TAXONOMY OF *JUNIPERUS DELTOIDES* FORMA *YALTIRIKIANA* IN TURKEY: LEAF TERPENOIDS AND SNPS FROM nrDNA AND petN

Robert P. Adams
Baylor University, Biology Department, One Bear Place, #97388, Waco, TX 76798, USA, email Robert_Adams@baylor.edu

and
Tuğrul Mataraci
Tarabya Bayırı Cad., Prof. Sitesi C Blok D:1 Tarabya-Sarıyer İstanbul, Turkey

ABSTRACT

Comparisons of SNPs of nrDNA and petN-psbM of *J. deltoides, J. oxycedrus,* and *J. o.* f. *yaltirikiana* revealed that f. *yaltirikiana* is part of *J. deltoides* (Turkey), not *J. oxycedrus* (France and Spain). Leaf terpenoids showed a similar pattern, supporting the recognition of *J. o.* f. *yaltirikiana* as ***J. deltoides*** R. P. Adams **f. *yaltirikiana*** (Meral Avci & Ziel.) R. P. Adams **comb. nov.** *Phytologia 93(3): 293-303 (December 1, 2011).*

KEY WORDS: *Juniperus deltoides, J. d.* f. *yaltirikiana, J. d.* var. *spilinanus*, *J. oxycedrus,* SNPs, nrDNA, petN-psbM, terpenes, taxonomy.

Recently, Avci and Zielinski (2008) described a new columnar form, *J. oxycedrus* L. f. *yaltirikiana* Meral Avci & Ziel. The occurrence of *J. oxycedrus* in Turkey seems problematic, as recent studies (Adams, 2004; Adams, et al., 2005) utilizing nrDNA sequencing, RAPDs, leaf terpenoids and morphology, clearly indicated that *J. oxycedrus* (*sensu stricto*) is restricted to the western Mediterranean; another, sibling species, *J. deltoides* R. P. Adams occupies the eastern Mediterranean region, including Turkey. Adams (2011) recognized both *J. deltoides* and *J. oxycedrus* in his monograph of *Juniperus*. Adams et al. (2010) analyzed the putative *J. oxycedrus* var. *spilinanus* Yalt., Eliçin & Terzioğlu and found the taxon to be *J. deltoides* [*J. d.* var. *spilinanus* (Yalt., Eliçin & Terzioğlu) Terzioğlu]. The purpose of the present study was to compare leaf terpenoids, SNPs from nrDNA and petN-psbM and morphology of *J. o.* f. *yaltirikiana* with *J. oxycedrus* (France, Spain) and *J. deltoides* (Turkey) to determine if the taxon is conspecific with *J. oxycedrus* or *J. deltoides*.

MATERIALS AND METHODS

Plant material*: J. deltoides, Adams 9430-9432*, Turkey; *J. d.* var. *spilinanus*, *Adams 10264-10266,* Turkey. *J. oxycedrus*, *Adams 9039, 9040*, France, *9053* Spain; *J. o.* f. *yaltirikiana*, *Adams 12393-12395,* Zonguldak Prov., Turkey. Voucher specimens are deposited at Baylor University (BAYLU).

Isolation 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 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.

Chemical Analyses - Oils from each of the taxa were analyzed and average values reported. 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 5 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.

Data Analysis - Terpenoids (as percent total oil) were coded and compared among the species by the Gower metric. Principal coordinate analysis was performed by factoring the associational matrix. Associational measures were computed using absolute compound value differences (Manhattan metric), divided by the maximum observed value for that compound over all taxa (Adams, 1975). Principal coordinate analysis was performed by factoring the associational matrix.

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.

PCR amplification ITS (nrDNA), 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 (petN-psbM) 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 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 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).

RESULTS AND DISCUSSION

Sequencing nrDNA revealed 17 nucleotide mutational events, including two mutations that occurred in two individuals among the taxa. A minimum spanning network based on the 17 SNPs is shown in figure 1 (left). No variation was found within or among *J. deltoides*, *J. d.* var. *spilinanus* or *J. oxycedrus* (Fig. 1, left). However, *J .oxycedrus* (France, Spain) was separated by 15 nrDNA SNPs from *J. deltoides* (Turkey) and *J. d.* var. *spilinanus* (Fig. 1, left). The three *J. o.* f. *yaltirikiana* individuals were either identical to *J. deltoides* or differed by a single mutation (Fig. 1).

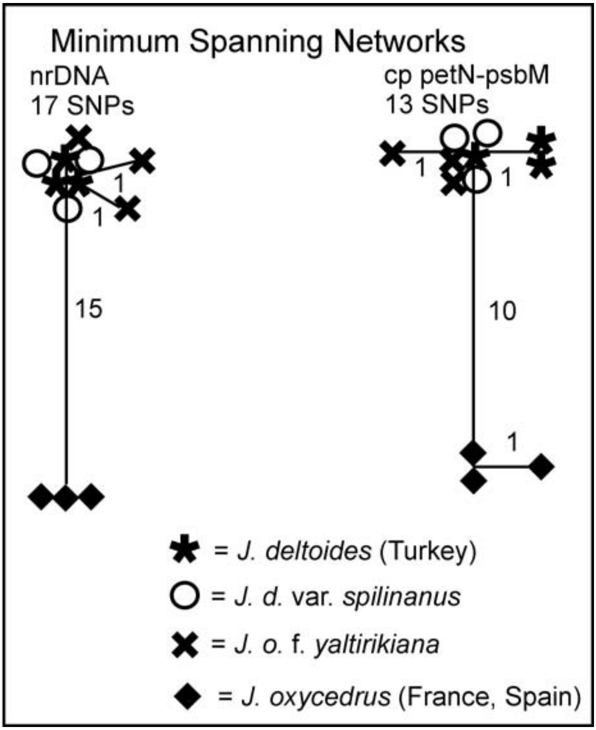

Figure 1. Minimum spanning networks based on nrDNA and petN-psbM SNPs. The numbers next to the lines are the number of SNPs.

Sequencing petN-psbM revealed 13 nucleotide mutational events that included three mutations that occurred in only single individuals among the taxa. *Juniperus oxycedrus* (France, Spain) was separated by 10 SNPs from *J. deltoides* - *J. d.* var. *spilinanus* (Fig. 1, right). The three *J. o.* f. *yaltirikiana* individuals were either identical to *J. deltoides* or differed by a single mutation (Fig. 1). It is clear from nrDNA and petN-psbM data that *J. oxycedrus* f. *yaltirikiana* is conspecific with *J. deltoides*.

Leaf terpenoids

The terpenoids of *J. o.* f. *yaltirikiana* are more like *J. deltoides* than *J. oxycedrus* (Table 1). Note the concentrations of β-phellandrene, trans-pinocarveol, myrtenal, carvone, α-terpinyl acetate, 2-tridecanone, α-muurolene, α-copaen-11-ol, α-calacorene, cadalene, germacrene B, dodecanoic acid, salvial-4-(14)-en-1-one, hexadecane, unknown sesquiterpene (AI1619), unknown C15-dienol acetate (AI1674), (2E,6E)-farnesol, (2E,6Z)-farnesol, 1-octadecene, nootkatone, sandaracopimara-8(14),15-diene, epi-13-manoyl oxide, sandaracopirmarinal, 1-docosene and phytol acetate (Table 1). It is interesting that some f. *yaltirikiana* compounds are quantatively more similar to *J. oxycedrus* than *J. deltoides*: α-pinene, p-cymene, cis-p-menthal-2,8-dien-1-ol, caryophyllene oxide, humulene epoxide II, epi-α-cadinol, α-cadinol, germacra-4(15),5,10(14)-triene-1-al, heptadecane, and tricosane (Table 1). The leaf oil of f. *yaltirikiana* contains several unique (to this data set) compounds: tetradecane, dodecanol, β-atlantol, benzophenone, muurola-4,10(14)-dien-1-β-ol, geranyl linalool, methyl linoleate, heneicosane, and abietal (Table 1).

Morphology

The f. *yaltirikiana* appears to differ from *J. deltoides* only by their columnar shapes. Adams (1982) found that *J. scopulorum* var. *columnaris* Fassett growing near a burning coal seam had columnar shapes. The coal seam has been burning since about 1880. Murphy and Holden (1979) propagated 25 columnar trees from the site and grew them in a smoke-free area. None of the cuttings produced columnar trees, but rather the typical, pyramidal trees of *J. scopulorum*. They concluded that ethylene from the burning coal induced the columnar shape. But, of course, other gasses from the burning coal (SO_2, NO and CO) may affect plant growth. Columnar trees of *Juniperus scopulorum* are also found downwind of coal burning plants, Butte, MT (Adams, 2011).

Figure 2. T. Mataraci with f. *yaltirikiana*.

Avci (2005) reported that hard coal is being burned to produce power in the area where the columnar junipers (f. *yaltirikiana*) occur. It seems possible that the burning coal gasses may be responsible for the columnar form. One could take cuttings and grow the clones in a smoke-free region as did Murray and Holden (1979) to determine if the columnar shape is genetic or environmentally controlled.

In view of the data presented in this study, it is apparent that *J. o.* f. *yaltirikiana* is not related to *J. oxycedrus* (*sensu stricto*), but to *J. deltoides*. To reflect this evolutionary relationship, *J. o.* f. *yaltirikiana* is recognized as:

Juniperus deltoides R. P. Adams **forma *yaltirikiana*** (M. Avci & Ziel.) R. P. Adams, **comb. nov**. **Basionym**: *Juniperus oxycedrus* L. forma *yaltirikiana* M. Avci & Ziel, Phytologia Balcanica 14: 38. 2008. Type: NW Turkey, E of Zonguldak, between Göbü and Türkali villages, 100-150 m, 17 Aug 2007, *M. Avci s.n.* (holotype: ISTO 32573).

Distribution: The taxon is known from the type locality. Additional specimens were collected from the type locality by T. Mataraci, ibid (*Adams 12393-12395*)

Key to forms and varieties of *J. deltoides*

Base of the leaf as wide as blade, stomatal bands generally not sunken. Leaves up to 17 mm long, 2.4 wide; erect-growing, spreading or prostrate shrub or tree.

1. Erect, large shrub or small tree to 12 m,
    2. Monopodial branching; pyramidal to round crown trees, leaves 9-17x1-2.4 mm.……………………………………....var. *deltoides*
    2. Fastigiate branching; strict trees, leaves up to 12mm long, 1.5 mm wide……………………………………………….f. *yaltirikiana*
1. Prostrate shrub or small tree to 0.5-0.6 m; leaves 6-10x1-1.5mm…… ……………………………………………………..var. *spilinanus*

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LITERATURE CITED

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

Adams, R. P. 1982. The effects of gases from a burning coal seam on morphological and terpenoid characters in *Juniperus scopulorum* (Cupressaceae). Southwestern Naturalist. 27: 279-286.

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. 2004. *Juniperus deltoides*, a new species, and nomenclatural notes on *Juniperus polycarpos* and *J. turcomanica* (Cupressaceae). Phytologia 86: 49-53.

Adams, R. P. 2007. Identification of essential oil components by gas chromatography/ mass spectrometry. 4th ed. Allured Publ., Carol Stream, IL.

Adams, R. P. 2011. Junipers of the world: The genus *Juniperus*. 3rd Ed., Trafford Publ., Vancouver, B.C., Canada.

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., J. A. Morris, R. N. Pandey and A. E. Schwarzbach. 2005. Cryptic speciation between *Juniperus deltoides* and *Juniperus oxycedrus* in the Mediterranean. Biochem. Syst. Ecol. 53: 771-787.

Adams, R. P., S. Terzioğlu and T. Mataraci. 2010. Taxonomy of *Juniperus oxycedrus* var. *spilinanus* in Turkey: Leaf terpenoids and SNPS from nrDNA and petN. Phytologia 92: 156-166.

Avci, M. and J. Zielinski. 2008. *Juniperus oxycedrus* f. *yaltirikiana* (Cupressaceae) a new form from NW Turkey. Phytologia Balcanica 14: 37-40.

Murphy, J. M. and D. J. Holden. 1979. The ethylene from burning lignite as a probable cause of columnarity in North Dakota junipers. Prairie Nat. 11: 11-22.

Yaltırık, F., G. Eliçin and S. Terzioğlu. 2007. *Juniperus oxycedrus* L. subsp. *oxycedrus* var. *spilinanus* Yalt., Eliçin & Terzioğlu: A new variety from Turkey. Turk. J. Bot. 31: 37-40.

Table 1. Comparisons of the per cent total oil for leaf oils components of *J. deltoides*, *J. d.* var. *spilinanus* and *J. d.* f. *yaltirikiana* compared to *J. oxycedrus*, France. Components that tend to separate the taxa are highlighted in boldface. AI = Retention Index on DB-5 column. Compositional values less than 0.1% are denoted as traces (t). Unidentified components less than 0.5% are not reported.

AI	Compound	delt	spil	yalt	oxy
802	hexanal	0.6	0.1	0.5	t
855	(E)-2-hexenal	0.9	0.3	0.7	0.4
927	tricyclene	0.1	0.2	t	0.1
930	α-thujene	t	0.1	t	t
939	**α-pinene**	**32.7**	**34.1**	**46.3**	**53.2**
953	α-fenchene	0.3	t	t	0.1
954	camphene	0.6	0.3	0.4	0.6
960	thuja-2,4(10)-diene	0.4	0.6	t	t
975	sabinene	0.2	1.3	0.2	0.5
979	**1-octen-3-ol**	**-**	**-**	**-**	**0.1**
979	β-pinene	3.0	0.8	3.5	2.1
991	myrcene	3.8	0.9	3.7	2.8
1002	**δ-2-carene**	**0.9**	**0.3**	**0.2**	**t**
1003	α-phellandrene	1.8	1.1	t	t
1011	**δ-3-carene**	**3.7**	**0.1**	**t**	**5.1**
1017	α -terpinene	0.1	0.2	t	t
1025	**p-cymene**	**2.3**	**2.6**	**0.7**	**0.3**
1029	limonene	6.0	6.4	1.4	3.5
1030	**β-phellandrene**	**11.5**	**10.5**	**6.3**	**0.8**
1050	(E)-β-ocimene	-	t	t	t
1060	γ-terpinene	0.2	0.2	0.2	0.1
1070	cis-sabinene hydrate	-	0.1	-	-
1089	terpinolene	2.0	0.8	2.0	0.7
1099	linalool	0.7	-	0.4	t
1101	n-nonanal	0.5	0.1	t	t
1122	**cis-p-menth-2-en-1-ol**	**0.3**	**0.4**	**t**	**-**
1123	**trans-p-mentha-2,8-dien-1-ol**	**t**	**-**	**-**	**-**
1126	α -campholenal	1.3	1.2	1.0	0.8
1126	chrysanthenone	t	t	-	-
1137	trans-pinocarveol	1.3	1.0	0.9	0.4

AI	Compound	delt	spil	yalt	oxy
1138	**cis-p-mentha-2,8-dien-1-ol**	**0.1**	**0.2**	**-**	**-**
1141	cis-verbenol	0.4	0.4	t	t
1145	trans-verbenol	1.8	3.0	0.5	0.6
1163	trans-pinocamphone	0.1	-	-	-
1165	pinocarvone	0.6	0.3	0.3	t
1170	p-mentha-1,5-dien-8-ol	1.1	0.6	1.1	0.5
1175	cis-pinocamphone	0.1	-	-	-
1177	terpinen-4-ol	0.6	0.4	0.4	0.3
1181	naphthalene	0.3	-	0.3	0.1
1183	p-cymen-8-ol	1.0	0.6	0.4	t
1189	α-terpineol	1.2	0.3	1.7	0.6
1196	**myrtenal**	**0.6**	**0.6**	**0.2**	**t**
1205	verbenone	0.7	0.7	0.6	0.3
1217	**trans-carveol**	**0.5**	**0.9**	**0.4**	**0.1**
1229	cis-carveol	t	0.2	-	-
1242	**cumin aldehyde**	**0.1**	**0.1**	**t**	**-**
1243	**carvone**	**0.3**	**0.6**	**t**	**-**
1253	piperitone	t	0.2	-	-
1257	linalyl acetate	t	-	-	0.3
1264	(2E)-decenal	0.2	0.1	-	-
1289	bornyl acetate	0.9	0.5	1.4	0.7
1298	trans-pinocarvyl acetate	0.1	-	-	-
1298	carvacrol	t	0.2	t	-
1299	**(2E,4Z)-decadienal**	**0.4**	**-**	-	**-**
1317	**(2E,4E)-decadienal**	**0.8**	**-**	**0.1**	**0.1**
1342	trans-carvyl acetate	t	0.1	-	-
1346	trans-piperitol acetate	-	-	-	-
1349	α-**terpinyl acetate**	**-**	**-**	-	**0.2**
1373	α-ylangene	-	t	-	-
1377	α-**copaene**	**0.2**	**-**	**0.3**	**-**
1381	geranyl acetate	t	-	-	-
1388	β-bourbenene	0.2	0.2	t	0.3
1400	**tetradecane**	**-**	**-**	**0.3**	**-**
1408	longifolene	0.6	-	-	-
1419	(E)-caryophyllene	1.2	0.7	0.6	0.4
1431	cis-thujopsene	0.1	-	-	-

AI	Compound	delt	spil	yalt	oxy
1455	α-humulene	0.8	0.5	0.3	0.3
1465	**dodecanol**	**-**	**-**	**0.3**	**-**
1480	γ-muurolene	t	-		0.1
1485	germacrene D	0.7	-	3.7	2.3
1486	ar-curcumene	-	0.2	-	-
1494	trans-muurola-4(14),5-diene	-	0.1		-
1496	**2-tridecanone**	**-**	**-**	-	**0.3**
1500	**α-muurolene**	**0.4**	**1.1**	**0.4**	**-**
1514	γ-cadinene	0.4	0.5	0.8	0.7
1514	**cubebol**	**-**	**0.2**	-	**-**
1523	δ-cadinene	0.4	0.8	1.6	0.4
1541	α-**copaen-11-ol**	**0.1**	**0.2**	**0.2**	**-**
1546	α-**calacorene**	**0.5**	**0.5**	**0.3**	**-**
1561	**germacrene B**	**-**	**-**	**-**	**0.1**
1566	**β-calacorene**	**0.3**	**-**	-	**-**
1563	**(E)-nerolidol**	**-**	**1.2**	-	**-**
1567	**dodecanoic acid**	**-**	**-**	**-**	**0.4**
1583	**caryophyllene oxide**	**3.2**	**3.9**	**0.2**	**0.4**
1595	**salvial-4(14)-en-1-one**	**-**	**-**	-	**0.4**
1600	**hexadecane**	**-**	**-**	-	**0.3**
1601	cedrol	0.1	t	-	t
1608	humulene epoxide II	1.1	1.7	0.2	0.3
1608	**β-atlantol**	**-**	**-**	**0.4**	**-**
1619	**sesquiterpene alcohol, M226**	**-**	**-**	-	**0.3**
1626	**benzophenone**	**-**	**-**	**0.2**	**-**
1627	1-epi-cubenol	0.1	0.2	-	-
1630	**muurola-4,10(14)-dien-1-β-ol**	**-**	**-**	**0.2**	**-**
1640	**epi-α-cadinol**	**-**	**0.1**	**0.6**	**0.6**
1651	β-eudesmol	-	-	-	t
1654	**α-cadinol**	**-**	**-**	**0.7**	**1.6**
1661	cis-calamenen-10-ol	-	0.2	-	-
1674	**C15-dienol acetate, M+224**	**-**	**-**	-	**1.6**
1677	**cadalene**	**0.1**	**0.2**	**t**	**-**
1686	**germacra-4(15),5,10(14)-**				
	triene-1-al	**-**	**-**	**1.1**	**1.6**
1700	heptadecane	-	-	0.2	0.3

AI	Compound	delt	spil	yalt	oxy
1717	**(2E, 6E)-farnesol**	**-**	**-**	-	**0.3**
1746	**(2E, 6Z)-farnesol**	**-**	**-**	-	**0.4**
1790	**1-octadecene**	**-**	**-**	-	**t**
1800	**octadecane**	**-**	**-**	**t**	**t**
1807	**nootkatone**	**-**	**-**	-	**0.1**
1900	**nonadecane**	**-**	**-**	t	**0.1**
1966	**sandaracopimara-8(14),15-diene**	**-**	**-**	-	**0.1**
1998	manoyl oxide	1.3	5.5	2.5	6.2
2000	eicosane	-	-	t	-
2014	palustradiene (=abieta-8,13-diene)	-	0.2	-	-
2017	**epi-13-manoyl oxide**	**-**	**-**	-	**0.1**
2023	abieta-8,12-diene	-	0.3	-	0.1
2026	**geranyl linalool**	**-**	**-**	**0.7**	**-**
2057	abietatriene	0.1	1.4	0.8	1.2
2088	**abietadiene**	**-**	**3.4**	**t**	**1.3**
2095	**methyl linoleate**	**-**	**-**	**0.3**	**-**
2100	**heneicosane**	**-**	**-**	**0.8**	**-**
2154	**abieta-8(14),13(15)-diene**	**-**	**0.2**	**-**	**0.2**
2185	**sandaracopimarinal**	**-**	**-**	**-**	**0.2**
2190	**1-docosene**	**-**	**-**	**-**	**0.1**
2200	**docosane**	**-**	**-**	**0.4**	**0.1**
2218	**phytol acetate**	**-**	**-**	**-**	**0.1**
2300	**tricosane**	**-**	**-**	**0.2**	**0.2**
2312	abieta-7,13-dien-3-one	-	0.1	-	-
2313	**abietal**	**-**	**-**	**0.3**	**-**