VARIATION AMONG THE SMOOTH-LEAF MARGINED *JUNIPERUS* OF MEXICO: ANALYSIS OF nrDNA, 4CL, ABI3 AND petN-psbM SNPs

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ABSTRACT

Analyses of sequence data from three nuclear genes and one chloroplast gene region for *Juniperus blancoi, J. b. var. huehuentensis, J. mucronata, J. scopulorum* and *J. virginiana* revealed that the *J. blancoi - huehuentensis - mucronata* complex is closely allied but distinct from *J. scopulorum* and very distinct from *J. virginiana.* The combined DNA data support *J. mucronata* as a sibling species to *J. blancoi* or as a variety of *J. blancoi.* Considerable differentiation was found in the *J. blancoi* population from El Oro (Carmona) and additional sampling is needed to resolve that variation. *Phytologia 91(3): 571-580 (December, 2009).*

KEY WORDS: *Juniperus blancoi, J. b. var. huehuentensis, J. b. var. mucronata, J. mucronata, J. scopulorum, J. virginiana*, nrDNA(ITS), 4-coumarate: CoA Ligase, Abscisic acid-insensitive 3, petN, psbM, SNPs, taxonomy.

The smooth-leaf margined junipers of Mexico consist of *Juniperus blancoi* Mart. *J. b. var. huehuentensis* R. P. Adams et al., *J. mucronata* R. P. Adams and *J. scopulorum* Sarg. (Adams, 2008). These junipers are difficult to key out and their similarities in morphology are probably indicative of their close relationship. *Juniperus mucronata* was named for its mucronate tip leaves found on plants in a single population (Adams, 2000). Farjon (2005) reduced it to *J. blancoi var. mucronata* (R. P. Adams) Farjon without comment. The leaf essential oils of the aforementioned taxa differ mainly quantatively (Adams, 2000; Adams et al., 2006).

RAPD analyses (Adams et al., 2006) indicated (Fig. 1) that *J. mucronata* is closely allied with *J. blancoi* and that *J. blancoi* var.

huehuentensis is very closely allied with *J. blancoi*. The volatile leaf oils and RAPD data supported the recognition of *J. mucronata* at the species level. In addition, the heartwood of *J. mucronata* is bright purple, which is quite distinct from the other smooth-leaf margined junipers.

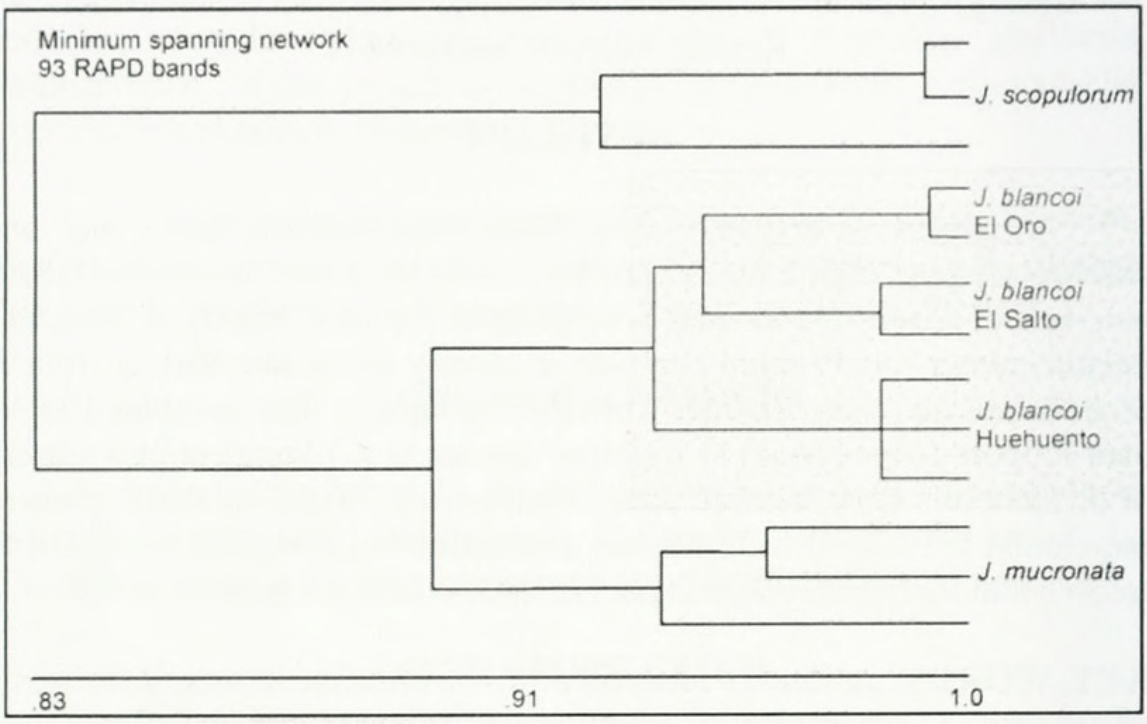

Figure 1. Minimum spanning network based on 93 RAPD bands. Adapted from Adams et al. (2006).

In an effort to better understand the affinities of these taxa, sequence data has been obtained from three nuclear gene regions: nrDNA, 4-coumarate CoA ligase (4CL) and abscisic acid-insensitive 3, (ABI3) and a cpDNA region, petN-spacer-psbM. The 4CL and ABI3 genes have been recently utilized in the Cupressaceae (Adams et al., 2009).

Peng and Wang (2008) utilized 4CL sequences to study *Thuja* species and *Thujopsis dolabrata*; they found the 4CL gene to be composed of 4 exons and 3 introns. Intron 2 was reported as 640 bp (EU183423). Aligning the GenBank sequences for *Thuja plicata* (EU183418, EU183417) and *Thujopsis dolabrata* (EU183423) enabled

us to design primers to span intron 2, and resulted in 708 - 709 bp of sequence data.

Lazarova, Zeng and Kermode (2001) reported on the occurrence of an abscisic acid-insensitive 3 (ABI3) gene homologue from *Chamaecyparis nootkatensis* (CnABI3). The ABI3 gene is composed of six exons and five introns, with the intron sizes of 105, 113, 110, ca. 1000 and 142 bp. Primers were designed in exon 4 and exon 5 to amplify intron 4 (see Materials and Methods below) and resulted in 1483-1485 bp of sequence data.

The cp region petN-spacer-psbM was used in phylogenetic studies of the Cupressaceae (Adams et al., 2009). This region resulted in 846-856 bp of sequence data.

The purpose of the present study is examine new sequence data to further clarify the nature of differentiation among the smooth-leaf margined junipers of Mexico.

MATERIALS AND METHODS

Specimens used in this study (GenBank accessions: nrDNA, petN-psbM): *J. blancoi, Adams 6849-6851,* 2 km El Oro, s of Carmona, Mexico, MX (GU120309, GU120312); *J. b. var. huehuentensis, Adams 10247-10249*, Cerro Huehuento, Durango, MX (GU120310, GU120313); *J. mucronata, Adams 8701-8703,*10 km w of Yepachic, Chihuahua, MX on hwy 16; *J. scopulorum, Adams 10895-10897*, 11 km e of Kamas, UT, U.S.A. on UT 150 (EF608963, EF608988); *J. virginiana, Adams 6753-6755*, I35 and Hewitt exit, Hewitt, TX, U.S.A. (EF6088980, FJ46734) 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 from juniper leaves by use of a Qiagen mini-plant kit as per manufacturer's instructions.

Amplification and sequencing

ITS (nrDNA), 4CL and petN-psbM 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 or K (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 (see Adams, Bartel and Price, 2009 for buffer enhancers used).

Primers (5'-3'):

ITS: ITSA-42F GAT TGA ATG ATC CGG TGA AGT
ITSB+57R ATT TTC ATG CTG GGC TCT

These primers are modified from Blattner (1999).

4CL: 4CL49F AAAGAGCTCATCAAATACAA
4CL814R GAAGAGCTTCCAGCTCAG

4CL primers are from conserved sequences in exon 2 and exon 3 of *Thuja plicata* (EU183418, EU183417) and *Thujopsis dolabrata* (EU1834232) and span intron 2.

CnABI3: CnABI11F AACAATAAGAGCAGGATGTA
CnABI357R CCAGTTTTGGTATCAGAGTA

Additional internal primers utilized:

CnABIint533R CAATATTATCACGCATTTG
CnABIint541R CACAGGAGCAATATTATCAC
CnABIint741R TTACTTGAAACAATCTATTTATGT

CnABI3 primers are from sequences in exon 4 and exon 5 of *Chamaecyparis nootkatensis* (AJ131113) and span intron 4.

petN - psbM:

petN5F: AAC GAA GCG AAA ATC AAT CA
psbM111R: AAA GAG AGG GAT TCG TAT GGA

petN and psbM primers were based on conserved sequences from *Juniperus* species.

The PCR reaction was subjected to purification by agarose gel electrophoresis (1.5% agarose, 70 v, 55 min.). In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit. The gel purified DNA band with the appropriate 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.). Alignments were made using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/). Minimum spanning networks were constructed from SNPs data using PCODNA software (Adams et al., 2006).

RESULTS AND DISCUSSION

Sequencing the nrDNA (ITS region) resulted in 1139 to 1140 bp of sequence data with 14 mutational events that included 2 indels consisting of a single bp each. A minimum spanning network is shown in figure 1. *Juniperus virginiana* is the most distinct taxon with 9 SNPs separating it from *J. scopulorum* (Fig. 2). The *J. scopulorum* individuals are separated by 3 SNPs from the *J. blancoi - mucronata -*

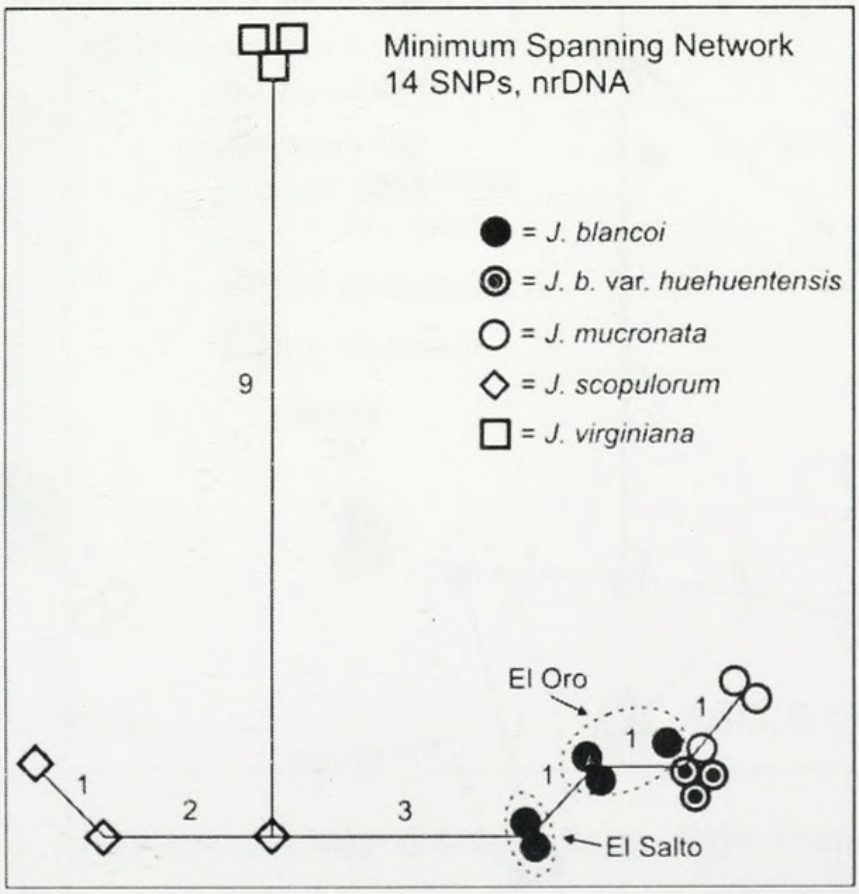

Figure 2. Minimum spanning network based on 14 SNPs from nr DNA.

huehuentensis complex (Fig. 2). Within the *J. blancoi - mucronata - huehuentensis* complex there is minor variation. Notice that there is some diversity between *J. blancoi* from El Salto and El Oro. Overall, *J. blancoi, J. mucronata* and *J. huehuentensis* are essentially indistinguishable in their nrDNA data.

Sequencing the petN-psbM region of cp DNA resulted in 846 to 847 bp of data with 17 SNPs found that included 7 indels. A minimum spanning network again shows (Fig. 3) *J. virginiana* to be the most distinct taxon.

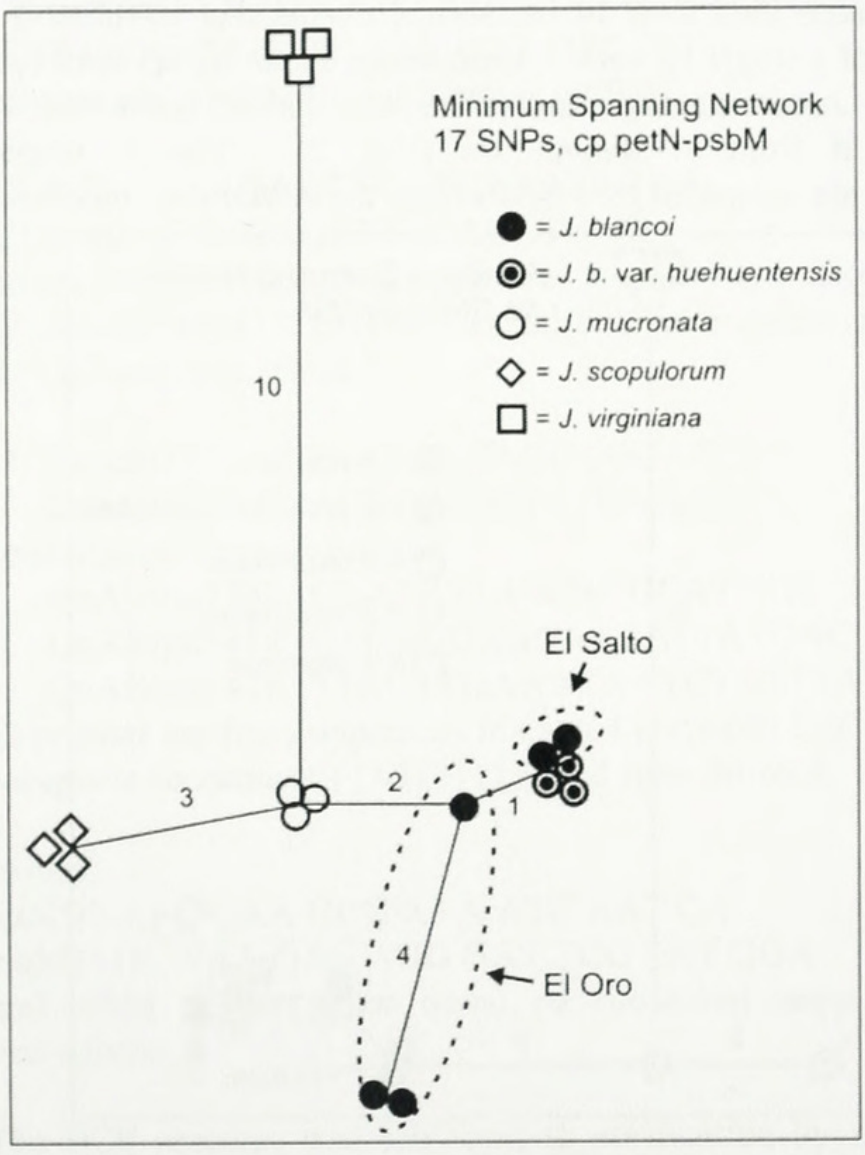

Figure 3. Minimum spanning network based on 17 SNPs from the cp petN-psbM region.

All the indels were single bases except a 7 bp deletion in *J. blancoi* individuals 6850 and 6851 from El Oro, MX (but not in 6849 from El Oro nor in the two plants from El Salto). The taxa are very uniform, except for the *J. blancoi* individuals. Two individuals of *J. blancoi* from El Oro had 4 SNPs that differentiated these plants from another *J. blancoi* plant from El Oro.

Analyses of the 4-coumarate: CoA ligase intron 2 (4CL) region resulted in 708 to 709 bp of sequence data that contained only 5 SNPs. A single indel (1 bp deletion) was found in one *J. virginiana* sample. The minimum spanning network shows little variation (Fig. 4, left) except for the separation of *J. virginiana* by 4 SNPs from other taxa.

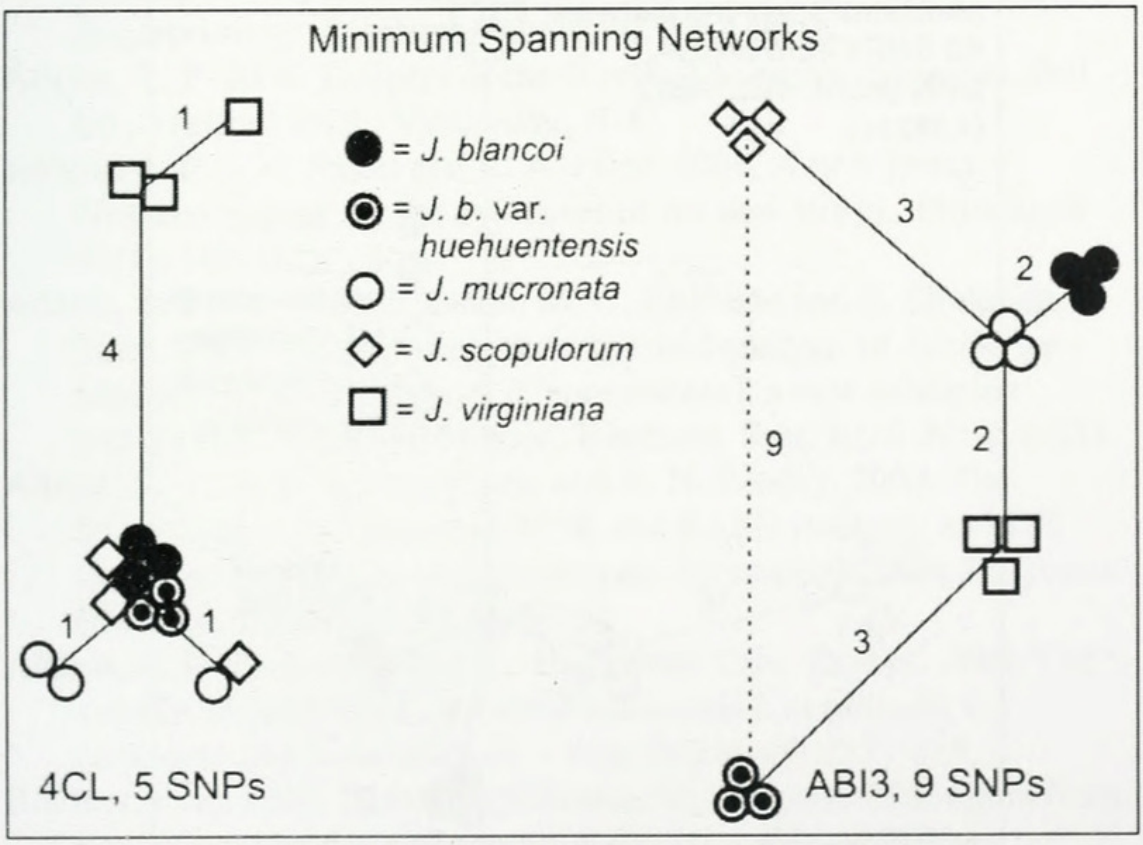

Figure 4. Minimum spanning networks using SNPs from 4CL and ABI3.

Sequencing the abscisic acid-insensitive 3 (ABI3) gene, intron 4 was difficult, but yielded 1483 - 1488 bp of data with 9 SNPs. The 9 SNPs include 4 indels and each indel occurred in all 3 plants of each

taxon. A minimum spanning network based on the 9 SNPs (including indels) reveals (Fig. 4, right) a different pattern than found in any of the other data sets. Each of the taxa are separated by 2 - 3 SNPs. ABI3 in *J. virginiana* has its greatest affinity to ABI3 from *J. mucronata* and *J. b.* var. *huehuentensis*.

An analysis using the SNPs from nr DNA, petN-psbM, 4CL and ABI3 sequences (4,182 bp of data) yielded 45 SNPs. The analysis gave 3 major groups: *J. virginiana*, *J. scopulorum* and the *blancoi - huehuentensis - mucronata* complex (Fig. 5). Notice that *J. blancoi* is more distinct from *J. b.* var. *huehuentensis* (8 SNPs) than from *J. mucronata* (5 SNPs). These data support the treatment of *J. mucronata* as a variety of *J. blancoi* or as a sibling species to *J. blancoi*.

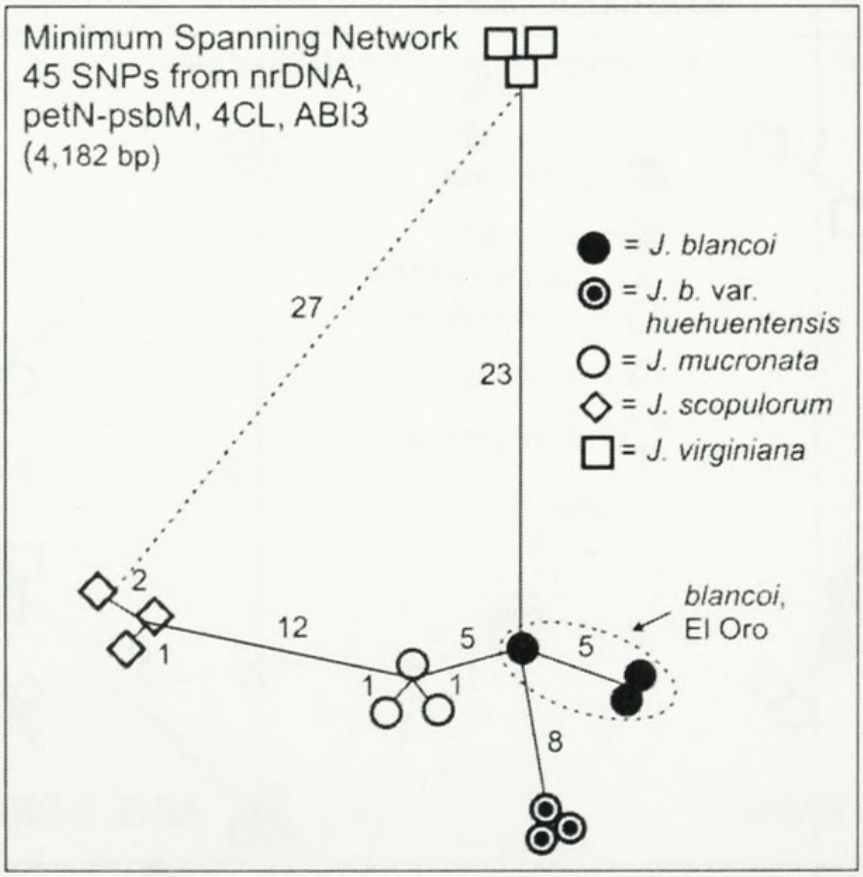

Figure 5. Minimum spanning network based on 45 SNPs from nr DNA, petN-psbM, 4CL and ABI3 sequences.

The diversity found in the El Oro samples (5 SNPs) is sufficient to warrant additional studies of *J. blancoi* populations.

ACKNOWLEDGEMENTS

Thanks to Andrea Schwarzbach and Billie Turner for manuscript reviews. Thanks to Alicia Mastretta for making available a copy of her thesis for examination. Thanks to Tonya Yanke for lab assistance. This research was supported in part with funds from Baylor University.

LITERATURE CITED

Adams, R. P. 1975. Statistical character weighting and similarity stability. Brittonia 27: 305-316.

Adams, R. P. 2000. Systematics of smooth leaf margin *Juniperus* of the western hemisphere based on leaf essential oils and RAPD DNA fingerprinting. Biochem. Syst. Ecol. 28: 149-162.

Adams, R. P. 2008. Junipers of the World: The genus *Juniperus*. 2nd Ed., Trafford Publ., Vancouver, B. C.

Adams, R. P. J. A. Bartel and R. A. Price. 2009. A new genus, *Hesperocyparis*, for the cypresses of the new world. Phytologia 91(1): 160-185.

Adams, R. P., M. S. G. Elizondo, M. G. Elizondo and E. Slinkman. 2006. DNA fingerprinting and terpenoid analysis of *Juniperus blancoi* var. *huehuentensis* (Cupressaceae), a new subalpine variety from Durango, Mexico. Biochem. Syst. Ecol. 34: 205-211.

Adams, R. P., A. E. Schwarzbach, and R. N. Pandey. 2003. The Concordance of Terpenoid, ISSR and RAPD markers, and ITS sequence data sets among genotypes: An example from *Juniperus*. Biochem. Syst. Ecol. 31: 375-387.

Adams, R. P., E. von Rudloff, L. Hogge and T. A. Zanoni. 1980. The volatile terpenoids of *Juniperus monticola* f. *monticola*, f. *compacta* and f. *orizabensis*. J. Nat. Prods. 43: 417 - 419.

Blattner, F. R. 1999. Direct amplification of the entire ITS region from poorly preserved plant material using recombinant PCR. BioTechniques 27: 1180-1186.

Farjon, A. 2005. World checklist & Bibliography of Conifers. ed. 2., Kew Press, London.