TAXONOMY OF *JUNIPERUS OXYCEDRUS VAR. SPILINANUS* IN TURKEY: LEAF TERPENOIDS AND SNPS FROM nrDNA AND petN

Robert P. Adams
Biology Department, Baylor University, Box 727
Gruver, TX, 79040, USA
Robert_Adams@baylor.edu

Salih Terzioğlu
Karadeniz Techincal University, Faculty of Forestry
Department of Forest Botany, TR-61080
Trabzon, Turkey
and
Tuğrul Mataracı
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.* var. *spilinanus* revealed that *J. o.* var. spilinanus is allied with *J. deltoides* (Turkey), not *J. oxycedrus* (France and Spain). Leaf terpenoids showed the same pattern, supporting the recognition of *J. o*. var. *spilinanus* as ***J. deltoides*** R. P. Adams **var. *spilinanus*** (Yalt. et al.) Terzioğlu, **comb. nov.** *Phytologia 92(2): 156-166 (August 2, 2010).*

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

Recently, Yaltırık et al. (2007) described a new shrubby variety, *J. oxycedrus* L. var. *spilinanus* Yalt., Eliçin & Terzioğlu from Spildağı National Park of western Turkey. The occurrence of *J. oxycedrus* in Turkey seems probablematical as recent studies (Adams, 2004; Adams, et al., 2005) utilizing nrDNA sequencing, RAPDs, leaf

terpenoids and morphology, clearly indicate 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 (Fig. 1). The same pattern was found in nr

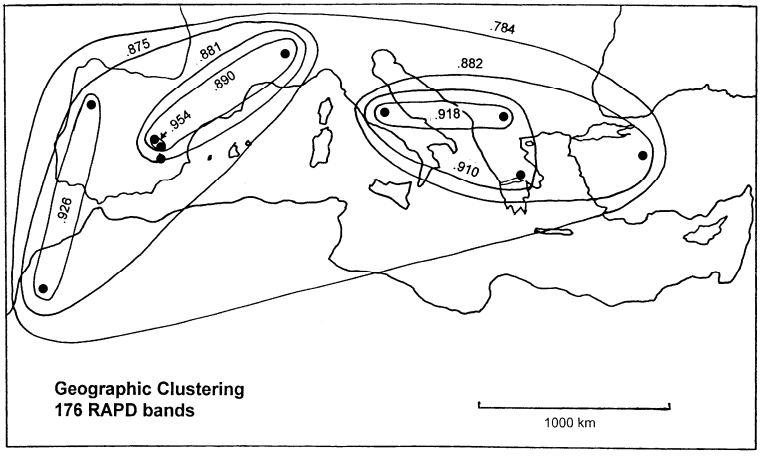

Figure 1. Differentiation of J. oxycedrus (left) and J. deltoides (right) based on 176 RAPD bands. (from Adams et al., 2005).

DNA sequences, leaf terpenoids and morphology (Adams et al., 2005). In his monograph of *Juniperus*, Adams (2008) recognized both *J. deltoides* and *J. oxycedrus.*

The purpose of the present study was to compare leaf terpenoids, SNPs from nrDNA and petN-psbM and morphology of the new *J. o.* var. *spilinanus* with *J. oxycedrus* (France, Spain) and *J. deltoides* (Turkey).

MATERIALS AND METHODS

Plant material*: J. deltoides, Adams 9430-9432*, Turkey; *J. oxycedrus*, *Adams 9039, 9040*, France, *9053* Spain; *J. o.* var. *spilinanus*, *Mataraci 1, 2, 3, (=Adams 10264-10266*; *=KATO 18791-18793)* Turkey. Voucher specimens are deposited at the Herbariums, Baylor University (BAYLU) and Karadeniz Technical University Faculty of Forestry (KATO).

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).

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). Principlal coordinate analysis was performed by factoring the associational matrix based on the formulation of Gower (1966) and Veldman (1967).

RESULTS AND DISCUSSION

Sequencing nrDNA revealed 12 nucleotide mutational events that included one mutation that occurred in only one individual among the taxa. The single nucleotide change was discarded from the SNPs leaving 11 nrDNA characters. A minimum spanning network based on the 11 SNPs is shown in figure 2 (left). No variation was found within

or among *J. deltoides*, *J. o.* var. *spilinanus* or *J. oxycedrus* (Fig. 2, left). However, *J .oxycedrus* (France, Spain) was separated by 11 nrDNA SNPs from *J. deltoides* (Turkey) and *J. o.* var. *spilinanus* (Fig. 2, left).

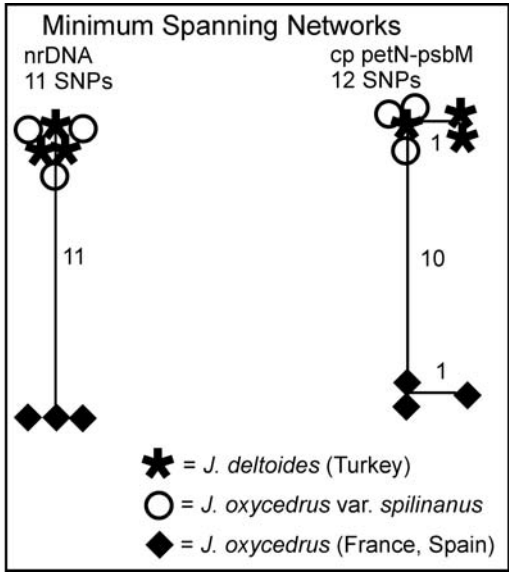

Figure 2. Minimum spanning networks based on nrDNA and petN-psbM SNPs. The numbers next to the lines are the number of SNPs.

Sequencing petN-psbM revealed 13 nucleotide mutational events that included a mutation that occurred in only one individual among the taxa. Discarding the singular event, resulted in 12 petN-psbM characters. *Juniperus oxycedrus* (France, Spain) was separated by 10 SNPs from *J. deltoides* - *J. o.* var. *spilinanus* (Fig. 2, right). A single SNP was found among *J. deltoides* and *J. oxycedrus*. It seems clear from nrDNA and petN-psbM data that *J. o.* var. *spilinanus* is conspecific with *J. deltoides*.

Leaf terpenoids

Overall, the leaf terpenoids of *J. oxycedrus* var. *spilinanus* are more like *J. deltoides* than *J. oxycedrus* (Table 1). Notice the concentrations of α-pinene, p-cymene, limonene, β-phellandrene, trans-pinocarveol, cis-p-menthal-2,8-dien-1-ol, myrtenal, carvone, (2E)-decenal, α-muurolene, α-calacorene, caryophyllene oxide, humulene epoxide II, and cadalene (Table 1).

The leaf terpenoids of *J. o.* var. *spilinanus* differ quantitatively from *J. deltoides* oil in the concentrations of myrcene (0.9, 3.8%) and δ-3-carene (0.1, 3.7%) and, based on this limited sampling, appear to have a few compounds not found in the oils of either *J. deltoides* or *J. oxycedrus*: ar-curcumene, trans-muurola-4(14), 5-diene, cubebol, and (E)-nerolidol.

Several of the diterpenoids are found in larger concentration in *J. o.* var. *spilinanus* than in *J. deltoides*, with amounts more similar to that of *J. oxycedrus*: manoyl oxide, abietatriene, abietadiene, and abieta-8(14),13(15)-diene (Table 1). However, the concentrations of these closely related diterpenes may be influenced by a few genes in that pathway, so one should be careful to not give too much weight to this similarity.

Morphology

Juniperus o. var. spilinanus is primarily separated from *J. oxycedrus* (Yaltirik et al., 2007) in being a shrub and having detached leaves with sagittate bases. The shape of detached leaf bases depends on how the leaf is attached to the stem. In *Juniperus,* sections *Juniperus* (approx. 12 species) and *Caryocedrus* (1 species) the awn-like leaves are attached directly to the stem and exfoliate by an abscission layer at the base of the leaf. In contrast, in *Juniperus* section *Sabina* (approx. 60 species with scale-like leaves) the leaf base clasps the stem and is attached to the stem. The shape of detached leaf bases has not previously been utilized in *Juniperus* taxonomy before Yaltirik et al. (2007). Examination of leaf bases of *J. oxycedrus* (sensu stricto), *J. o.* var. *spilinanus* and *J. deltoides* on leaves detached from herbarium specimens revealed that all three taxa have very similar detached leaf

bases. All of the detached leaf bases examined varied from a blunt to sagittate shape for the brown pre-abscission layer. Generally, the detached leaf base had a semi-circle shape that gave a sagittate shape. Both *J. deltoides* and *J. o.* var. *spilinanus* had leaves that scarcely narrowed at the base of attachment, in contrast to *J. oxycedrus* (France, Spain) that had leaves that narrowed at the base. In addition, both *J. deltoides* and *J. o.* var. *spilinanus* had stomatal bands scarcely sunken, whereas J. oxycedrus (*sensu stricto*) had sunken stomatal bands. Both *J. deltoides* and *J. o.* var. *spilinanus* had seed cones with raised cone scale tips, compared to seed cones globose, without raised cone scale tips for *J. oxycedrus*. *Juniperus o.* var. *spilinanus* fits well with *J. deltoides* in the European *Juniperus* key of Adams (p. 85, 2008).

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

J. deltoides* var. *spilinanus (Yalt. et al.) Terzioğlu, **comb. nov.**

Basionym: *Juniperus oxycedrus* L. subsp. *oxycedrus* var. *spilinanus* Yalt. et al., Turk. J. Bot. 31: 38, 2007. Type: Spildağı National Park, Turkey.

Distribution: The taxon is known from the type (KATO 13371) and Isotype (KTUB 533); Turkey, Manisa: Spildağı National Park, in *Pinus brutia* Ten. forest *P. nigra* J.F. Arnold subsp. *nigra* var. *caramanica* (Loudon) Rehder forests, on stony North and South slopes, 800-1400 m a.s.l. The specimens analyzed were collected by T. Mataracı, ibid (Adams 10264-10266 and KATO 18791 - 18793).

Table 1. Comparisons of the per cent total oil for leaf oils components of *J. oxycedrus*, France, *J. deltoides*, Turkey and *J. oxycedrus* var. *spilinanus*, shrubs, Turkey. Components that tend to separate the populations 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	oxy	spil	delt
802	hexanal	t	0.1	0.6
855	(E)-2-hexenal	0.4	0.3	0.9
927	tricyclene	0.1	0.2	0.1
930	α-thujene	t	0.1	t
939	**α-pinene**	**53.2**	**34.1**	**32.7**
953	α-fenchene	0.1	t	0.3
954	camphene	0.6	0.3	0.6
960	thuja-2,4(10)-diene	t	0.6	0.4
975	sabinene	0.5	1.3	0.2
979	1-octen-3-ol	0.1	-	-
979	β-pinene	2.1	0.8	3.0
991	**myrcene**	**2.8**	**0.9**	**3.8**
1002	δ-2-carene	t	0.3	0.9
1003	α-phellandrene	t	1.1	1.8
1011	**δ-3-carene**	**5.1**	**0.1**	**3.7**
1017	α -terpinene	t	0.2	0.1
1025	**p-cymene**	**0.3**	**2.6**	**2.3**
1029	**limonene**	**3.5**	**6.4**	**6.0**
1030	**β-phellandrene**	**0.8**	**10.5**	**11.5**
1050	(E)-β-ocimene	t	t	-
1060	γ-terpinene	0.1	0.2	0.2
1070	cis-sabinene hydrate	-	0.1	-
1089	terpinolene	0.7	0.8	2.0
1099	linalool	t	-	0.7
1101	n-nonanal	t	0.1	0.5
1122	cis-p-menth-2-en-1-ol	-	0.4	0.3
1123	**trans-p-mentha-2,8-dien-1-ol**	**-**	**-**	**t**
1126	α -campholenal	0.8	1.2	1.3
1126	chrysanthenone	-	t	t
1137	**trans-pinocarveol**	**0.4**	**1.0**	**1.3**

AI	Compound	oxy	spil	delt
1138	**cis-p-mentha-2,8-dien-1-ol**	**-**	**0.2**	**0.1**
1141	cis-verbenol	t	0.4	0.4
1145	trans-verbenol	0.6	3.0	1.8
1163	trans-pinocamphone	-	-	0.1
1165	pinocarvone	t	0.3	0.6
1170	p-mentha-1,5-dien-8-ol	0.5	0.6	1.1
1175	cis-pinocamphone	-	-	0.1
1177	terpinen-4-ol	0.3	0.4	0.6
1181	naphthalene	0.1	-	0.3
1183	p-cymen-8-ol	t	0.6	1.0
1189	α -terpineol	0.6	0.3	1.2
1196	**myrtenal**	**t**	**0.6**	**0.6**
1205	verbenone	0.3	0.7	0.7
1217	trans-carveol	0.1	0.9	0.5
1229	**cis-carveol**	**-**	**0.2**	**t**
1242	cumin aldehyde	-	0.1	0.1
1243	**carvone**	**-**	**0.6**	**0.3**
1253	piperitone	-	0.2	t
1257	linalyl acetate	0.3	-	t
1264	**(2E)-decenal**	**-**	**0.1**	**0.2**
1289	bornyl acetate	0.7	0.5	0.9
1298	trans-pinocarvyl acetate	-	-	0.1
1298	carvacrol	-	0.2	t
1299	**(2E,4Z)-decadienal**	**-**	**-**	**0.4**
1317	**(2E,4E)-decadienal**	**0.1**	**-**	**0.8**
1342	trans-carvyl acetate	-	0.1	t
1346	trans-piperitol acetate	-	-	-
1349	**α-terpinyl acetate**	**0.2**	**-**	**-**
1373	α-ylangene	-	t	-
1377	**α-copaene**	**-**	**-**	**0.2**
1381	geranyl acetate	-	-	t
1388	β-bourbenene	0.3	0.2	0.2
1408	longifolene	-	-	0.6
1419	(E)-caryophyllene	0.4	0.7	1.2
1431	cis-thujopsene	-	-	0.1
1455	α-humulene	0.3	0.5	0.8
1480	γ-muurolene	0.1	-	t

AI	Compound	oxy	spil	delt
1485	germacrene D	2.3	-	0.7
1486	**ar-curcumene**	**-**	**0.2**	**-**
1494	**trans-muurola-4(14),5-diene**	**-**	**0.1**	**-**
1496	**2-tridecanone**	**0.3**	**-**	**-**
1500	**α-muurolene**	**-**	**1.1**	**0.4**
1514	γ-cadinene	0.7	0.5	0.4
1514	**cubebol**	**-**	**0.2**	**-**
1523	δ-cadinene	0.4	0.8	0.4
1541	**α-copaen-11-ol**	**-**	**0.2**	**0.1**
1546	**α-calacorene**	**-**	**0.5**	**0.5**
1561	germacrene B	0.1	-	-
1566	**β-calacorene**	**-**	**-**	**0.3**
1563	**(E)-nerolidol**	**-**	**1.2**	**-**
1567	dodecanoic acid	0.4	-	-
1583	**caryophyllene oxide**	**0.4**	**3.9**	**3.2**
1595	**salvial-4(14)-en-1-one**	**0.4**	**-**	**-**
1600	**hexadecane**	**0.3**	**-**	**-**
1601	cedrol	t	t	0.1
1608	**humulene epoxide II**	**0.3**	**1.7**	**1.1**
1619	**sesquiterpene alcohol, M+226**	**0.3**	**-**	**-**
1627	1-epi-cubenol	-	0.2	0.1
1640	epi-α-cadinol	0.6	0.1	-
1651	β-eudesmol	t	-	-
1654	**α-cadinol**	**1.6**	**-**	**-**
1661	cis-calamenen-10-ol	-	0.2	-
1674	**C10-dienol acetate, M+224**	**1.6**	**-**	**-**
1677	**cadalene**	**-**	**0.2**	**0.1**
1686	**germacra-4(15),5,10(14)-triene-1-al**	**1.6**	**-**	**-**
1700	**heptadecane**	**0.3**	**-**	**-**
1717	**(2E, 6E)-farnesol**	**0.3**	**-**	**-**
1725	**(E,E)-farnesol**	**0.6**	**-**	**-**
1746	**(2E, 6Z)-farnesol**	**0.4**	**-**	**-**
1790	**1-octadecene**	**t**	**-**	**-**
1800	**octadecane**	**t**	**-**	**-**
1807	**nootkatone**	**0.1**	**-**	**-**
1900	**nonadecane**	**0.1**	**-**	**-**

AI	Compound	oxy	spil	delt
1966	**sandaracopimara-8(14),15-diene**	**0.1**	**-**	**-**
1998	**manoyl oxide**	**6.2**	**5.5**	**1.3**
2014	palustradiene (=abieta-8,13-diene)	-	0.2	-
2017	**epi-13-manoyl oxide**	**0.1**	**-**	**-**
2023	abieta-8,12-diene	0.1	0.3	-
2057	**abietatriene**	**1.2**	**1.4**	**0.1**
2088	**abietadiene**	**1.3**	**3.4**	**-**
2154	**abieta-8(14),13(15)-diene**	**0.2**	**0.2**	**-**
2185	**sandaracopimarinal**	**0.2**	**-**	**-**
2190	**1-docosene**	**0.1**	**-**	**-**
2200	**docosane**	**0.1**	**-**	**-**
2218	**phytol acetate**	**0.1**	**-**	**-**
2300	**tricosane**	**0.2**	**-**	**-**
2312	abieta-7,13-dien-3-one	-	0.1	-

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