Taxonomy of the serrate leaf *Juniperus* of North America: Phylogenetic analyses using nrDNA and four cpDNA regions.

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ABSTRACT

The serrate leaf *Juniperus* of North America were analyzed by nrDNA (ITS), petN-psbM, trnS-trnG, trnD-trnT, trnL-trnF sequencing (4411 bp). The varieties of *J. ashei* (var. *ashei*,var. *ovata*) were found to be in separate clades, supporting the recognition of ***Juniperus ovata*** (R. P. Adams) R. P. Adams, ***comb. & stat. nov***. *Juniperus zanonii* has been treated as a *J. monticola* f. *compacta*, but its DNA was very distinct and it was well supported in a clade with *J. saltillensis*, not with *J. monticola*. In the single seeded group, *J. arizonica* was the most distinct species with 8 mutational events (MEs) and *J. angosturana* - *J. pinchotii*, the least distinct (1 ME). Yet, *J. angosturana* and *J. pinchotii* are quite different in their morphology and leaf essential oils (Adams 2011). In the three western US junipers, *J. grandis* was separated by only 4 MEs from *J. osteosperma* and 7 MEs from *J. occidentalis*. The varieties of *J. deppeana* were mostly unresolved showing their close relationship (Adams and Schwarzbach 2013c). Variation in the nrDNA and 4 cp DNAs sequences were not completely correlated with species delimitation. Hybridization (past and present) and incomplete lineage sorting among the closely related taxa appear to present problems in DNA analysis.
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KEY WORDS: *Juniperus deppeana* varieties, *Cupressaceae*, DNA, nrDNA (ITS), petN-psbM, trnS-trnG, trnD-trnT, trnL-trnF, systematics, taxonomy.

The genus *Juniperus* is composed of approximately 75 species in three sections: *Caryocedrus* (1 species, Adams and Schwarzbach, 2012a), *Juniperus* (14 species, Adams and Schwarzbach, 2012a) and *Sabina* (approx. 60 species). Section *Sabina* is divided into three major clades (Mao et al., 2010, Adams 2011):
1. Serrate-leaf junipers of North America (21 species, Adams and Schwarzbach, 2011),

2. Turbinate-seed cones, single-seeded, entire-leaf junipers, eastern hemisphere (16 species, Adams and Schwarzbach, 2012b, 2013a, Zanoni and Adams, 1976, 1979) and

3. Multi-seeded, entire-leaf junipers, both eastern and western hemispheres (23 species, Adams and Schwarzbach, 2012c, 2013b).

Recently, Adams and Schwarzbach (2006, 2013c) and Adams and Nguyen (2005) have reported on the taxonomy of *J. deppeana* and its varieties. The focus of the present study was to integrate the data from Adams and Schwarzbach (2011) and Adams and Schwarzbach (2013c) to give complete coverage of all the species, major varieties and formas (Adams 2011) of the serrate junipers North America using data obtained from extended sequencing of nrDNA (ITS), petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF.

MATERIALS AND METHODS

Specimens used in this study: *J. angosturana, Adams 6881-6885*, 21 km e of Cerritos, San Luis Potosi, MX, *J. arizonica, Adams 7635-7638*, Rock Hound State Park, NM, *J. ashei var. ashei, Adams 10398, 10399*, Bosque Blvd., Waco, TX, *J. ashei var. ovata, Adams 7470, 7473*, Ozona, TX, *J. californica, Adams 10154, 10155*, Hesperia, CA, *J. coahuilensis, Adams 10241, 10242*, km 18, n of Durango, MX, *J. comitana, Adams 6858-6862*, 14 km s Comitan, Chiapas, MX, *J. deppeana* var. *deppeana, Adams 10539-10541*, El Chico National Park, Hidalgo, MX; *Adams 7632-7634*, Sacramento Mtns., e of Alamogordo, NM, USA; Adams *10640-10642*, Oak Creek Canyon-Flagstaff, AZ; *J. deppeana* var. *gamboana, Adams 6863-6867*, Comitan, Chiapas, MX; *J. deppeana* var. *patoniana, Adams 6836-6839*, km 152, w. of Durango (city), Durango, MX (P); *J. deppeana* var. *robusta, Adams 10255-10256*, w of La Ciudad, Durango, MX; *J. deppeana* f. *sperryi, Adams 10626*, Bridge Spring, Davis Mtns., TX, USA; *Adams 11312*, Munds Mtn., AZ; *J. deppeana* f. *zacatecensis, Adams 6840-6842*, 18 km w. Sombrette, Zacatecas, MX; *J. durangensis, Adams 6832-6835*, 52 km w El Salto, Dur., MX, *J. flaccida, Adams 6893-6896*, 22 km e San Roberto Jct., Nuevo, Leon, MX, *J. grandis, Adams 11964-11968*, Meyers, CA, *J. jaliscana, Adams 6846-6848*, 19 km e Mex. 200 on road to Cuale, Jalisco, MX, *J. martinezii, Adams 5950-5954*, 42 km n Lagos de Moreno, Jalisco, MX, *J. monosperma, Adams 10931-10934*, Reserve, NM, *J. monticola f. monticola, Adams 6874-6878*, El Chico Natl. Park, Hidalgo, MX, *J. occidentalis, Adams 8592-8596*, Sisters, OR, *J. osteosperma, Adams 6811-6815*, Salt Lake City, UT, *J. pinchotii, Adams 10463-10467*, Meridian, TX, *J. poblana, Adams 6868-6872*, 62 km s Oaxaca, MX, *J. saltillensis, Adams 6886-6890*, 14 km e San Roberto Jct., Nuevo Leon, MX, *J. standleyi, Adams 6852-6856*, 24 km nw Huehuetango, Guatemala, *J. zanonii, Adams 6898-6902*, Cerro Potosi, Nuevo Leon, MX, *J. virginiana, Adams 10231-10232*, Knoxville, TN, USA. Voucher specimens are deposited at BAYLU herbarium Baylor University.

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 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, trnD-T, trnL-F, trnS-G) 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 $MgCl_2$ 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. R6-1 (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. Minimum spanning networks were constructed from mutational events (ME) data using PCODNA software (Adams et al., 2009; Adams, 1975, Veldman, 1967).

RESULTS AND DISCUSSION

Sequencing the five gene regions (nrDNA (ITS), petN-psbM, trnS-trnG, trnD-trnT, trnL-trnF) resulted in 4411 bp of data. A Bayesian tree based on these data (Fig. 1) shows the diversity in this section. *Juniperus californica* is the most distinct in its DNA. The varieties of *J. deppeana* are mostly unresolved reflecting their close relationship (Adams and Schwarzbach 2013c). The varieties of *J. ashei* (var. *ashei*, var. *ovata*) are in separate clades, supporting a taxonomic change (see below). *Juniperus arizonica*, treated as *J. coahuilensis* var. *arizonica* (see Adams 2004), is clearly very distinct in its DNA (at least in these 5 DNA regions sequenced) from *J. coahuilensis* (as well as *J. monosperma*, *J. pinchotii* and *J. angosturana*, Fig. 1. *Juniperus zanonii* has been treated as *J. monticola* f. *compacta*, but its DNA is very distinct and it is well supported in a clade with *J. saltillensis*, not with *J. monticola* (Fig. 1).

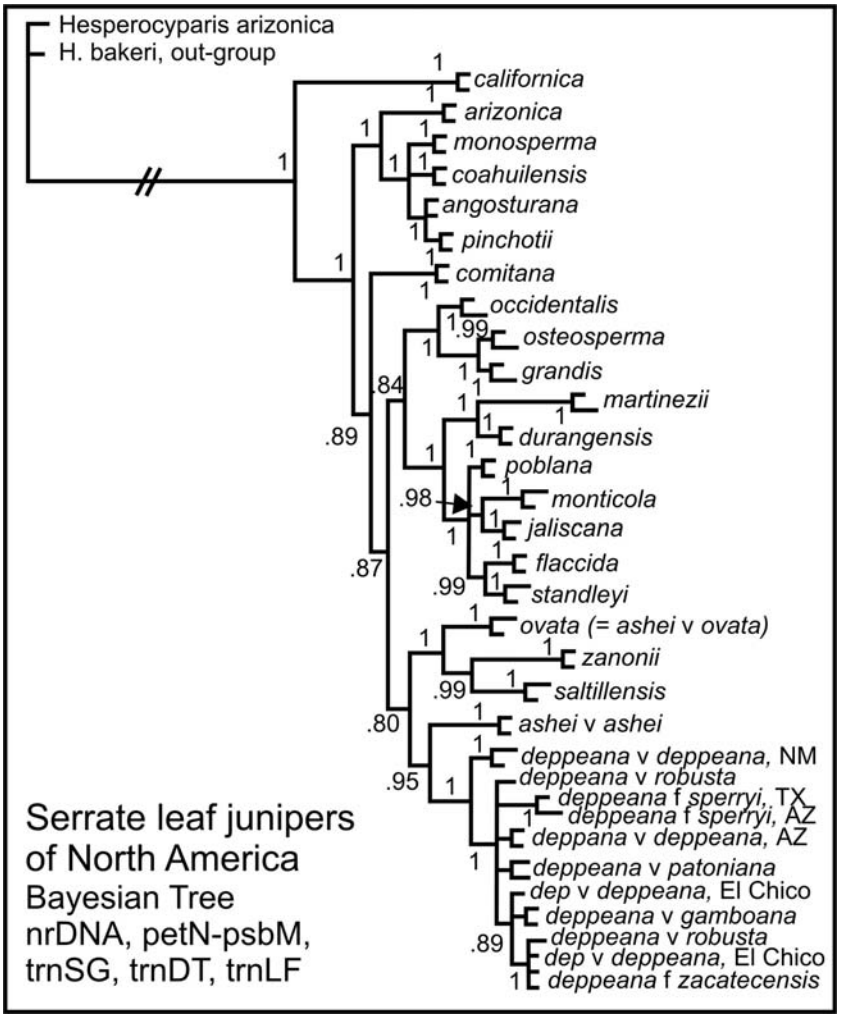

Figure 1. Bayesian tree of the serrate leaf junipers of North America. Numbers are posterior probabilities.

The Bayesian tree gives good information on the phylogeny within this group, but not about the magnitude of the mutational events (MEs) among the taxa. Analysis of the MEs (nucleotide substitutions plus indels) among these taxa (with *J. virginiana* as an outgroup) revealed multiple 191 MEs (found in more than one sample) and 21 single occurrence MEs. A minimum spanning network was constructed using the 191 MEs. Most of the taxa have accumulated many MEs. But three groups exhibit little variation: three western junipers; single seeded group; and *J. deppeana* group (Fig. 2).

Just as seen in the Bayesian tree (Fig. 1), *J. zanonii* is not near *J. monticola*, but it nearest neighbor is *J. saltillensis* (16 MEs, Fig. 2). In the single-seeded group, *J. arizonica* is the most distinct (8 MEs) and *J. angosturana* - *J. pinchotii*, the least distinct (1 ME, Fig. 2). Yet, *J. angosturana* and *J. pinchotii* are quite distinct in their morphology and leaf essential oils (Adams 2011). In the three western US junipers, *J. grandis* is separated by only 4 MEs from *J. osteosperma* and 7 MEs from *J. occidentalis* (Fig. 2). It seems evident that the nrDNA and 4 cp DNAs are not completely correlated with species delimitation.

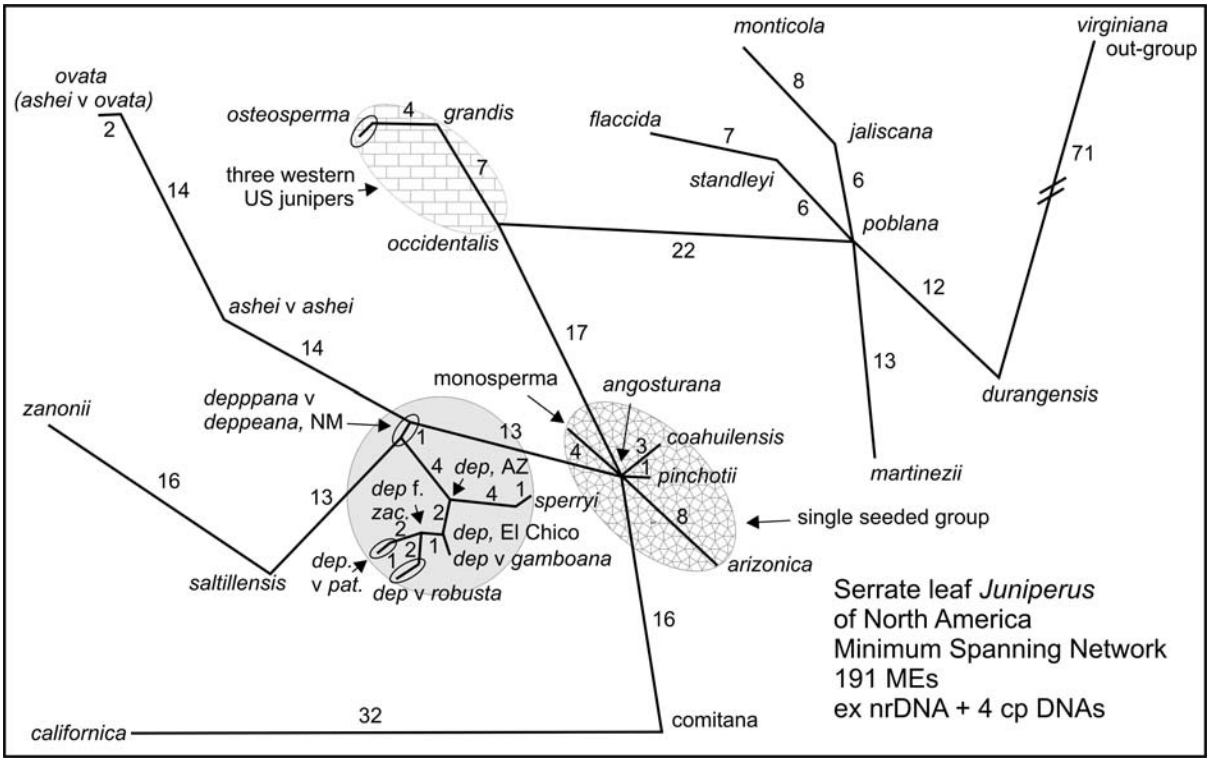

Figure 2. Minimum spanning network of serrate leaf *Juniperus* of North America. Numbers on the lines are the number of MEs (Mutational Events).

The varieties and forms of *J. deppeana* differ by 1 to 4 MEs and, on this basis, scarcely support varietal recognition. However, these taxa do differ in their morphology and leaf oils (Adams and Schwarzbach, 2013c; Zanoni and Adams, 1976, 1979).

Perhaps the most unusual taxa are *J. ashei* var. *ashei* and *J. a.* var. *ovata* that differ by 14 MEs (Fig. 2). These taxa overlap in their ranges near Ozona and New Braunfels, TX and have been shown to hybridize around New Braunfels (Adams, 2008). In view of the morphological and terpenoid differences (Adams 2011) and DNA differences, it seems appropriate to recognize *J. ashei* var. *ovata* as:

Juniperus ovata (R. P. Adams) R. P. Adams, ***stat. & com. nov.,*** oval gland juniper,
Basionym: *Juniperus ashei* Buch. var. *ovata* R. P. Adams, Phytologia 89(1): 17 (2007). TYPE: U. S. A., Texas, Crockett Co., 5 km w. Ozona, 6 Dec. 1994, *R. P. Adams 7463* (holotype: BAYLU, Paratypes: *R. P. ADAMS 7664, 7465, 7466, 7467* (BAYLU).

A summary of the level of support for taxonomic taxa based on the present DNA sequence data is presented in table 1. The treatments of Adams (2011) and Farjon (2005, 2020) differ for *J. grandis, J. martinezii, J. poblana* and *J. zanonii* (Table 1). In each of these cases, the DNA sequences in the present

study support the taxonomy of Adams (2011), except for *J. grandis*, in which specific status is only moderately supported. Notice that *J. grandis* and *J. osteosperma* differ by only 4 MEs (Table 1), but they are in strongly supported, separate clades (Fig. 1).

Table 1. Comparison of Adams and Farjon taxonomic treatments of taxa in this study. DNA sequencing support: ++ strong support; + support; +/- equivocal. NA = not analyzed, -- not mentioned. Nomenclatural changes are in boldface.

Adams(2011)	Farjon (2005, 2010)	Supported, this study
J. angosturana R. P. Adams	*J. angosturana*	+/- *J. angosturana*
J. arizonica (R. P. Adams) R. P. Adams	*J. arizonica*	++ *J. arizonica*
J. ashei Buchholz	*J. ashei*	++ *J. ashei*
var. *ovata* R. P. Adams	var. *ovata*	**++ *J. ovata***
J. californica Carriere	*J. californica*	++ *J. californica*
J. coahuilensis (Martinez) Gaussen ex R. P. Adams	*J. coahuilensis*	+/- *J. coahuilensis*
J. comitana Martinez	*J. comitana*	++ *J. comitana*
J. deppeana Steudel var. *deppeana*	*J. d.* var. *deppeana*	+ *J. d.* var. *deppeana*
J. deppeana Steudel var. *deppeana*	*J. d.* var. *pachyphlaea*	+ *J. d.* var. *deppeana*
forma *elongata* R. P. Adams	--	NA
forma *sperryi* (Correll) R. P. Adams	var. *sperryi*	+ f. *sperryi*
forma *zacatacensis* (Mart.) R. P. Adams	var. *zacatacensis*	+/- f. or var. *zacatacensis*?
var. *gamboana* (Mart.) R. P. Adams	*J. gamboana*	+/- var. *gamboana*
var. *patoniana* (Martinez) Zanoni	var. *robusta*	+/- f. or var. *patoniana*?
var. *robusta* Martinez	var. *robusta*	+/- f. or var. *robusta* ?
J. durangensis Martinez	*J. durangensis*	++ *J. durangensis*
var. *topiensis* R. P. Adams & S. Gonzalez	--	NA
J. flaccida Schlecht.	*J. flaccida*	++ *J. flaccida*
J. grandis R. P. Adams	*J. occidentalis* var. *australis*	+ *J. grandis*
J. jaliscana Martinez	*J. jaliscana*	++ *J. jaliscana*
J. martinezii Perez de la Rosa	*J. flaccida* var. *martinezii*	++ *J. martinezii*
J. monosperma (Engelm.) Sarg.	*J. monosperma*	+ *J. monosperma*
J. monticola Martinez forma *monticola*	*J. monticola*	++ *J. monticola*
forma *compacta* Martinez	forma *compacta*	++ *J. zanonii* in part
forma *orizabensis* Martinez	forma *orizabensis*	NA
J. occidentalis Hook.	*J. occidentalis*	++ *J. occidentalis*
J. occidentalis f. *corbetii* R. P. Adams	--	NA
J. osteosperma (Torr.) Little	*J. osteosperma*	+ *J. osteosperma*
J. pinchotii Sudworth	*J. pinchotii*	+/- *J. pinchotii*
J. poblana (Martinez) R. P. Adams	*J. flaccida* var. *poblana*	++ *J. poblana*
J. saltillensis M. T. Hall	*J. saltillensis*	++ *J. saltillensis*
J. standleyi Steyermark	*J. standleyi*	++ *J. standleyi*
J. zanonii R. P. Adams	*J. monticola* f. *compacta*	++ *J. zanonii*

Farjon (2005, 2010) recognized *J. d. f. sperryi* as *J. d*. var. *sperryi* and the DNA data shows the two accessions of *sperryi* in a well-supported clade (Fig. 1). They differ by 4 MEs from *J. d.* var. *deppeana* (AZ, Fig. 2). However, the furrowed bark is so distinctive that it is easy to recognize *sperryi* among quadrangular bark trees, so the furrowed bark trees should be commonly reported. But, in fact, furrowed bark trees (*sperryi*) are very rarely reported (Adams and Schwarzbach, 2013c). *Juniperus d*. f. *sperryi* has yet to be found in a uniform population of trees with furrowed bark, but is found as one or a very few trees, interspersed with trees having quadrangular bark (*J. deppeana*). Every aspect of the scattered

occurrence of *sperryi* points to the presence of one or a few genes that is (are) expressed in *J. deppeana*. This pattern is typical of a *forma* not a variety.

As a group, the serrate-leaf junipers are closely related and found in deserts and semi-arid mountains of the southwestern United States and Mexico. It seems likely that many taxa are products of hybridization. Hybridization (past and present) and incomplete lineage sorting among the closely related taxa appear to present problems in the DNA analysis.

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