VARIATION IN LEAF ESSENTIAL OILS, DNA SEQUENCES AND MOPHOLOGY IN *JUNIPERUS DURANGENSIS*

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ABSTRACT

The leaf essential oil of *J. durangensis* is dominated by α-pinene (57.7%) and δ-3-carene (14.2%) with moderate amounts of verbenene, β-pinene, myrcene, limonene, β-phellandrene, terpinolene, linalool and elemol. The oil of the multi-seeded (5-9) Topia plants is similar to typical *J. durangensis*, except differing by containing several compounds not found in other *J. durangensis* oils: 1,8-cineole (2.8%, trace in other oils), cis-p-menth-2,8-dien-1-ol, germacrene B, patchouli alcohol, hexadecanol and sandaracopimarinal. The multi-seeded Topia junipers are recognized as a new variety, ***Juniperus durangensis*** var. ***topiensis*** R. P. Adams and S. Gonzalez, **var. nov**. *Phytologia 94(1): 40-52 (April 2, 2012).*

KEY WORDS: *Juniperus durangensis*, *J. durangensis var. topiensis,* terpenes, nrDNA, petN-psbM, trnD-trnT, trnL-trnF, trnS-trnG, SNPs, Cupressaceae, geographic variation.

Juniperus durangensis is in the serrate leaf margined junipers and appears to be most closely related to *J. martinezii* Peréz de la Rosa (Fig. 1). *Juniperus durangensis* Mart. is a tree or large shrub to 5 m that generally branches near the base and its seed cones contain 1-3(4) seeds (Adams, 2011).

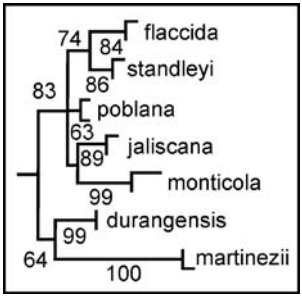

Figure 1. Clade from the serrate leaf margined junipers, from Adams and Schwarzbach (2011) showing the putative relationship of *J. durangensis* to *J. martinezii*.

It is often found on rhyolite, a nutrient poor rocky volcanic substrate, in Sierra Madre Occidental, a mountain range of western Mexico from Sonora and Chihuahua southward to Aguascalientes (Fig. 2). Adams (2009) reported DNA analysis of the multi-seeded (5-9 seeds), shrubs of *J. durangensis* growing near Topia, Durango. He considered those plants as conspecific with *J. durangensis*, differing by only 2 SNPs (in nrDNA and trnC-trnD). However, a subsequent visit to Topia, revealed that there are important differences between these plants and typical *J. durangensis*.

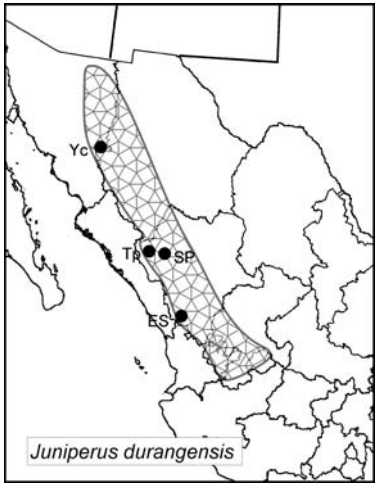

Figure 2. Distribution of *J. durangensis* with sampled areas.

Particularly noticeable is the large number of seeds per cone (Table 1) and multi-stemmed branching shrub habit in the Topia plants (Fig. 3). In addition, the plants were growing on lower elevations on the western flank of the Sierra Madre Occidental on tuffs (compacted volcanic ash or dust), not on rhyolite. Due to the morphological and edaphic differences seen at Topia, the previous study was expanded to additional populations of *J durangensis* and additional characters (leaf

essential oils, additional gene sequences and morphological data). These data are reported in this paper.

Table 1. Comparison of seeds/cone and habit in *J. durangensis*.

	seeds/cone	habit
Typical	1-3 (4)	small tree/shrub
Topia Tp, (Fig. 2)	(3) 5-9, 6.5 avg.	shrub
Santiago Papasquiraro(SP)	1.7 - 5 very variable	small tree/shrub

Figure 3. *J. durangensis* shrub habit growing at Topia.

MATERIALS AND METHODS

Specimens collected: *J. durangensis*, *Adams 8464, 8466, 8468,* 3 km sw of Yecora, 28º 22' N, 108º 57' W, 1570 m, Sonora, *Adams 6832-6834*, 52 km (road) w of El Salto, on Mex 40, 23º 41' N, 105º 44' W, 2700 m, Durango, MX; *Adams 11922, 11929, 11930*, 80 km (air), 137 km (road) east of Topia, 38 km (air), 116 km (road) w of Santiago Papasquiraro, 25º 03' 25.5" N, 105º 47' 45.2" W, 2670 m, Durango, *Adams 11923-25*, Topia, 25º 12.894' N, 106º 33.891' W, 1840 m, Durango, MX; *J. martinezii, Adams 5950, 5951, 8709,* 42 km n of Lagos de Moreno on Mex 80, thence east 10 km on road to La Quebrada Ranch, 21º 36' N, 101º 36' W, 2985 m, Jalisco, MX. Voucher specimens are deposited at BAYLU.

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

The oils were analyzed on a HP5971 MSD mass spectrometer, scan time 1/ sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see Adams, 2007 for operating details). Identifications were made by library searches of our volatile oil library (Adams, 2007), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

DNA Analysis - 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 using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia CA). PCR 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, trnDT, trnSG) 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 and Schwarzbach (2011) for the ITS, petN-psbM, trn D-trnT and trnS-trnG primers utilized. 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. (South 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 and NJ trees 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., 2009). Associational measures were computed using absolute compound value differences (Manhattan metric), divided by the maximum observed value for that compound over all taxa (= Gower metric, Gower, 1971; Adams, 1975). Principal coordinate analysis was performed by factoring the associational matrix based on the formulation of Gower (1966) and Veldman (1967).

RESULTS AND DISCUSSION

The composition of the leaf essential oil of *J. durangensis* from west of El Salto is very similar to the previous report (Adams et al. 1985) being dominated by α-pinene (57.7%) and δ-3-carene (14.2%) with moderate amounts of verbenene, β-pinene, myrcene, limonene, β-phellandrene, terpinolene, linalool and elemol. Several previously unknown compounds have now been identified. The oil of the Topia plants is also high in α-pinene (54.6%) and δ-3-carene (15.8%) with moderate amounts of β-pinene, myrcene, limonene, β-phellandrene, terpinolene and (E)-caryophyllene. In addition, its oil contains several compounds not found in other *J. durangensis* oils: 1,8-cineole (2.8%, trace in other oils), cis-p-menth-2,8-dien-1-ol, germacrene B, patchouli alcohol, hexadecanol and sandaracopimarinal (Table 2). The oils from the population east of Topia and Yecora are more similar to typical *J. durangensis* from El Salto, then to the Topia oil (Table 2), but they are much lower in δ-3-carene (7.1, 4.3%). An unusual aspect of the oils of *J. durangensis* is the number of components present in low concentrations that vary from present to absent across the populations. Some of these may due to enzyme non-specificity and/or free radical reactions. The differentiation of the Topia plants' oil is about the level one would expect in different varieties of *Juniperus*.

Sequencing nrDNA resulted in 1272 bp of aligned sequences which contained 9 single mutations and 10 multiple occurring mutations (informative). A minimum spanning network base on these 10 informative SNPs (Fig. 4) shows the separation of the closely related *J. martinezii,* but no grouping among the populations of *J. durangensis*.

Sequencing trnL-trnF (cpDNA) resulted in 695 bp of identical aligned sequences. Sequencing trnD-trnT (cpDNA) resulted in 656 bp of identical aligned sequences. Sequencing trnS-trnG (cpDNA) resulted in 818 bp of aligned sequences with only one SNP that separated *J. martinezii* from all *J. durangensis* samples.

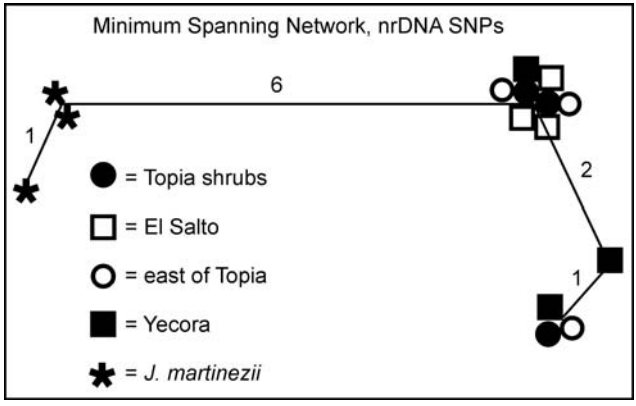

Figure 4. Minimum spanning network based on 10 SNPs from nrDNA. The numbers next to the links are the number of SNPs.

Sequencing petN-psbM yielded 847 bp of aligned sequences with 9 SNPs. Figure 5 shows that *J. martinezii* is separated by 6 SNPs from *J. durangensis.* The Topia plants are separated by 3 SNPs from other *J. durangensis* plants.

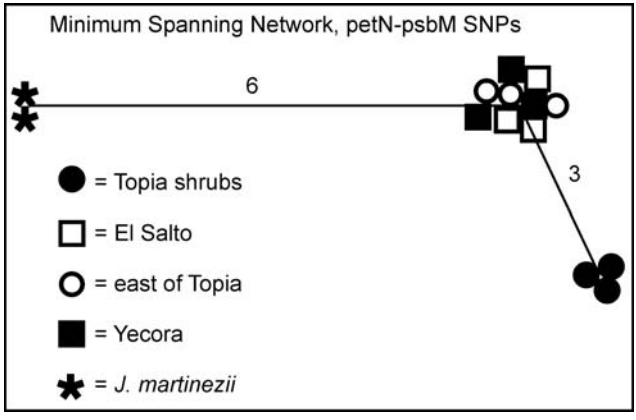

Figure 5. Minimum spanning network based on 9 SNPs found in petN-psbM (cpDNA).

Clearly the Topia junipers are closely related to typical *J. durangensis*. However, considering its much more shrubby habit, the difference in the number of seeds/ cone (considered one of the most important taxonomic characters in *Juniperus*), and its different habitat at Topia, it seems appropriate to recognize the junipers at Topia as a variety of *J. durangensis*:

Juniperus durangensis var. **topiensis** R. P. Adams & S. Gonzaléz, **var. nov**. TYPE: Mexico, Durango, Topia, 25º 12.894' N, 106º 33.891' W, 1840 m, *Adams 11923.*
(HOLOTYPE: BAYLU)., Fig. 6.

Junipero durangensi similis sed differt strobilis seminibus 5-9, et habitu fruticoso parvo multiramoso et ramulis laxiusculis.

Similar to *Juniperus durangensis*, but differing in having 5-9 seeds per cone and being a small, multi-branched shrub with less crowded branchlets.

Juniperus durangensis var. *topiensis* is currently known only from the type locality where, it is common on hillsides around Topia at about 1800-1900 m.

Other specimens studied: TOPOTYPES: *Adams 11924, 11925,* BAYLU, *S. Gonzaléz 7268a, 7268b*, BAYLU, CIIDIR, others to be distributed).

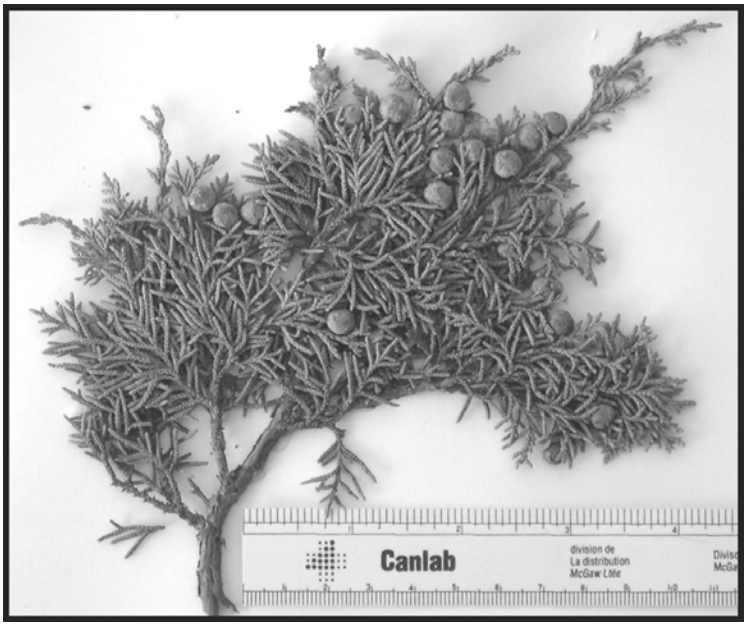

Figure 6. Holotype of *J. durangensis* var. *topiensis*.

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LITERATURE CITED

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

Adams, R. P. 1991. Cedarwood oil - Analysis and properties. pp. 159-173. in: Modern Methods of Plant Analysis, New Series: Oil and

Waxes. H.-F. Linskens and J. F. Jackson, eds. Springler- Verlag, Berlin.
Adams, R. P. 2007. Identification of essential oil components by gas chromatography/ mass spectrometry. 4th ed. Allured Publ., Carol Stream, IL.
Adams R. P. 2009. Variation in *Juniperus durangensis* and related junipers (Cupressaceae): Analysis of nrDNA and petN SNPs. Phytologia 91: 353-358.
Adams, R. P. 2011. The junipers of the world: The genus *Juniperus*. 3rd ed. Trafford Publ., Victoria, BC.
Adams, R. P. J. A. Bartel and R. A. Price. 2009. A new genus, *Hesperocyparis*, for the cypresses of the new world. Phytologia 91: 160-185.
Adams, R. P. and A. E. Schwarzbach. 2011. DNA barcoding a juniper: the case of the south Texas Duval county juniper and serrate junipers of North America. Phytologia 93: 146-154.
Adams, R. P., T. A. Zanoni and L. Hogge. 1985. The volatile leaf oils of two rare junipers from western Mexico: *Juniperus durangensis* and *J. jaliscana*. J. Nat. Prod. 48: 673-674.
Gower, J. C. 1966. Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika 53: 326-338.
Gower, J. C. 1971. A general coefficient of similarity and some of its properties. Biometrics 27: 857-874.
Veldman D. J., 1967. Fortran programming for the behavioral sciences. Holt, Rinehart and Winston Publ., NY.

Table 2. Leaf essential oil composition of populations of *J. durangensis*: west of El Salto (w El Salto), Topia shrubs, east of Topia, and Yecora. Compounds in boldface appear to separate taxa and were used in numerical analyses. KI = Kovats Index (linear) on DB-5 column. Compositional values less than 0.1% are denoted as traces (t). Unidentified components less than 0.5% are not reported. For unknown compounds, four ions are listed, with the largest ion underlined.

KI	Compound	w El Salto	Topia shrubs	east of Topia	Yecora
921	tricyclene	0.2	0.1	0.1	0.1
924	α-thujene	t	t	-	t
932	α-pinene	57.7	54.6	54.3	58.8
945	α-fenchene	0.7	0.6	0.3	0.2
946	camphene	0.6	0.8	0.5	0.7
953	thuja-2,4-diene	t	0.1	0.1	t
961	**verbenene**	**1.6**	**0.3**	**1.7**	**4.0**
969	sabinene	t	t	t	t
974	β-pinene	1.7	2.7	2.2	2.9
988	myrcene	2.3	4.1	3.0	3.9
1002	α-phellandrene	t	t	t	0.1
1008	**δ-3-carene**	**14.2**	**15.8**	**7.1**	**4.3**
1014	α-terpinene	t	t	t	t
1020	p-cymene	0.2	t	0.3	0.3
1024	sylvestrene	t	t	t	t
1024	limonene	1.6	2.8	1.4	1.4
1025	β-phellandrene	1.7	2.8	1.3	1.4
1026	**1,8-cineole**	**t**	**2.8**	**t**	**t**
1044	(E)-β-ocimene	t	t	0.5	0.7
1054	γ-terpinene	0.2	0.1	0.2	0.2
1085	p-mentha-2,4(8)-diene	t	t	t	t
1086	terpinolene	1.8	2.1	0.8	1.1
1087	**p-cymenene**	**0.1**	**t**	**0.8**	**0.2**
1094	**unknown, <u>96</u>, 109, 137, 152**	**0.6**	**t**	**1.4**	**0.2**
1098	**linalool**	**1.1**	**t**	**0.2**	**0.4**
1100	n-nonanal	-	0.1	0.1	0.1
1102	isopentyl-isovalerate	-	-	t	-
1113	endo-fenchol	0.1	t	t	-

KI	Compound	w El Salto	Topia shrubs	east of Topia	Yecora
1113	3-methyl-3-buten-methyl-butanoate	-	-	0.3	-
1122	α-campholenal	0.2	0.2	0.4	0.2
1135	trans-pinocarveol	0.3	0.2	0.5	0.2
1133	**cis-p-menth-2,8-dien-1-ol**	**-**	**t**	**-**	**-**
1137	cis-verbenol	-	-	t	-
1140	trans-verbenol	-	t	0.6	t
1141	camphor	0.3	0.1	-	0.4
1144	neo, iso-pulegol	0.7	-	-	-
1145	camphene hydrate	-	t	-	0.1
1148	citronellal	0.1	-	-	-
1154	karahanaenone	0.2	-	-	-
1155	iso, iso-pulegol	0.2	-	-	-
1158	trans-pinocamphone	-	t	t	t
1160	pinocarvone	t	-	t	-
1165	borneol	0.2	-	-	-
1166	δ-terpineol	0.1	-	-	-
1166	p-mentha-1,5-dien-8-ol	-	-	0.4	0.2
1172	cis-pinocamphone	t	t	t	0.1
1174	terpinen-4-ol	0.4	t	0.1	0.1
1178	naphthalene	-	0.1	t	t
1179	p-cymen-8-ol	0.1	t	0.2	t
1186	α-terpineol	0.2	0.2	0.3	0.1
1195	myrtenol	t	t	0.1	0.1
1195	methyl chavicol	-	-	0.3	0.1
1204	verbenone	0.2	0.1	0.3	0.1
1215	trans-carveol	t	t	t	-
1218	endo-fenchyl acetate	-	0.1	0.2	0.1
1223	citronellol	0.1	-	-	-
1232	thymol, methyl ether	t	-	0.8	t
1235	trans-chrysanthenyl acetate	-	-	-	1.2
1241	carvacrol, methyl ether	-	-	0.2	-
1254	linalyl acetate	-	t	-	t
1257	methyl citronellate	0.7	t	0.4	t
1274	pregeijerene B	-	-	-	t
1274	neo, iso-pulegol	-	-	0.2	-
1283	iso, iso-pulegol	-	-	t	t
1287	bornyl acetate	0.2	0.4	0.4	1.8

KI	Compound	w El Salto	Topia shrubs	east of Topia	Yecora
1298	trans-pinocarvyl acetate	-	-	t	-
1322	methyl geranate	0.1	0.2	-	-
1324	myrtenyl acetate	-	-	0.1	0.7
1326	unknown, 43, 92, 119, 152	-	-	-	0.7
1345	α-cubebene	-	-	0.1	0.2
1346	α-terpinyl acetate	-	-	-	t
1403	methyl eugenol	-	0.1	-	0.4
1407	longifolene	t	-	-	-
1417	(E)-caryophyllene	0.9	1.6	0.3	0.9
1448	cis-muurola-3,5-diene	t	-	-	-
1451	trans-muurola-3,5-diene	t	-	-	0.3
1452	α-humulene	0.7	t	-	0.4
1461	cis-cadina-1(6),4-diene	t	-	-	-
1465	cis-muurola-4(14),5-diene	t	-	-	-
1475	trans-cadina-1(6),4-diene	t	t	-	0.4
1478	γ-muurolene	-	t	t	-
1480	germacrene D	0.8	0.6	-	0.4
1493	trans-muurola-4(14),5-diene	0.1	t	-	0.7
1493	epi-cubebol	t	t	-	0.4
1500	α-muurolene	t	t	t	0.2
1513	β-curcumene	0.1	-	-	-
1513	γ-cadinene	0.4.	0.2	t	0.6
1513	endo-1-burbonanol	0.4	0.2	-	-
1513	cubebol	-	-	-	0.5
1514	trans-calamenene	t	-	-	0.5
1522	δ-cadinene	0.8	0.5	0.5	0.6
1528	zonarene	-	-	-	0.2
1544	α-calacorene	-	-	-	0.3
1548	elemol	1.0	0.3	4.3	0.1
1555	elemicin	-	-	t	-
1559	**germacrene B**	**-**	**0.3**	**-**	**-**
1561	(E)-nerolidol	-	-	-	0.1
1574	germacrene-D-4-ol	-	t	0.2	0.1
1582	caryophyllene oxide	0.1	0.6	0.2	1.1
1607	β-oplopenone	-	-	t	-
1608	humulene epoxide II	0.1	-	-	0.4
1627	1-epi-cubenol	0.2	0.7	-	1.0
1630	γ-eudesmol	-	t	1.6	-

KI	Compound	w El Salto	Topia shrubs	east of Topia	Yecora
1638	epi-α-cadinol	0.2	t	t	0.3
1638	epi-α-muurolol	0.3	t	t	0.4
1644	α-muurolol	t	t	t	t
1649	β-eudesmol	t	0.3	2.1	t
1652	α-eudesmol	t	0.2	1.3	-
1652	α-cadinol	0.6	0.3	1.2	0.5
1658	**patchouli alcohol**	**-**	**0.1**	**-**	**-**
1670	bulnesol	-	-	0.5	-
1688	shyobunol	t	0.6	0.2	0.1
1874	**hexadecanol**	**-**	**0.1**	**-**	**-**
1978	manoyl oxide	0.2	0.3	0.2	0.4
2026	geranyl linalool	0.2	-	-	-
2055	abietatriene	0.1	0.2	t	0.3
2184	**sandaracopimarinal**	**-**	**0.1**	**-**	**-**
2298	4-epi-abietal	0.2	-	0.2	0.2