea E. SchwarzbachDepartment of Health and Biomedical Sciences, University of Texas - Rio Grande Valley,
Brownsville, TX 78520, USA.**ABSTRACT**

Analysis of nrDNA, petN-psbM, trnS-trnG, trnD-trnT, and trnF-trnL revealed that *J. flaccida* is diverse, but quite separated from *J. poblana* and *J. aff. poblana* from Nayarit by at least 10 Mutational events (MEs, SNPs + indels), and is actually a bit closer to *J. standleyi* (8 MEs). A divergent *J. poblana* from Oaxaca is merely 2 MEs distant from the Nayarit trees. The *J. poblana* samples from the type locality (Amozoc, Pue.) vary by only 1 ME among samples. Leaf oils, on the other hand, show the Nayarit juniper quite differentiated from other *J. poblana*. In addition, the habit (large trees with a strong central axis with branchlets long, pendulous and very planate) and habitat of the Nayarit trees support the recognition of it as a different taxon closely related to *J. poblana*. However, additional evidence is needed in order to resolve its taxonomic status. Published on-line www.phytologia.org *Phytologia* 100(1): 19-26 (Mar 16, 2018). ISSN 030319430.

KEY WORDS: *J. flaccida*, *J. poblana*, Cupressaceae, Nayarit, nrDNA, petN-psbM DNA, terpenes.

Juniperus flaccida Schltld. and *J. poblana* (Martínez) R.P. Adams are closely related species with large, multi-seeded cones and weeping (flaccid) foliage that make them difficult to differentiate morphologically (Adams 2014). Recently, we reported (Adams et al. 2017a) on the discovery of large, beautiful trees in a new population of putative *Juniperus poblana* from Nayarit, Mexico. The trees have a very strong central axis and long, pendulous foliage. They are magnificent on the rocky areas where they occur.

Recently, analysis of DNA sequences (Adams et al. 2017a), placed those Nayarit trees in a clade with *J. poblana*, but the Nayarit trees were in a well-supported sub-clade. However, samples of *J. poblana* exhibited considerable variation and, because no samples were included from Amozoc (the type locality), it seemed premature to make a decision about the taxonomic status of the Nayarit trees.

Additional samples were collected of *J. flaccida* from the Chihuahuan desert and *J. poblana*, from the type locality, Amozoc. The volatile leaf oils of the taxa were analyzed and revealed (Adams et al. 2017b) that the oils from the Nayarit trees were quite distinct (Fig. 1). Notice that the first coordinate (34%) largely separates the Nayarit trees from all the other OTUs (Fig. 1). This finding was surprising, as the DNA sequencing placed the Nayarit trees in an unresolved polytomy with *J. poblana* (Adams et al. 2017a).

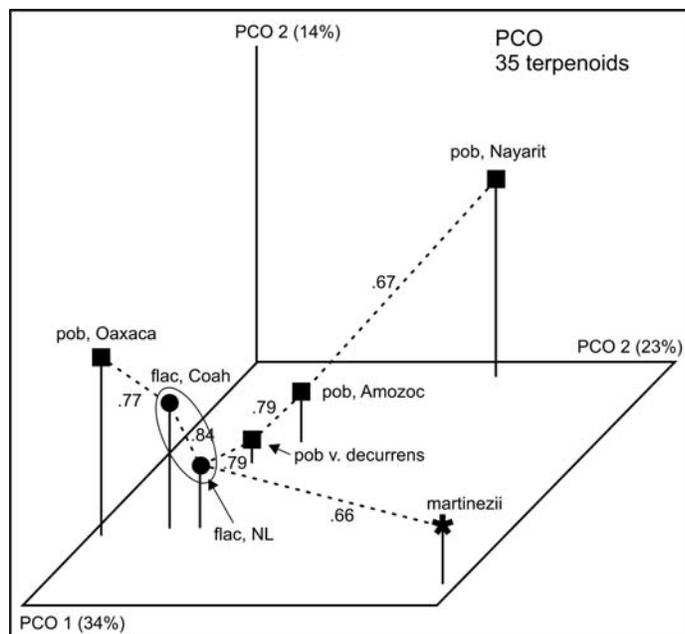


Figure 1. PCO based on 35 terpenoids from the volatile leaf oils of *J. poblana*, *J. flaccida*, *J. martinezii*, and the Nayarit trees. The dotted lines are the minimum spanning network. The numbers next to the dotted lines are the similarities (1 to 0.0). NL = Nuevo Leon. Coah = Coahuila.

The purpose of this study was to further explore the position of the magnificent Nayarit trees of putative *J. poblana* through DNA sequencing of additional samples of *J. flaccida* and *J. poblana*.

MATERIALS AND METHODS

Plant material and populations studied:

- J. flaccida*, Short trees, 1.5-3 m tall; bark on branches papery and exfoliating, inner bark smooth, reddish; no seed cones, similar to *J. flaccida*, but in a very dry habitat in the Chihuahuan desert region, Mexico, Durango, Mpio. Lerdo, Sierra del Rosario, nearly atop the mountain, with *Yucca* and oak scrub; on limestone, 25° 38' 44" N, 103° 54' 40" W, 2700 m, 8 Apr 2008, Coll. M. S. Gonzalez-Elizondo et al. 7375 a,b; Lab Acc. Robert P. Adams 14616, 14617.
- J. aff. poblana*, uncommon young trees (saplings) 2 m, in oak woodland dominated by *Quercus resinosa*, Mexico, Nayarit, Mpio. El Nayar, SW of Mesa del Nayar on road to Ruiz, Km 86.8; S of bridge of arroyo del Fraile, E of El Maguey, 22° 10' 08" N, 104° 43' 51" W, 1150 m, 19 Jan. 2016, Coll. M. S. Gonzalez-Elizondo and M. Gonzalez-Elizondo 8381 with L. López, A. Torres Soto; Lab Acc. Robert P. Adams 14896
- J. aff. poblana*, large, single stemmed trees, foliage long and pendulous, abundant trees, up to 25 m high, on strongly rocky slope, forest of *Juniperus-Clusia* with elements of mesophytic forest (*Magnolia*) and tropical forest (*Bursera*, *Opuntia*, *Pilosocereus purpusii*) as well as *Agave attenuata* and *Yucca jaliscensis*, Mexico, Nayarit, Mpio. El Nayar, SW of Mesa del Nayar on road to Ruiz; NE of El Maguey, 22° 07' 40" N, 104° 47' 47" W, 1430 m, 19 Jan. 2016, Coll. M. S. Gonzalez-Elizondo and M. Gonzalez-Elizondo 8379a,b,c,d, with L. López, A. Torres Soto; Lab Acc. Robert P. Adams 14897-14900,
- J. flaccida*, Adams 6892-6896, 23 km E. of San Roberto Junction on Mex. 60, Nuevo Leon, Mexico;
- J. flaccida*, Reserva Ecológica Municipal de Sierra y Cañon de Jimulco, 25° 07' 38" N, 103° 16' 15" W., 2118 m, 17 Jan 2017, Torreon, Coahuila, Mexico, Coll. Manuel Rodríguez Muñoz et al. #1,2,3,4,5, Lab Acc. Adams 15203 - 15207;
- J. martinezii*, Adams 5950-5952, 8709, 40 km n of Lagos 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. poblana* var. *decurrens*, R. P. Adams 11926, 11927, 11928, small trees, to 5 m tall, with strong central axis, foliage very, very, weeping, common, about 2 km S. of Valle de Topia. All leaves decurrent, and

prickly and are not merely juvenile leaves. 25° 14' 11" N; 106° 26' 55.7" W, 1818 m, 30 Jun 2009, Durango, Mexico;
J. poblana, Adams 6868-6870, 62 km S. of Oaxaca, Mexico on Mex. 190.
Voucher specimens are deposited at BAYLU and CIIDIR when applicable.

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 from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions.

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 (petN-psbM), D (maldehy) or K (nrDNA) (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 MgCl₂ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010). 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.) or Sequencher v. 5 (genecodes.com). Sequence datasets were analyzed using Geneious v. R7 (Biomatters. Available from <http://www.geneious.com/>), the MAFFT alignment program. Further analyses utilized the Bayesian analysis software Mr. Bayes v.3.1 (Ronquist and Huelsenbeck 2003). For phylogenetic analyses, appropriate nucleotide substitution models were selected using Modeltest v3.7 (Posada and Crandall 1998) and Akaike's information criterion.

RESULTS AND DISCUSSION

Sequencing nrDNA, petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF resulted in 4,351 bp of concatenated sequence data. A Bayesian tree shows (Fig. 2) *J. poblana* (Oaxaca), *J. poblana* var. *decurrens* (Adams and Schwarzbach, 2015) (red shaded), *J. flaccida*, (yellow) and *J. poblana* (Amozoc, type locality) and the Nayarit junipers (blue) are all grouped in one clade with high support.

Within this clade, three sub-clades are supported: *J. poblana* (Oaxaca), *J. poblana* var. *decurrens* (red shaded), *J. flaccida*, (yellow) and *J. poblana* (Amozoc, type lcn.) and the Nayarit junipers (blue) (Fig. 2). Notice that all the Nayarit junipers have little or no variation in their DNA sequences (Fig. 2). In addition, two samples of *J. poblana*, Amozoc, show few or no differences from the Nayarit junipers (Fig. 2).

To visualize the variation in molecular events (MEs = SNPs + indels) among individuals, a minimum spanning network (MSN) was constructed using both nucleotide substitutions and indel data. Whereas, the Bayesian tree is a flattened 2-dimensional representation, the MSN has more of a 3-dimensional perspective. In addition, one can see exactly the magnitude of differences separating the groups. A few related, but taxonomically distinct species are included in the MSN. Notice that *J. standleyi*, *J. monticola*, *J. jaliscana*, *J. durangensis* and *J. martinezii* are each separated from adjacent taxa by 6 to 11 MEs (Fig. 2). This metric (6-11) is useful to keep in mind, when we consider specific levels among *J. flaccida*, *J. poblana* and the Nayarit junipers.

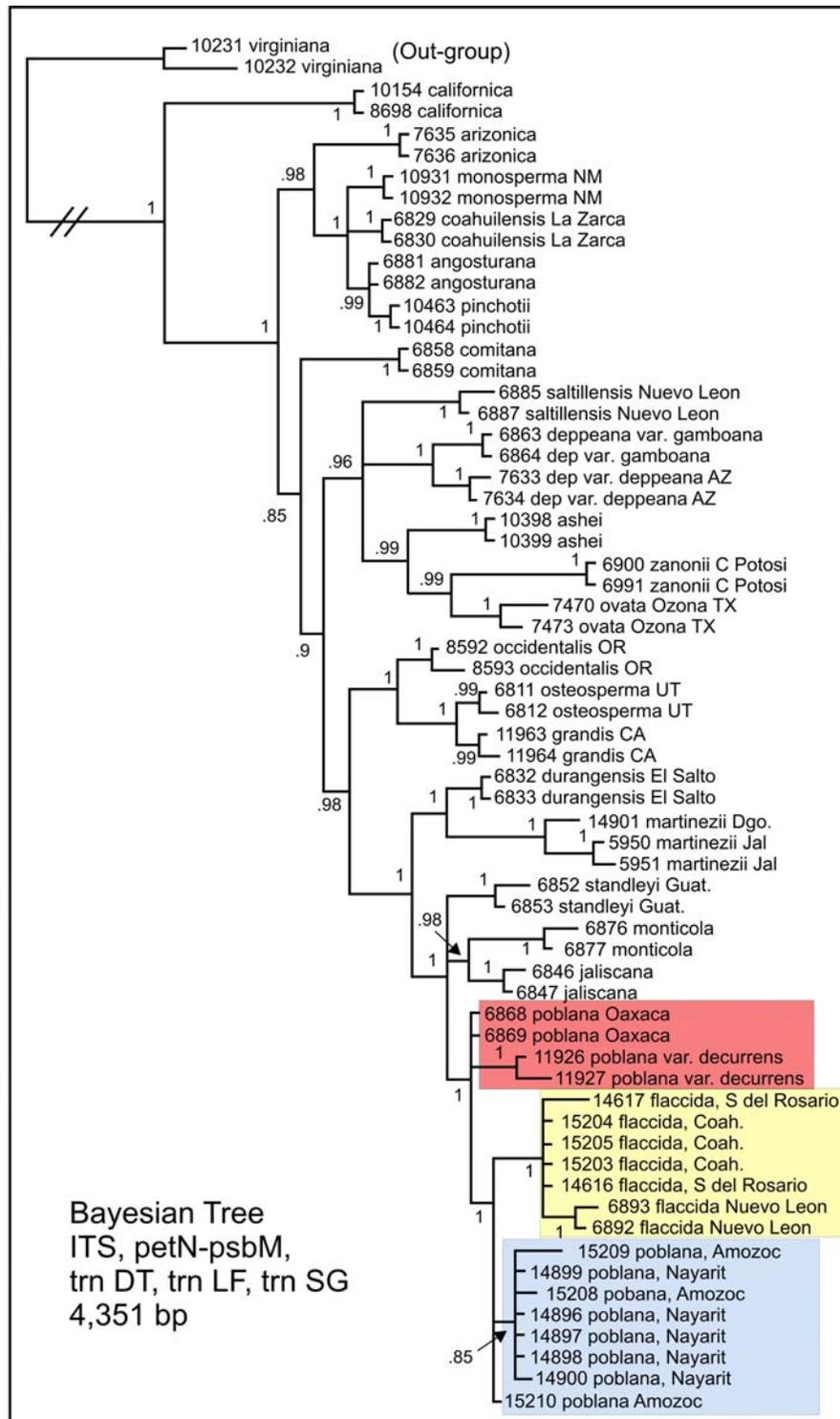


Figure 2. Bayesian tree based on sequences from ITS (nrDNA), petN-psbM, trn D-trn T, trn L- trn F, and trn S -trn G. Numbers at the branch points are posterior probabilities. *J. pohlana*, Oaxaca, and *J. pohlana* var. *decurrens* are in red shading. *J. flaccida* individuals are in yellow, and *J. pohlana*, Nayarit and Amozoc are in blue shading. See text for discussion.

The MSN shows *J. flaccida* is diverse, but quite separated from *J. poblana* and the Nayarit junipers by at least 10 MEs, but actually a bit closer to *J. standleyi* (8 MEs, Fig. 3). No variation is seen among the five (5) Nayarit junipers in their DNA sequences (Fig. 2). The 'divergent' *J. poblana* from Oaxaca (as per the Bayesian tree, Fig. 1), is merely 2 MEs distant from the Nayarit trees. The *J. poblana* samples from the type locality (Amozoc, Pue.) vary by only 1 ME among samples (Fig. 3). The *J. poblana* var. *decurrens* samples are separated from *J. poblana* by 4 MEs (Fig. 3). This divergence of 4 MEs is about half of the amount (6-11 MEs) typically found between species (in this study), and thus, the DNA divergence supports the recognition of *J. poblana* var. *decurrens* as a variety.

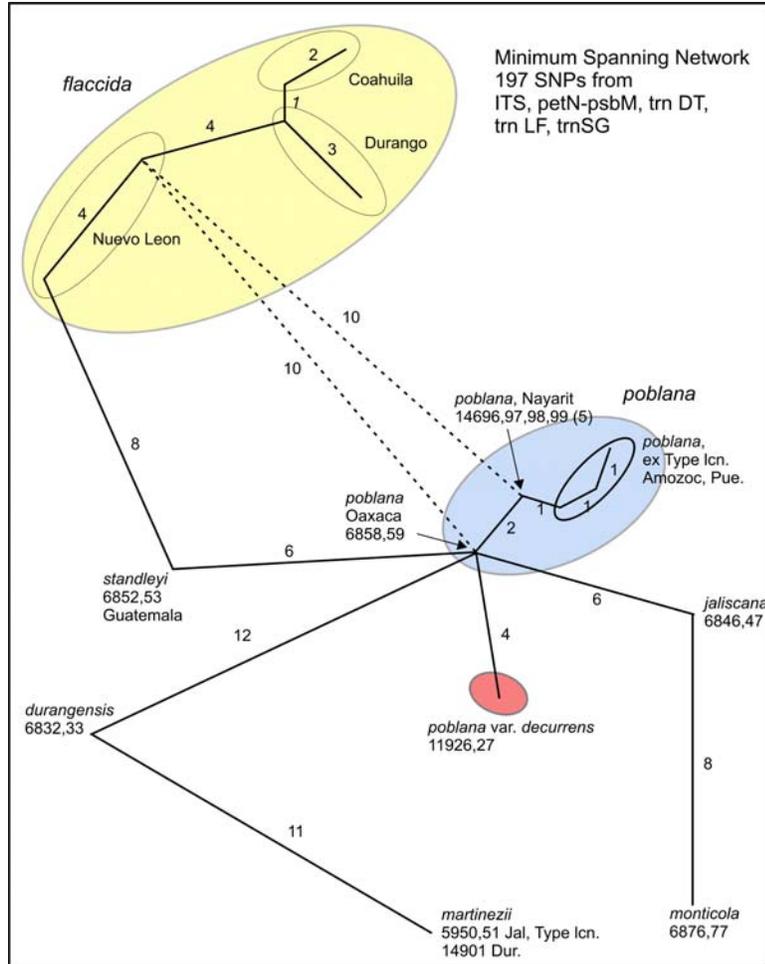


Figure 3. Minimum spanning network based on SNPs and indels in ITS (nrDNA), petN-psbM, trn D-trn T, trn L- trn F, and trn S -trn G. The numbers next to the lines are the number of MEs (mutational events = SNPs + indels). The two dashed lines are the 2nd shortest links between *J. flaccida* and *J. poblana*.

It is interesting to examine a minimum spanning network based on the leaf terpenoids (Fig. 4). Notice that the leaf oil from the Nayarit juniper is quite divergent from *J. poblana*, Amozoc oil (Fig. 4). So, in contrast to the ITS and cp DNA, the oil data shows the Nayarit junipers to have some genetic differentiation.

In addition, differentiation is seen (Fig. 4) within *J. poblana* as the oil of *J. poblana* (Oaxaca) is more similar to the oil of *J. flaccida* than other the oils of other *J. poblana*.

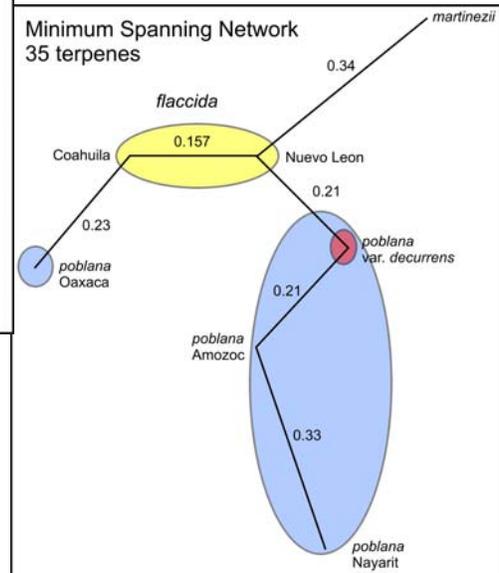


Fig. 4. Minimum spanning network based on 35 leaf terpenoids. The numbers next to the lines are distances scaled as: [1.0 - terpene similarity].

The Nayarit junipers are clearly differentiated in their habit (Fig. 5), having a strong central axis and long trunk vs. *J. poblana* south of Oaxaca (Figs. 6, 7) and *J. p. var. decurrens* (Fig. 8).



Fig. 5. Nayarit juniper as large tree with a strong central axis.



Fig. 6. *J. poblana*, habit, at KM 62 S of Oaxaca.



Fig. 7. *J. poblana*, habit, at Km 62, S of Oaxaca.



Fig. 8. *J. poblana* var. *decurrens*, habit.

The foliage is also more robust and planate (Fig. 9), than in *J. poblana*, Oaxaca (Fig. 10) and *J. p.* var. *decurrens* (Fig. 11). Notice the lack of blue bloom (glaucous) on the cones of the Nayarit juniper (Fig. 9).



Fig. 9. Foliage of the Nayarit juniper.



Fig. 10. Foliage and cones, *J. poblana*, S of Oaxaca.

Fig. 11. Foliage and cones, *J. poblana* var. *decurrens*.



In summary, the Nayarit juniper differs in its leaf volatile oils, habit, aspects of its morphology, ecology and habitat (with elements of mesophytic and tropical deciduous forest), and is geographically disjunct, which support its recognition as a different lineage. However, molecular data show it closely clustering with *J. poblana*. Therefore, additional evidence is needed in order to resolve its taxonomic status.

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