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DNA fingerprinting and terpenoid analysis of *Juniperus blancoi* var. *huehuentensis* (Cupressaceae), a new subalpine variety from Durango, Mexico

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Abstract

During a survey of the flora of the Mexican state of Durango, a subalpine, shrubby–prostrate form of *Juniperus blancoi* Martinez was discovered on the summit of Cerro Huehuento. A comparison among populations of *J. blancoi* growing in different environments indicate that this subalpine taxon is differentiated in its morphology and RAPD DNA banding from *J. blancoi* populations growing at lower elevations. A new variety is recognized, *J. blancoi* Mart. var. *huehuentensis* R. P. Adams, S. González and M. González Elizondo. In addition to being shrubby–prostrate in habit, *J. blancoi* var. *huehuentensis* has the female cones attached to the lower sides of planate branchlets. A comparison of the leaf essential oils of *Juniperus scopulorum*, *Juniperus mucronata*, *J. blancoi* and *J. blancoi* var. *huehuentensis* revealed that the oil of var. *huehuentensis* exhibited few differences from typical *J. blancoi*.

Keywords: *Juniperus blancoi* var. *huehuentensis*; Cupressaceae; Morphology; RAPD; Systematics; Subalpine; Leaf terpenoids

1. Introduction

Juniperus blancoi Martinez was described from the Sierra Madre Occidental in Durango, México (Martinez, 1946). Later it was reported from Sonora and the state of México (Zanoni and Adams, 1979), as well as from Michoacán and Tlaxcala (Zamudio and Carranza, 1994). *J. blancoi* belongs to the group of entire leaf margined junipers in the section *Sabina* (Adams, 2000, 2004). The other species of this group known from Mexico are *Juniperus mucronata* R. P. Adams and *Juniperus scopulorum* Sarg.

In spite of its wide geographical range, *J. blancoi* is not common, known only from small, widely separated populations (Zanoni and Adams, 1975). According to Zamudio and Carranza (1994), *J. blancoi* may have been dispersed

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from cultivated trees in a small region of the states of México and Michoacán. Only one population is known from Michoacán at the extreme NE of the state, in a riparian area of an open *Cupressus* and *Abies* forest.

J. blancoi usually grows at 2580–2800 m elevation as a riparian tree along streams or in wet soils in flood plains. It has also been recorded as growing on slopes around a valley in El Oro, state of Mexico, as well as in Tlaxcala, on deep soil of a slope at 3000 m elevation.

A prostrate to low shrub (Fig. 1) referable to *J. blancoi*, was collected on bed rock and very thin soil on the upper slopes and at top of one of the highest peaks of the Sierra Madre Occidental (Cerro Huehuento), at 3150–3270 m. This form seems adapted to the extreme climatic conditions of the high elevation in which it grows. It differs from the typical *J. blancoi* in its prostrate to shrubby habit, forming broad patches, having the female cones on the underside of flattened branches.

The purposes of this study were to examine additional characters to determine if the 'subalpine' form represented genetic differentiation or merely environmental plasticity, and if there is genetic differentiation, is it sufficient to justify the recognition of a formal variety. To examine these questions, both the leaf volatile essential oils and DNA fingerprinting (RAPDs, Random Amplification of Polymorphic DNAs) were analyzed.

2. Material and methods

Specimens used in this study were *J. blancoi*: Adams 6849–6851 and 6903–6904, 2580 m, 7 km S of Carmona (s of El Oro) Mexico, Mexico; Adams 10257–10259, 2581 m, 7 km S of El Salto, Durango, Mexico; 10247–10251, 3227 m, Cerro Huehuento, Durango, Mexico; *J. mucronata*, Adams 8701–8705, 1180 m, 19 km W of Maicoba, Chihuahua/Sonora border, Mexico; *J. scopulorum*, Adams 7063–7066, 1761 m, Soda Springs, Idaho, USA. Voucher specimens are deposited at BAYLU, Baylor University and CIIDIR, Durango, Mexico.

2.1. Isolation and analyses of essential oils

Fresh leaves (200 g fresh wt.) were steam distilled for 2 h using a modified circulatory Clevenger apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored at −20 °C until analyzed. The extracted leaves were oven dried (48 h, 100 °C) for determination of oil yields.

The essential oils were analyzed on a Hewlett–Packard 5972 MSD, directly coupled to an HP5980 gas chromatograph. EI mass spectra were collected at 70 eV ionization voltage over the mass range *m/z* 41–425. Oil samples of 0.1 μl (10% solution in diethyl ether) were injected and split 1:10. Analytical conditions: column: J & W DB-5 (0.26 mm × 30 m, 0.25 μm coating thickness), carrier gas: Helium at 1 ml/min; injector temperature: 220 °C; oven programming: initial temperature: 60 °C, gradient: 3 °C/min, final temperature: 246 °C. The percentages of each compound are TIC values. Identifications were made by library searches of our volatile oil library (Adams, 2001) coupled with retention time data of reference compounds.

Fig. 1. *Juniperus blancoi* (left), showing the effects of stream forces during floods and the prostrate–shrubby *J. blancoi* juniper (right) at the peak of Cerro Huehuento.

2.2. Sampling for RAPD data

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 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 GCA 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; 265, CAG CTG TTC A; 268, AGG CCG CTT A; 327, ATA CGG CGT C; 338, CTC TGG CGG T; 346, TAG GCG AAC G; 347, TTG CTT GGC G; 413, GAG GCG GCG A.

PCR was performed in a volume of 15 μl containing 50 mM KCl, 10 mM Tris−HCl (pH 9), 2.0 mM MgCl2, 0.01% gelatin and 0.1% Triton X-100, 0.2 mM of each dNTP, 0.36 μM primers, 0.3 ng genomic DNA, 15 ng BSA and 0.6 unit of Taq DNA polymerase (Promega). A 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.). The thermal cycle was 94 °C (1.5 min) for initial strand separation (denaturation), 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. Amplification products were analyzed by electrophoresis on 1.5% agarose gels, 75 V, 55 min, and detected by staining with ethidium bromide. The gels were photographed under UV light with Polaroid film 667.

Bands that occurred once or that did not show fidelity within the replicated samples of each taxon were eliminated. It should be noted that these bands contain very useful information for the study of genetic variance and individual variation, but are merely ‘‘noise’’ in the present taxonomic study. Bands were scored in four classes: very bright (=6); medium bright (=5), faint (=4) and absent (=0). See Adams and Demeke (1993) for details on electrophoresis and RAPD band scoring.

Similarity 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).

3. Results and discussion

The leaf essential oils of the subalpine *J. blancoi* (BH) are similar (Table 1) to those of typical *J. blancoi* (BL), *J. mucronata* (MU) and *J. scopulorum* (SC) (see also Adams, 2000). All of these closely related taxa are high in sabinene (25.5−42.5%, Table 1) and moderately high in terpinen-4-ol (5.6−9.9%, Table 1). The subalpine *J. blancoi* has only one unique compound (naphthalene). Shyobunol (sesquiterpene), sempervirol, 4-*epi*-abietal and *trans*-totarol (diterpenes) were only found in *J. blancoi* (BL) and not in the subalpine form (Table 1). Overall, the oils are very similar among these four taxa. It might be noted that the newly discovered pregeijerene B (Cool and Adams, 2003) is common in these junipers (Table 1).

The RAPDs analysis (Fig. 2) revealed that the subalpine *J. blancoi* plants tightly cluster together, then with the other *J. blancoi* plants, then with *J. mucronata*. *J. scopulorum* clustered last, showing the very close similarity between *J. blancoi* and *J. mucronata*.

The prostrate−shrubby habit of the subalpine form of *J. blancoi* might just be an environmentally induced, subalpine ecotype. However, the typical tree forms of *Juniperus deppeana* var. *robusta* grow interspersed with the subalpine *J. blancoi* on the upper slopes of Cerro Huehuento. If the shrubby nature of the subalpine *J. blancoi* was due to frost damage to growing tips, it seems likely that the *J. deppeana* trees would also be shrubby. Furthermore, no individuals of the ‘‘typical’’ tree forms of *J. blancoi* were found on the lower slopes of Cerro Huehuento or nearby. The development of the female cones on the underside of the branches is perhaps an adaptation to protect the cones against the weather. Transplant studies are needed to ascertain if the shrubby type is due to the environment. The differences in DNA data are not due to environmental plasticity, but may be the result of selection to fit this harsh environment.

The RAPDs DNA data do not support the separation of the subalpine *J. blancoi* taxon as a distinct species. But the distinct morphology (seemingly not environmentally induced) of the plants from Cerro Huehuento supports their recognition as a variety of *J. blancoi*. The subalpine plants, geographically isolated from the lower elevation *J. blancoi* populations, represent a combination of genes that are not seen in the lower populations, so these subalpine plants are recognized as:

Juniperus blancoi Martinez var. *huehuentensis* R. P. Adams, S. González and M. González Elizondo, var. nov.

Table 1
Comparisons of the percent total oil for leaf essential oils for *J. blancoi* (BL), *J. blancoi* f. *huehuentensis* (BH), *J. mucronata* (MU), and *J. scopulorum* (SC)

KI	Compound	BL	BH	MU	SC
899	unknown, FW125, 43, 55, 67	0.4	0.6	0.9	0.2
926	tricyclene	–	–	t	t
931	α-thujene	1.1	1.8	2.2	1.3
939	α-pinene	1.3	2.4	3.0	5.9
953	α-fenchene	–	t	t	t
953	camphene	–	t	0.1	0.1
976	sabinene	25.5	39.0	42.5	32.1
980	β-pinene	t	0.1	0.5	0.3
991	myrcene	1.6	3.0	3.9	1.4
996	**hexanoic acid, 4-methyl, methyl ester**[a]	**0.2**	**0.5**	**1.5**	–
1001	**δ-2-carene**	**0.5**	**0.6**	**0.5**	–
1005	α-phellandrene	0.1	0.2	–	0.1
1011	**δ-3-carene**	–	–	**0.1**	**0.1**
1018	α-terpinene	1.2	3.0	1.7	1.2
1026	*p*-cymene	0.1	0.3	0.4	0.7
1031	**limonene**	**1.2**	**1.5**	**3.6**	**7.5**
1031	β-phellandrene	0.2	1.5	0.9	1.0
1034	2-heptylacetate	0.1	–	–	–
1050	(*E*)-β-ocimene	0.1	0.3	0.6	0.1
1062	γ-terpinene	1.9	4.7	2.8	2.1
1068	*cis*-sabinene hydrate	1.6	1.4	2.3	1.8
1088	terpinolene	0.5	1.5	1.5	1.2
1091	2-nonanone	2.4	0.6	1.6	0.2
1097	*trans*-sabinene hydrate	2.1	0.9	1.6	2.1
1098	linalool	1.0	0.5	2.4	0.7
1102	*n*-nonanal	0.3	0.3	0.2	–
1114	*trans*-thujone(=β-thujone)	–	t	–	0.1
1121	*cis*-*p*-menth-2-en-1-ol	0.8	1.0	0.6	0.5
1140	*trans*-*p*-menth-2-en-1-ol	0.5	0.4	0.2	0.3
1143	camphor	–	0.2	–	0.2
1148	camphene hydrate	–	–	–	t
1177	terpinen-4-ol	8.0	9.9	5.6	8.0
1179	**naphthalene**	–	**0.5**	–	–
1183	*p*-cymen-8-ol	–	–	–	t
1184	dillether	–		–	–
1189	α-terpineol	0.5	0.5	0.2	0.3
1191	myrtenol	t	–	–	–
1193	*cis*-piperitol	0.1	0.2	t	0.1
1205	*trans*-piperitol	0.3	0.2	t	0.2
1228	citronellol	0.3	–	0.2	0.7
1252	piperitone	0.1	–	0.2	–
1252	*trans*-sabinene hydrate acetate	–	–	–	t
1257	4Z-decen-1-ol	0.4	0.2	0.3	0.2
1274	pregeijerene B	2.4	3.4	2.1	6.1
1285	bornylacetate	0.3	0.1	0.5	0.9
1291	2-undecanone	–	–	0.2	–
1401	methyleugenol	–	–	–	t
1418	(*E*)-caryophyllene	t	0.3	0.6	0.1
1450	*trans*-muurola-3,5-diene	t	t	t	–
1451	(*Z*)-methyliso-eugenol	t	t	t	t
1455	α-humulene	–	t	t	t
1466	9-*epi*-(*E*)-caryophyllene	–	–	–	t
1477	γ-muurolene	–	t	t	t
1480	**germacrene D**	**0.1**	**0.3**	**0.1**	–
1493	4-*epi*-cubebol	–	t	0.1	t
1499	α-muurolene	t	0.2	0.2	t
1513	**γ-cadinene**	–	**0.3**	**0.4**	**0.1**

Table 1 (*continued*)

KI	Compound	BL	BH	MU	SC
1524	δ-cadinene	t	0.7	0.8	0.3
1535	α-copaen-11-ol	0.3	0.1	t	0.2
1538	α-cadinene	–	t	t	t
1549	elemol	6.5	3.9	1.8	4.2
1561	germacrene B	t	0.3	t	0.3
1564	(*E*)-nerolidol	–	t	–	–
1574	germacrene D-4-ol	0.2	1.0	3.2	1.1
1606	β-oplopenone	t	0.7	0.2	0.5
1630	γ-eudesmol	0.2	0.8	t	0.6
1640	*epi*-α-cadinol	t	0.5	1.1	0.4
1640	*epi*-α-muurolol	t	0.5	t	0.3
1645	α-muurolol (=torreyol)	t	0.2	t	–
1649	β-eudesmol	0.9	0.8	0.3	0.5
1652	α-eudesmol	0.7	0.9	0.8	0.5
1653	α-cadinol	0.7	0.9	0.8	0.6
1666	bulnesol	0.4	0.3	0.2	0.3
1689	**shyobunol**	**0.3**	–	–	–
1789	8-α-acetoxyelemol	13.0	2.3	1.3	7.4
2055	**manool**	**12.6**	**0.3**	**0.2**	–
2283	**sempervirol**	**0.2**	–	–	–
2288	**4-*epi*-abietal**	**0.1**	**t**	–	–
2314	***trans*-totarol**	**0.1**	–	–	–

Components that tend to separate the species are highlighted in boldface.
Compositional values less than 0.1% are denoted as traces (t).
Unidentified components less than 0.5% are not reported.
KI = Kovat's Index on DB-5(=SE54) column.
[a] Tentatively identified.

Frutex dioicus 0.5–1.5 m × 2–4 m cortice brunneis exuviatis; foliis flavovirentibus ramulis flabellatis applanatis apicibus cernuis; strobilis femineis plerumque 2-lobatis ubi maturis (one annis) cyaninis atque glaucis, infra in ramulosis locatis; pollinis liberatis mediis Martiis ad mediis Maiis. Type: México. Durango: Cerro Huehuento, cima, al S de Huachichiles, 24°4′30 N, 105°44′22″ W, S. González 6832 with E. Lozano and M. Lozano (holotype CIIDIR, isotypes BAYLU, others to be distributed), Huehue juniper, subalpine blancoi juniper.

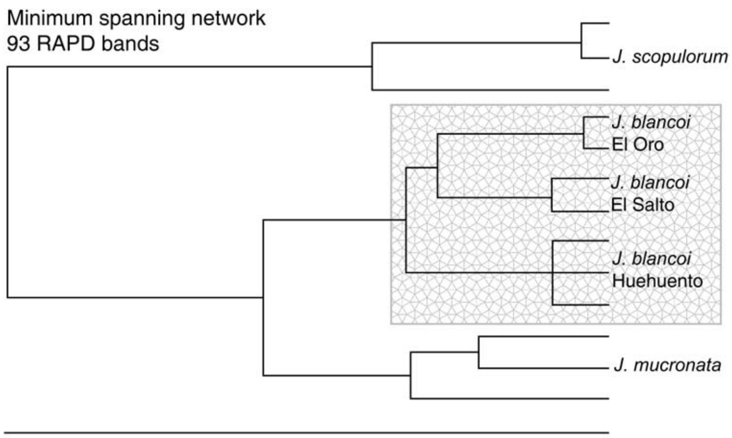

Fig. 2. Minimum spanning network based on 93 RAPD bands. Note the clustering of the Huehuento subalpine juniper with typical *J. blancoi* junipers.

Dioecious shrub, 0.5−1.5 m × 2−4 m wide, brown bark exfoliating in thin plates; foliage yellowish green, branchlets in flattened sprays, the ends lax or pendulous; most female cones bi-lobed, dark blue and glaucous when ripe (1 year), on the underside of the branches; pollen shedding mid-March to mid-May.

Other specimens examined: Durango: common on rock at top of Cerro Huehuento, 24° 04.587′N, 105° 44.463′W, 3227 m, 8.5.2004, R. P. Adams 10247 (BAYLU, CIIDIR); same locality, R. P. Adams 10248, 10249, 10250, 10251 (BAYLU, CIIDIR).

The new variety is very similar to *J. blancoi* Martinez var. *blancoi* which is a dioecious tree or shrubby tree, 1.5−15 m tall, with one main stem 15−45 (−60) cm diam., sometimes damaged by flood water (Fig. 1) and branched close to the base, and then appearing to be a shrub; bark brown, exfoliating in thin plates, reddish beneath, foliage lax or pendulous, branchlets ∼1 cm diam., scaly with bronze color beneath scales, ultimate branchlets generally planate; mature fruit with two lobes or globose (if one seeded), dark blue and glaucous upon maturity. *J. blancoi* var. *blancoi* is known from Durango, Michoacán, the state of Mexico and Tlaxcala, forming small, isolated populations.

Specimens of *J. blancoi* Martinez var. *blancoi* examined:

Durango: El Salto, E. C. Blanco A-500 (MEXU!, holotype); 3.2 m S of town square of El Salto on logging road to Pueblo Nuevo, ‘‘tascate’’, along bottom of arroyo (eight plants only) with pine−oak−juniper woods with *J. deppeana* var. *patoniana* and *J. deppeana* var. *robusta*, 26.12.1973, T. Zanoni 2769 (MEXU); Arroyo de Las Adjuntas, 1½−2 m N of Las Adjuntas (7.2 m W of El Salto on Rt. 40), scattered in arroyo bottom of pine−*J. deppeana* var. *robusta* forest, 8800−9000 ft, 26.12.1973, T. Zanoni 2775; on the banks of a running stream, approx. 7 km from El Salto town square on road to Pueblo Nuevo, 23° 45.241′N, 105° 22.851′W, 2580 m, 9.5.2004, female, past pollination, R. P. Adams 10257 (BAYLU, CIIDIR); same locality, male, pollen shed in spring, 9.5.2004, R. P. Adams 10258 (BAYLU, CIIDIR); on the banks of a running stream. approx. 6 km from El Salto town square on road to Pueblo Nuevo, about 23° N, 105° W, 2580 m, 9.5.2004, R. P. Adams 10259 (BAYLU, CIIDIR).

Michoacán: Ejido San Pedro Tarímbaro, mpio. Tlalpujahua, orilla de canal, 2800 m, 5.02.1993, tree 6−7 m, female, S. Zamudio 9008 (IEB, MEXU); same locality, árbol 6−8 m, male, S. Zamudio 9009 (IEB, MEXU).

Estado de México: El Oro, S of Carmona, R. P. Adams 6849, 6850, 6851, 6903, 6904 (BAYLU); 0.5 km N of El Salvador on road to Carmona; Adams 1486 (CS); Villa Victoria, en la desviación a Agangueo, mpio. San Felipe del Progreso, *Juniperus*, forest, tree 8−9 m, 40−60 cm DAP, 11.06.1992, E. Carranza 4099 (MEXU).

Tlaxcala: 3 km al W de San Felipe de Hidalgo, mpio. Nanacamilpa, *Pinus*-*Quercus* woods, 3000 m; tree 10−12 m, 16.11.1995, A. Cruz, C. Torres y M. Suarez s.n. (MEXU).

It should be noted that Zanoni and Adams (1979) cite *J. blancoi* from Sonora: Canon de Bavispe, White 3112 (ARIZ, MICH); Rancho de La Nacha, White 4093 (ARIZ, MICH). These specimens appear to be *J. scopulorum*.

The group of junipers in Mexico with entire leaf margin has thin, reddish brown bark; pendulous branchlets; and female cones dark blue to blackish, with a light to heavy coat of bloom, the pulp soft and fleshy. A key based on morphological traits to separate the Mexican junipers with entire leaf margin is presented:

Key to the junipers with entire leaf margins in México

1a	Seed cones globose, not bi-lobed, maturing in 2 years; twigs (6−15 mm diam.) with bark exfoliating in plates, reddish-copper beneath; leaves light to dark green, often glaucous blue or blue−gray.	*J. scopulorum*
1b	Seed cones bi-lobed, maturing in 1 year, twig (6−15 mm diam.) rarely exfoliating in plates and with reddish-copper beneath, leaves dark yellowish green to green.	
2a	Leaves mucronate tipped; heartwood bright purple.	*J. mucronata*
2b	Leaves with acute to obtuse tips (rarely mucronate on whip leaves in *J. blancoi*); heartwood red−brown with a purplish cast.	
3a	Tree or shrubby tree; female cones not on the underside of planate branches; foliage not dense.	*Juniperus blancoi* var. *blancoi*
3b	Prostrate shrub forming patches, one to several meters in diameter; female cones on the underside of planate branches; foliage dense.	*J. blancoi* var. *huehuentensis*

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