



ELSEVIER

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

biochemical
systematics
and ecology

Biochemical Systematics and Ecology 33 (2005) 771–787

www.elsevier.com/locate/biochemsyseco

Cryptic speciation between *Juniperus deltoides* and *Juniperus oxycedrus* (Cupressaceae) in the Mediterranean

Robert P. Adams^{a,*}, Julie A. Morris^b, Ram N. Pandey^c,
Andrea E. Schwarzbach^b

^a Biology Department, Baylor University, P.O. Box 97388, Waco, TX 76798, USA

^b Biology Department, Kent State University, Kent, OH 44242, USA

^c Department of Biomedical Sciences, Wright State University, 3640

Colonel Glenn Highway, Dayton, OH 45435, USA

Received 14 August 2004; accepted 6 January 2005

Abstract

Analyses of individuals classically treated as *Juniperus oxycedrus* L. var. *oxycedrus* from Morocco, Portugal, Spain, France, Italy, Greece and Turkey, using DNA sequencing of nrDNA (ITS 1, 5.8S, ITS 2) plus RAPDs, leaf terpenoids and morphology revealed that two cryptic, genetically distinct but morphologically almost identical species are present. These species, *J. oxycedrus* L. var. *oxycedrus* and *Juniperus deltoides* R.P. Adams, are about as different from each other as *Juniperus navicularis* and *Juniperus macrocarpa* are from *J. oxycedrus* var. *oxycedrus*. Examination of herbarium specimens revealed that the two species are largely allopatric with *J. deltoides* occurring from Italy eastward through Turkey into the Caucasus Mts. and Iran. *J. oxycedrus* var. *oxycedrus* appears to be largely concentrated west of Italy (France, Spain, Portugal, Morocco). Cryptic speciation is discussed.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: *Juniperus deltoides*; *Juniperus oxycedrus*; Cupressaceae; Cryptic speciation; ITS; RAPD; Terpenes

* Corresponding author. Tel.: +1 2547102911; fax: +1 2547102969.

E-mail address: robert_adams@baylor.edu (R.P. Adams).

1. Introduction

Cryptic species are species that are clearly genetically quiet differentiated, but show little or no morphological differences. With the advent of DNA sequencing, cryptic species are now being found in many organisms. It is not surprising to find new species in microscopic sized organisms such as protozoans, bacteria, single cell algae, etc. and this has been the case. Peterson (2000) reported 39 new species of *Penicillium* in working with a collection of 600 molds. Roy et al. (1998) identified cryptic species of rust fungi using nrDNA (ITS). Cryptic species of malaria parasites (*Plasmodium* sp.) were identified using the cytochrome *b* gene sequence (Perkins, 2000).

However, cryptic species have also been reported in larger organisms. The black mudfish (*Neochanna diversus*) from New Zealand, when analyzed using mtDNA sequences, was found to consist of two species (Gleeson et al., 1999). Parra-Olea and Wake's (2001) study revealed that individuals from three *Lineatriton lineolus* (salamander) populations formed three clades with differences comparable to other recognized species. Odrzykoski (1999) reported cryptic species of liverworts in *Conocephalum conicum* (L.) Dum. using isozymes and psbA sequencing. Cryptic speciation has been reported (Rubinoff and Powell, 2001) in the moth, *Syndemis afflictana* collected from Redwood and Monterey Pine hosts, using mtDNA cytochrome oxidase I (620 bp) and nDNA elongation factor I alpha (900 bp) sequences. Shaw (2001), using isozymes and DNA sequence data, found cryptic species in bryophytes. Three cryptic species were found within *Asplenium nidus* (Yatabe et al., 2001) using rbcL sequence data. Cryptic species of *Pellia* (*Hepaticae*) were discovered using intergenic sequences between nuclear tRNA tandem repeats (Fiedorow et al., 2001). Hoot and Taylor (2001) found cryptic species of *Isoetes* using ITS, LEAFY and atpB–rbcL sequence data. Analysis of 171 bp from β -tubulin and 921 bp from elongation factor – 1alpha, indicated that the California and South American populations of *Liriomyza huidobrensis* (leaf miner insects) are two clades of specific rank (Scheffer and Lewis, 2001).

The common raven (*Corvus corax*) has an extensive range covering most of the Northern Hemisphere (Omland et al., 2000) but shows little morphological variation. Omland et al. (2000) analyzed two mtDNA regions, the control (314 bp) and the cytochrome *b* (307 bp) region and three microsatellite loci. Their analyses revealed that the common raven is clearly composed of two species: the holarctic and California clades that are as different as either is to a Chihuahuan species, *C. cryptoleucus* (Omland et al., 2000).

Finally, it is worthwhile to note that cryptic species can be found in very large organisms. Roca et al. (2001) reported that the African elephant is actually composed of two species, based on sequences from four nuclear genes (1732 bp). It is becoming increasingly clear that there may be speciation and genetic differentiation that does not involve any or very little morphological characters.

Current estimates suggest that plant genomes contain between 20,000 and 30,000 genes (Somerville and Somerville, 1999). An even smaller subset of genes is likely

responsible for the visible differences in morphology used to classify species. In a classical study of two species of goldenrod (*Solidago*), Charles and Goodwin (1953) found 35 as the minimum number of genes for six key taxonomic characters (5.8 genes/trait). Their estimates are not very different from the modern estimates of quantitative trait loci (QTL), where 3.73 and 4.0 QTL/trait were reported for *Helianthus* (Kim and Rieseberg, 1999) and *Mimulus* (Bradshaw et al., 1998; Kearsley and Farquhar, 1998), respectively.

The number of independent genes involved in speciation is further complicated by pleiotropy (a single gene that has multiple effects) and epistasis (gene interactions). For example, many of the species specific QTL identified in sunflower (Kim and Rieseberg, 1999) and *Mimulus* (Bradshaw et al., 1998) mapped to the same genomic location, possibly indicative of pleiotropy. If this interpretation is correct, then due to pleiotropy, the number of genes contributing to species differences may actually be overestimated.

Juniperus carinata (Y.K. Yu & L.K. Fu) R.P. Adams (Adams, 2000a); *Juniperus erectopatens* (Cheng & L.K. Fu) (Adams, 1999); *Juniperus lutchuensis* Koidz. and *Juniperus morrisonicola* Hayata (Adams, et al., 2002); *Juniperus microsperma* (Cheng & L.K. Fu) R.P. Adams (Adams, 2000a) and *Juniperus mucronata* R.P. Adams (Adams, 2000b) have been identified as cryptic species in *Juniperus*. *J. carinata*, *J. erectopatens*, *J. microsperma* and *J. mucronata* are each known from only a single population. *J. lutchuensis* is endemic to the Ryukyu Islands and s. coast of Japan and *J. morrisonicola* is endemic to Taiwan. Each of the six aforementioned *Juniperus* species are cryptic from a sibling species and are very difficult to recognize based on morphology, but have been shown to be genetically quite differentiated from their sibling species.

Recently, Adams (2004) recognized a new cryptic species, *Juniperus deltoides* R.P. Adams, which was extracted from *Juniperus oxycedrus* L. var. *oxycedrus*. The morphology of *J. deltoides* is nearly identical to *J. oxycedrus* var. *oxycedrus* (Adams, 2004). *J. oxycedrus* L. var. *oxycedrus* as described in the literature (Farjon, 1998) is chiefly a Mediterranean species that grows from Morocco, Algeria, and Tunisia in north Africa into Portugal, Spain, France, Italy, Greece, the Balkans, Turkey and eastward into Iran. It has been divided into *J. oxycedrus* L. ssp. *oxycedrus*, *J. oxycedrus* ssp. *badia*, (H. Gay) Debeaux, *J. oxycedrus* ssp. *macrocarpa* (Sibth. & Sm.) Neill., and *J. oxycedrus* var. *trastagana* Franco. However, Adams (2000c) using leaf terpenoids and RAPDs (Random Amplified Polymorphic DNAs) recognized *J. macrocarpa* Sibth. & Sm. (= *J. oxycedrus* ssp. *macrocarpa*) and *Juniperus navicularis* Grand (= *J. oxycedrus* var. *trastagana* Franco), leaving *J. oxycedrus* L. var. *oxycedrus* as the prickly leaved juniper of the Mediterranean and *J. oxycedrus* var. *badia* H. Gay in central Spain.

This work is published to examine, in detail, several character suits that might distinguish between *J. deltoides* and *J. oxycedrus* var. *oxycedrus*. To examine the speciation of *J. deltoides* and *J. oxycedrus* var. *oxycedrus*, collections were made from areas ranging from Morocco to Turkey and these samples were examined using DNA sequencing, RAPDs, morphological characters, and leaf terpenoids. The results of these investigations are presented in this paper.

2. Materials and methods

Specimens used in this study and GenBank accessions are given in Table 1. Voucher specimens are deposited at BAYLU, Baylor University.

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 $-20\text{ }^{\circ}\text{C}$ until analyzed. The extracted leaves were oven dried (48 h, $100\text{ }^{\circ}\text{C}$) for determination of oil yields.

The essential oils were analyzed on a Hewlett-Packard 5973 MSD, directly coupled to a HP5980 gas chromatograph. EI mass spectra were collected at 70 eV ionization voltage over the mass range m/z 41–425. Oil samples of $0.1\ \mu\text{l}$ (10% solution in diethyl ether) were injected and split 1:10. Analytical conditions: column: J & W DB-5, ($0.26\ \text{mm} \times 30\ \text{m}$, $0.25\ \mu\text{m}$ coating thickness), carrier gas: helium at 1 ml/min; injector temperature: $220\text{ }^{\circ}\text{C}$; oven programming: initial temperature: $60\text{ }^{\circ}\text{C}$, gradient: $3\text{ }^{\circ}\text{C}/\text{min}$, final temperature: $246\text{ }^{\circ}\text{C}$. 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.

Table 1

List of specimens collected, collection numbers, and GenBank accession numbers used in this study

Taxon/collection #	Location	GenBank accession
<i>J. navicularis</i>		
Adams 8239 , 8240 , 8241–8243	1 km se. of Apostica, Portugal	AY046518, 045617
<i>J. macrocarpa</i>		
Adams 7205 , 7206 , 7207	15 km w. of Tarifa, Spain	AY045622, 0456621
<i>J. oxycedrus</i> var. <i>badia</i>		
Adams 7795 , 7800	18 km s. of Jaen, Spain	AY046525, 046526
<i>J. oxycedrus</i> var. <i>oxycedrus</i>		
Adams 7080 , 7081 , 7082	El Penon, Spain	AY046524, 046523
Adams 9053 , 9054	erect foliage, Ruidera, Spain	AY046569
Adams 9052 , 9055, 9056	Pendulous foliage, Ruidera, Spain	AY046568, 046570
Adams 9039 –9041	5 km n. of Peyruis, France	AY046567
Adams 9405, 9406 , 9407	20 km sse. of Marrakech, Morocco	AY046571
Adams 9448, 9449 , 9450	Vila Nova de Foz Coa, Portugal	AY046572
<i>J. deltoids</i>		
Adams 8787 , 8788	8 km w. of Lemos, Greece	AY046519, 046520
Adams 9430 –9432	30 km n. of Eskisehir, Turkey	AY046564
Adams 9436 –9438	14 km e. of Archova, Greece	AY046565
Adams 9445, 9446 , 9447	2 km w of Raiano, Italy	AY046566

All collections are deposited at BAYLU. Boldface collection number refers to GenBank accession.

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). Tenmer primers were purchased from the University of British Columbia (5'–3'): 116, TAC GAT GAC G; 153, GAG TCA CGA G; 184, CAA ACG GAC C; 204, TTC GGG CCG T; 218, CTC AGC CCA G; 239, CTG AAG CGG A; 250, CGA CAG TCC C; 265, CAG CTG TTC A; 338, CTG TGG CGG T; 347, TTG CTT GGC G; 375, CCG GAC ACG A; 389, CGC CCG CAG T; 413, GAG GCG GCG A; 478, CGA GCT GGT C.

PCR was performed in a volume of 15 μl containing 50 mM KCl, 10 mM Tris–HCl (pH 9), 2.0 mM MgCl_2 , 0.01% gelatin and 0.1% Triton X-100, 0.2 mM of each dNTPs, 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, then 40 cycles of 38°C (2 min), 72°C (2 min), and 91°C (1 min). Two additional steps were used: 38°C (2 min) and 72°C (5 min) for final extension.

Bands that occurred once or did not show fidelity within the two 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 4 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.

2.3. DNA sequencing

Leaf collections and DNA extraction methods were used as described for RAPDs. ITS amplifications were performed in 50 μl reactions using 0.6 ng of genomic DNA, 1.5 units Promega Taq polymerase, 5 μl Promega $10\times$ buffer (final concentration: 50 mM KCl, 10 mM Tris–HCl (pH 9), 2.0 mM MgCl_2 , 0.01% gelatin and 0.1% Triton X-100), 2 mM MgCl_2 , 0.4 mM each dNTP, 0.23 μM each primer (ITSA, ITSB), and 8% (by vol.) DMSO. ITSA = GGA AGG AGA AGT CGT AAC AAG G; ITSB = CTT TTC CTC CGC TTA TTG ATA TG. The sequences for the ITSA and ITSB primers were based on Blattner (1999). The following PCR conditions were used: MJ Research Programmable Thermal Cycler, 45 cycles, 94°C (1 min), 50°C (1 min), 72°C (1 min), with a final step of 72°C (5 min). The PCR reaction was subjected to purification by agarose gel electrophoresis (1.5% agarose, 1 h) and the ITS band (approx. 1100 bp) was excised and purified by use of a Qiagen QIAquick gel extraction kit.

Dideoxy terminating sequencing PCR reactions were performed in 10 μl :2 μl ABI Big Dye Sequencing Kit (Perkin–Elmer Corp.), 2 μl Half Term Dye Terminator

(Genpak, St. James, NY), 1–5 μl (~ 45 ng) template DNA, 1 μl primer (ITSA or ITSB = 0.29 μM), ddH₂O to add up to 10 μl . PCR program: 96 °C(30 s), 50 °C(15 s), 60 °C(4 min), 25 cycles. Sequencing was performed on an ABI 377 and ABI 3700 automated sequencer. Sequences for both strands were edited and a consensus sequence was produced using Sequencher version 4.1 (GeneCodes Corp., Ann Arbor, MI). The alignment was done manually in Sequencher. Gaps were coded as missing.

2.4. RAPD data analysis

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).

2.5. Terpenoid data analysis

For the terpenoid data, similarities were computed as quantitative matches as well as simple presence/absence matches. The presence/absence (+/–) matching was found to be more similar to the DNA data. Principal coordinate analysis (PCO) of the similarity matrices follows Gower (1966).

2.6. DNA sequence data analysis

PAUP* version 4.0b10 was used for phylogenetic analysis. A heuristic parsimony search with 100 random additions and TBR branch swapping was performed. The resulting tree was transformed into a haplotype tree by collapsing zero-length branches and displayed as unrooted network. A bootstrap analysis using a heuristic search strategy with 1000 replicates, simple addition and TBR branch swapping was performed to assess branch support.

3. Results and discussion

3.1. Leaf essential oils

Three unidentified compounds (>0.5% of the oil) were found (Table 2: KI 1619, M + 222, sesquiterpene alcohol, 41(68), 55(18), 69(100), 81(12), 93(10), 109(19), 123(18), 161(6), 179(18); KI 1674, M + 224, C10-dien-ol acetate, 43(100), 54(48), 67(32), 82(30), 109(7), 125(7), 138(8), 166(8); KI 2016, diterpene oxide, M + 290?, 43(40), 55(21), 69(16), 81(10), 91(7), 117(20), 129(18), 143(19), 159(100), 185(32), 213(17), 241(100), 257(40), 275(7)).

The leaf essential oils from the western, *J. oxycedrus*, locations (Morocco, Portugal, Spain, France) were generally dominated by α -pinene (45.3–50.3%), whereas the eastern, *J. deltooides*, populations (Italy, Greece, Turkey) had lower amounts (19.3–32.7%), with moderate amounts of α -phellandrene, *p*-cymene, β -phellandrene, limonene, myrcene, α -terpineol, (*E*)-nerolidol and manoyl oxide (Table 2). Several compounds were only found in the eastern, *J. deltooides*,

Table 2

Comparisons of the percent total oil for leaf oils components of *J. oxycedrus* var. *oxycedrus* from: Morocco (MO), Portugal (PO), Spain (SP), France (FR), Italy (IT), southern Greece (sGR), northern Greece (nGR) and Turkey (TK)

KI	Compound	MO	PO	SP	FR	IT	sGR	nGR	TK
802	Hexanal	t	t	t	t	0.2	0.2	t	0.6
855	(<i>E</i>)-2-hexenal ^a	t	0.5	0.2	0.4	0.6	0.7	0.5	0.9
927	tricyclene	t	0.1	0.1	0.1	t	t	0.1	0.1
930	α -thujene	t	t	0.2	t	t	t	—	t
939	α-pinene^a	45.3	47.3	40.9	53.2	19.3	19.7	27.4	32.7
953	α -fenchene	0.5	t	—	0.1	0.8	t	t	0.3
954	camphene	0.5	0.4	0.5	0.6	t	0.4	0.5	0.6
960	thuja-2,4(10)-diene	t	t	0.1	t	t	t	0.4	0.4
975	sabinene ^a	1.0	0.4	2.3	0.5	0.2	0.2	0.4	0.2
979	1-octen-3-ol	t	t	—	0.1	t	—	t	—
979	β -pinene ^a	1.9	2.6	2.3	2.1	2.5	2.7	2.7	3.0
991	myrcene ^a	2.5	3.0	5.0	2.8	2.5	2.8	2.9	3.8
1002	δ -2-carene ^a	0.3	0.2	0.3	t	—	—	0.4	0.9
1003	α -phellandrene ^a	0.5	0.9	9.2	t	0.8	0.3	1.6	1.8
1011	δ -3-carene ^a	13.9	—	—	5.1	13.6	3.7	—	3.7
1017	α -terpinene	0.2	—	0.3	t	0.1	t	t	0.1
1025	<i>p</i> -cymene ^a	1.8	0.6	7.3	0.3	0.8	0.4	2.2	2.3
1029	limonene^a	0.8	0.3	1.0	3.5	27.3	20.0	26.9	6.0
1030	β -phellandrene ^a	4.0	6.3	9.0	0.8	2.9	4.2	5.0	11.5
1050	(<i>E</i>)- β -ocimene	—	—	—	t	0.3	0.2	—	—
1060	γ -terpinene	0.4	—	0.5	0.1	0.2	0.1	0.2	0.2
1070	<i>cis</i> -sabinene hydrate	—	—	0.1	—	—	—	—	—
1088	<i>p</i> -mentha-2,4(8)-diene	—	—	—	—	t	—	—	—
1089	terpinolene ^a	1.3	0.7	3.3	0.7	2.0	1.2	1.2	2.0
1099	linalool ^a	t	0.2	0.3	t	0.2	0.4	0.3	0.7
1101	<i>n</i> -nonanal ^a	t	t	—	t	0.3	0.4	0.3	0.5
1122	<i>cis</i> - <i>p</i> -menth-2-en-1-ol	t	0.2	0.1	—	—	—	—	0.3
1123	<i>trans</i>-<i>p</i>-mentha-2,8-dien-1-ol	—	—	—	—	0.3	0.3	0.3	t
1126	α -campholenal ^a	0.6	0.3	0.5	0.8	0.3	0.3	1.5	1.3
1126	chrysanthenone	—	—	—	—	—	—	t	t
1137	<i>trans</i> -pinocarveol ^a	0.6	0.3	0.5	0.4	—	0.3	1.1	1.3
1138	<i>cis</i>-<i>p</i>-mentha-2,8-dien-1-ol	—	—	—	—	0.3	0.3	0.2	0.1
1141	<i>cis</i> -verbenol	—	t	—	t	—	—	0.4	0.4
1145	<i>trans</i> -verbenol ^a	t	0.4	0.6	0.6	0.3	0.5	1.9	1.8
1163	<i>trans</i> -pinocamphone	—	—	—	—	—	—	0.1	0.1
1165	pinocarvone	t	t	—	t	—	—	0.5	0.6
1169	borneol	—	0.2	—	—	—	—	—	—
1170	<i>p</i> -mentha-1,5-dien-8-ol ^a	0.5	0.2	—	0.5	0.3	0.3	0.5	1.1
1175	<i>cis</i> -pinocamphone	—	—	—	—	—	—	0.1	0.1
1177	terpinen-4-ol ^a	0.5	0.3	1.3	0.3	0.3	0.5	0.3	0.6
1181	naphthalene	t	t	0.2	0.1	0.1	0.3	0.1	0.3
1183	<i>p</i> -cymen-8-ol	t	0.1	0.4	t	t	0.3	0.3	1.0
1189	α -terpineol ^a	0.3	0.4	4.2	0.6	0.1	0.4	0.3	1.2
1196	myrtenal	—	t	0.2	t	—	—	0.5	0.6
1205	verbenone ^a	0.1	0.1	0.1	0.3	0.2	0.2	0.4	0.7
1217	<i>trans</i> -carveol ^a	—	—	0.1	0.1	0.8	1.1	0.8	0.5
1229	<i>cis</i>-carveol^a	—	—	—	—	0.3	0.3	0.1	t

(continued on next page)

Table 2 (continued)

KI	Compound	MO	PO	SP	FR	IT	sGR	nGR	TK
1235	thymol, methyl ether	—	—	0.1	—	—	—	—	—
1242	cumin aldehyde	—	—	—	—	—	—	t	0.1
1243	carvone^a	—	—	—	—	0.7	0.8	0.6	0.3
1253	piperitone	—	—	0.1	—	—	—	—	t
1257	linalyl acetate	t	—	—	0.3	—	—	—	t
1264	(2E)-decenal^a	—	—	—	—	0.2	0.2	0.2	0.2
1289	bornyl acetate ^a	0.7	0.9	—	0.7	0.3	0.7	0.9	0.9
1298	<i>trans</i> -pinocarvyl acetate	t	t	—	—	—	—	t	0.1
1299	(2E,4Z)-decadienal^a	—	—	—	—	0.1	0.2	t	0.4
1317	(2E,4E)-decadienal^a	—	—	—	0.1	0.2	0.2	0.1	0.8
1342	<i>trans</i> -carvyl acetate	t	—	—	—	t	—	0.1	t
1346	<i>trans</i> -piperitol acetate	t	—	—	—	—	—	—	—
1349	α-terpinyl acetate^a	t	0.2	t	0.2	—	—	—	—
1351	α -cubebene	t	—	0.1	—	—	—	—	—
1377	α-copaene^a	t	—	t	—	0.2	0.3	0.3	0.2
1381	geranyl acetate	—	—	—	—	—	0.1	t	t
1388	β -bourbenene ^a	0.5	0.3	0.2	0.3	0.1	0.2	0.1	0.2
1400	tetradecane	—	—	—	—	—	0.1	—	—
1408	longifolene	—	—	—	—	—	—	—	0.6
1419	(<i>E</i>)-caryophyllene ^a	0.4	0.2	0.2	0.4	0.9	0.3	1.5	1.2
1431	<i>cis</i> -thujopsene	—	—	—	—	—	0.1	t	0.1
1432	β -copaene	—	t	—	—	—	—	—	—
1455	geranyl acetone	—	t	—	—	—	—	—	—
1455	α -humulene ^a	0.3	0.3	0.1	0.3	0.6	0.2	1.9	0.8
1480	γ -muurolene	0.5	t	t	0.1	t	0.1	t	t
1485	germacrene D ^a	1.9	2.9	0.8	2.3	0.9	0.9	0.2	0.7
1486	ar-curcumene^a	—	—	—	—	0.3	0.9	1.7	—
1494	<i>trans</i> -muurola-4(14),5-diene	—	—	0.1	—	—	—	—	—
1496	2-tridecanone^a	1.1	0.6	—	0.3	—	—	—	—
1500	α -muurolene ^a	t	—	t	—	0.3	0.1	0.3	0.4
1505	cuparene	—	—	—	—	—	—	0.1	—
1514	butylated hydroxytoluene	t	0.2	—	—	0.2	0.4	—	0.1
1514	γ -cadinene ^a	1.2	0.5	0.3	0.7	0.5	1.1	0.9	0.4
1523	δ -cadinene ^a	0.5	0.3	t	0.4	0.6	1.2	0.8	0.4
1541	α-copaen-11-ol^a	—	—	—	—	0.3	1.2	0.3	0.1
1546	α-calacorene^a	—	—	—	—	0.5	1.0	0.5	0.5
1561	germacrene B	—	—	—	0.1	—	—	—	—
1566	β-calacorene^a	—	—	—	—	0.4	t	0.2	0.3
1563	(<i>E</i>)-nerolidol	—	—	1.9	—	—	2.2	—	—
1567	dodecanoic acid	—	—	—	0.4	—	0.8	—	—
1572	1 α -,10 α -epoxyamorph-4-ene	—	—	—	—	—	0.1	—	—
1576	germacrene D-4-ol	—	0.2	—	—	—	—	—	—
1583	ar-tumerol	—	—	—	—	—	0.3	0.3	—
1583	caryophyllene oxide ^a	0.4	0.3	0.1	0.4	3.9	—	1.9	3.2
1595	salvial-4(14)-en-1-one	—	0.3	—	0.4	—	—	—	—
1600	hexadecane	—	—	—	0.3	—	0.2	—	—
1601	cedrol ^a	—	—	—	t	t	0.4	0.7	0.1
1608	humulene epoxide II^a	0.3	0.3	0.5	0.3	1.5	0.8	0.9	1.1
1619	sesquiterpene alcohol, M + 226^a	0.5	1.0	0.2	0.3	—	—	—	—
1623	α -corocalene	—	—	—	—	—	0.1	—	—
1640	epi- α -cadinol	0.4	0.3	0.1	0.6	0.2	0.5	0.4	—

Table 2 (continued)

KI	Compound	MO	PO	SP	FR	IT	sGR	nGR	TK
1642	epi- α -muurolol	—	0.3	—	—	0.2	0.4	—	—
1651	β -eudesmol	—	0.2	—	t	—	—	—	—
1654	α -cadinol ^a	0.5	0.9	0.2	1.6	0.1	0.4	—	—
1661	<i>cis</i> -calamene-10-ol	—	—	—	—	t	0.3	0.1	—
1669	<i>trans</i> -calamene-10-ol	—	—	—	—	t	0.1	—	—
1674	C10-dienol acetate, M + 224^a	5.8	2.9	t	1.6	—	—	—	—
1677	cadalene^a	—	—	—	—	0.2	0.5	0.2	0.1
1686	germacra-4(15),5,10(14)-triene-1-ol	0.8	0.4	t	1.6	—	0.3	—	—
1689	2,3-dihydro-farnesol	—	1.0	—	—	—	—	—	—
1699	2-pentadecanone	0.9	0.2	—	—	—	—	—	—
1700	heptadecane	—	—	—	0.3	—	0.2	—	—
1717	(2 <i>E</i> , 6 <i>E</i>)-farnesol	t	0.6	0.2	0.3	—	0.1	—	—
1725	(<i>E,E</i>)-farnesol ^a	0.8	2.5	0.7	0.6	—	1.5	—	—
1746	(2 <i>E</i> , 6 <i>Z</i>)-farnesol ^a	t	1.2	0.3	0.4	—	0.3	—	—
1790	1-octadecene	—	—	—	t	—	—	—	—
1800	octadecane	—	—	—	t	—	0.1	—	—
1807	nootkatone	—	—	—	0.1	—	—	—	—
1900	nonadecane	—	—	—	0.1	—	—	—	—
1966	sandaracopimara-8(14),15-diene	—	—	—	0.1	—	0.1	—	—
1998	manoyl oxide ^a	0.7	7.3	2.4	6.2	3.6	9.9	1.5	1.3
2016	diterpene, M + 290^a	—	—	—	—	0.2	0.6	0.1	t
2017	epi-13-manoyl oxide	—	0.3	t	0.1	—	—	—	—
2023	abieta-8,12-diene	—	0.2	—	0.1	—	—	—	—
2057	abietatriene ^a	1.0	0.8	0.1	1.2	1.2	2.5	0.7	t
2088	abietadiene ^a	1.3	2.0	0.2	1.3	1.0	1.8	0.7	—
2154	abieta-8(14),13(15)-diene	—	0.4	—	0.2	—	—	—	—
2185	sandaracopimarinal	—	t	—	0.2	—	—	—	—
2190	1-docosene	—	—	—	0.1	—	—	—	—
2200	docosane	—	—	—	0.1	—	—	—	—
2218	phytol acetate	—	—	—	0.1	—	—	—	—
2300	tricosane	—	—	—	0.2	—	—	—	—
2314	abietal	—	0.2	—	—	—	0.6	0.5	—

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

^a Used in numerical analysis.

populations (Italy, Greece, Turkey): *cis-p*-mentha-2,8-dien-1-ol, *cis*-carveol, (2*E*)-decenal, (2*E*,4*Z*)-decadienal, ar-curcumenene, α -copaen-11-ol, α -calacorene, β -calacorene, cadalene, and the unknown diterpene (KI 2016). Some compounds were only found in the western, *J. oxycedrus*, populations (Morocco, Portugal, Spain, France): α -terpinyl acetate, 2-tridecanone, 2-pentadecanone, KI 1619, and KI 1674.

Principal coordinate analysis (PCO) of the similarity matrix based on the oil composition resulted in seven eigenroots of which the first three (40.5, 19.2, 9.9%) accounted for 69.6% of the variance among the eight populations. PCO revealed two major groups: the eastern, *J. deltoides*, and western, *J. oxycedrus*, populations

(Fig. 1). The plants from France, Morocco, Portugal and Spain form a relatively compact group, whereas there is more diversity in the Greece, Italy and Turkey group (Fig. 1). The oils of the northern Greece (nGR) plants appear more similar to oils from Italy than those from southern Greece (sGR, Fig. 1).

Geographic clustering of the minimum spanning network of similarities clearly shows the two major groupings (Fig. 2). The oils of the western group are very similar. Portugal and France link at 0.834, followed by Morocco (0.827), then Spain (0.790). In the eastern group, Italy links with northern Greece (0.755), then with southern Greece (0.730) followed by linkage to Turkey (0.719, Fig. 2). The eastern, *J. deltooides*, and western, *J. oxycedrus*, groups are linked via a similarity of 0.573 between Spain and Italy. Thus, just as in the PCO (Fig. 1), we can see (Fig. 2) that although the oils are more diverse in the eastern than in the western populations, the major trend is to form these two groups.

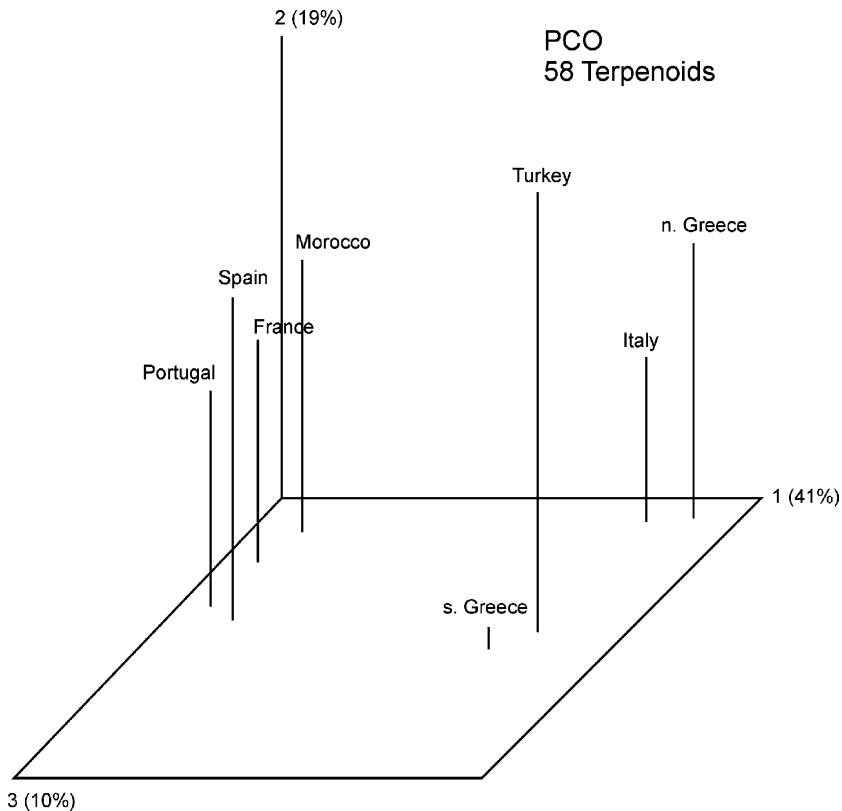


Fig. 1. Principal coordinate analysis based on 58 terpenoids. Note the clear separation between the *J. oxycedrus* western populations (PO, Portugal; SP, Spain; FR, France; MO; Morocco) and the *J. deltooides* eastern populations (sGR, s. Greece; n. GR, n. Greece; TK, Turkey; IT, Italy).

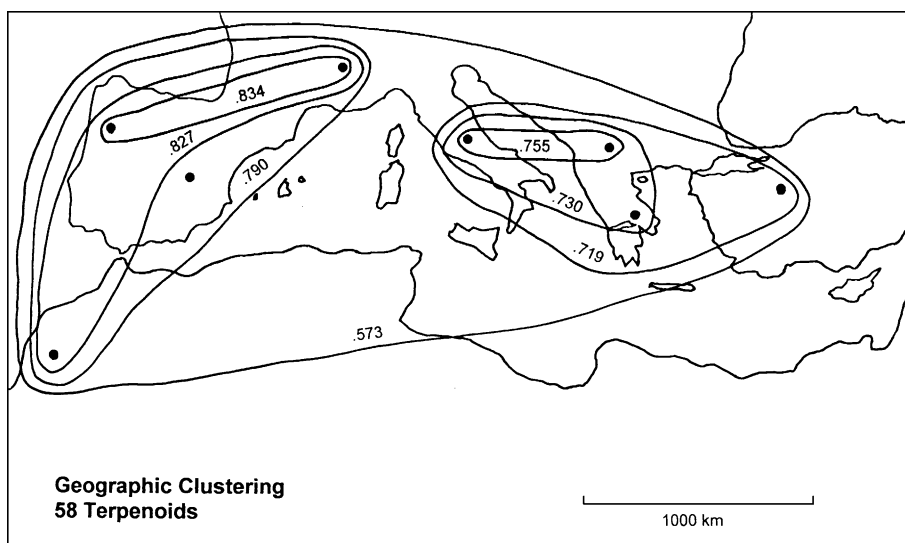


Fig. 2. Contoured terpenoid similarities. Populations of *J. deltooides* and *J. oxycedrus* are divided into eastern and western groups, respectively.

3.2. RAPDs data

Three taxa: *J. oxycedrus* var. *badia*, *J. macrocarpa* and *J. navicularis* were included in the analysis of *J. oxycedrus* from the eight populations to gain some taxonomic perspective. The minimum spanning network (Fig. 3) reveals that the eastern populations, *J. oxycedrus*, cluster are clearly quite differentiated from the western, *J. deltooides*, populations. In addition, *J. oxycedrus* var. *badia* clusters within *J. oxycedrus* supporting the status of *J. oxycedrus* var. *badia* at the varietal level. *J. navicularis* is clearly in the section with the western *J. oxycedrus* (Fig. 3).

PCO analysis of the RAPDs for individuals in the eight *J. deltooides*/*oxycedrus* populations appeared to asymptote after six eigenroots. The first three eigenroots accounted for 45.7, 10.7, 7.0% of the variance among individuals (63.4%). The major trend (45.7%) is the separation of the eastern, *J. deltooides*, and western, *J. oxycedrus*, populations (Fig. 4). As with the terpenoid data, the western populations of *J. oxycedrus* appear to be more uniform than the eastern populations of *J. deltooides*. Notice that the individuals of *J. oxycedrus* from Morocco and Portugal tend to co-mingle (Fig. 4) implying that these populations are not well differentiated. It is very likely that the Moroccan population arose from the Portugal population in the Pleistocene or earlier.

Geographic clustering of the RAPDs similarities (Fig. 5) shows the same basic pattern as with the terpenoid data (Fig. 2). The clustering in the eastern, *J. deltooides*, populations is exactly the same order as with the terpenoid data. Notice that the Italy population links with the northern Greece population, then southern Greece, just as seen with the terpenoids (Figs. 2, 5). However, in the western,

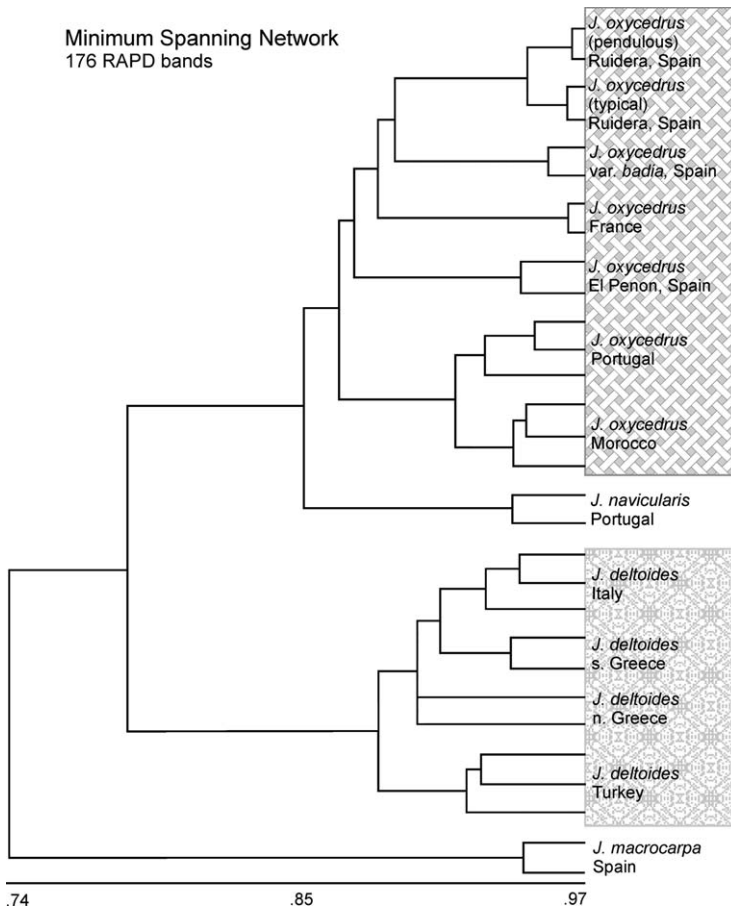


Fig. 3. Minimum spanning network based on 176 RAPD bands. Note that *J. deltooides* populations from the eastern Mediterranean are very distinct, even more distinct than those of *J. navicularis*.

J. oxycedrus, populations, one sees a strong linkage (0.926) between Morocco and Portugal populations (Fig. 5) and the Spain and France form a group (but just barely) before joining with Portugal–Morocco.

3.3. ITS sequence data

The aligned sequences are 1110 bp long and 15 different haplotypes were found. The PAUP search resulted in a single most parsimonious tree that was transformed into a haplotype tree by collapsing the zero-length branches. This haplotype tree based on ITS sequence data shows the basic pattern (Fig. 6) as seen in the terpenoids and RAPDs: *J. navicularis* is clearly quite distinct; *J. macrocarpa* is distinct; *J. deltooides* from Greece, Italy and Turkey form a group; *J. oxycedrus* from France,

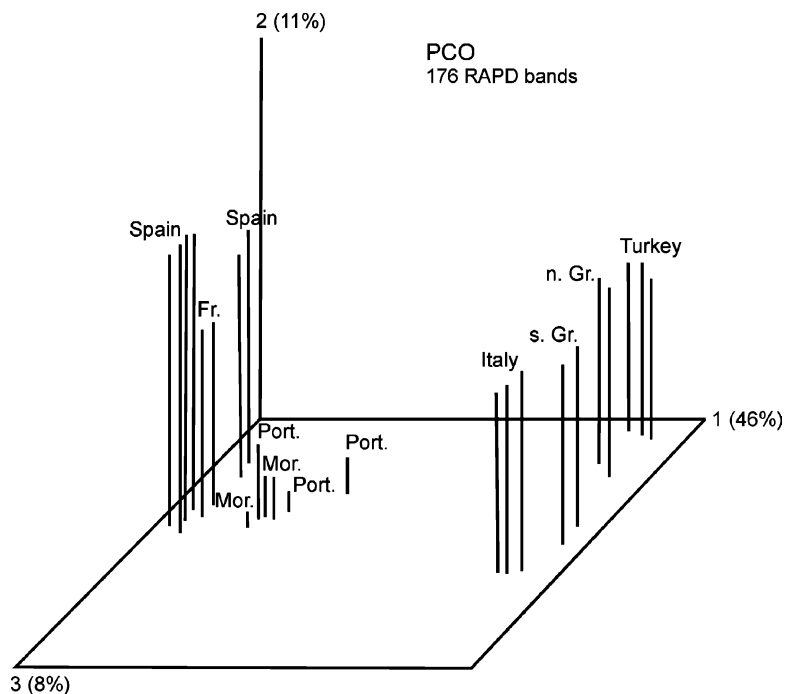


Fig. 4. Principal coordinates analysis based on 176 RAPD bands shows individuals cluster by population, then by region. Notice the grouping of the Portuguese and Moroccan populations.

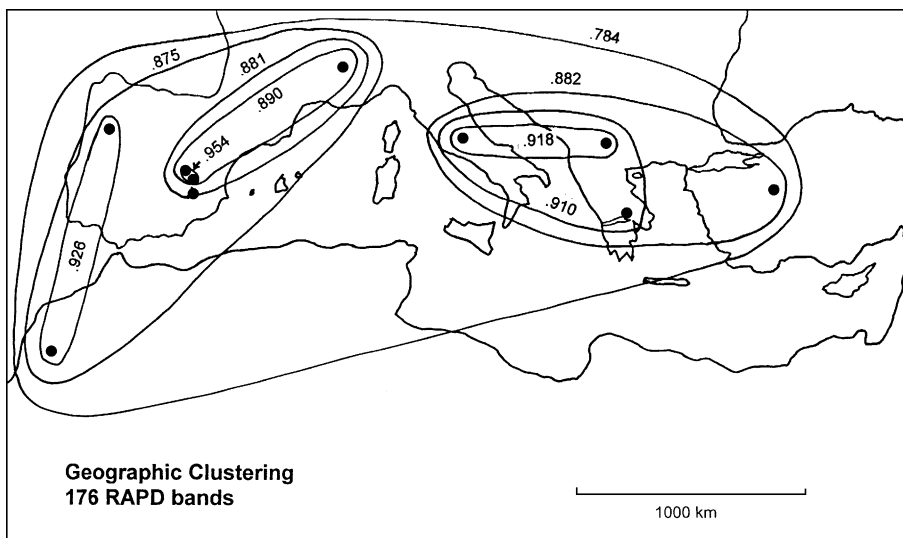


Fig. 5. Contour mapping of RAPD similarities showing the same pattern differentiation between eastern *J. deltoides* and western *J. oxycedrus* as seen in the terpenoids (Fig. 2). The same affinity between Italy and n. Greece is shown just as with the terpenoids.

Morocco, Portugal and Spain form a separate group. *J. oxycedrus* var. *badia* is intermingled with *J. oxycedrus* (Fig. 6). Bootstrap values are high for almost all branches. The separation of *J. deltooides* from the rest of the group is supported by a 93% bootstrap value.

3.4. Morphology

Examination of specimens revealed some subtle differences between *J. deltooides* and *J. oxycedrus*. Although the leaves were often longer and narrower in the western

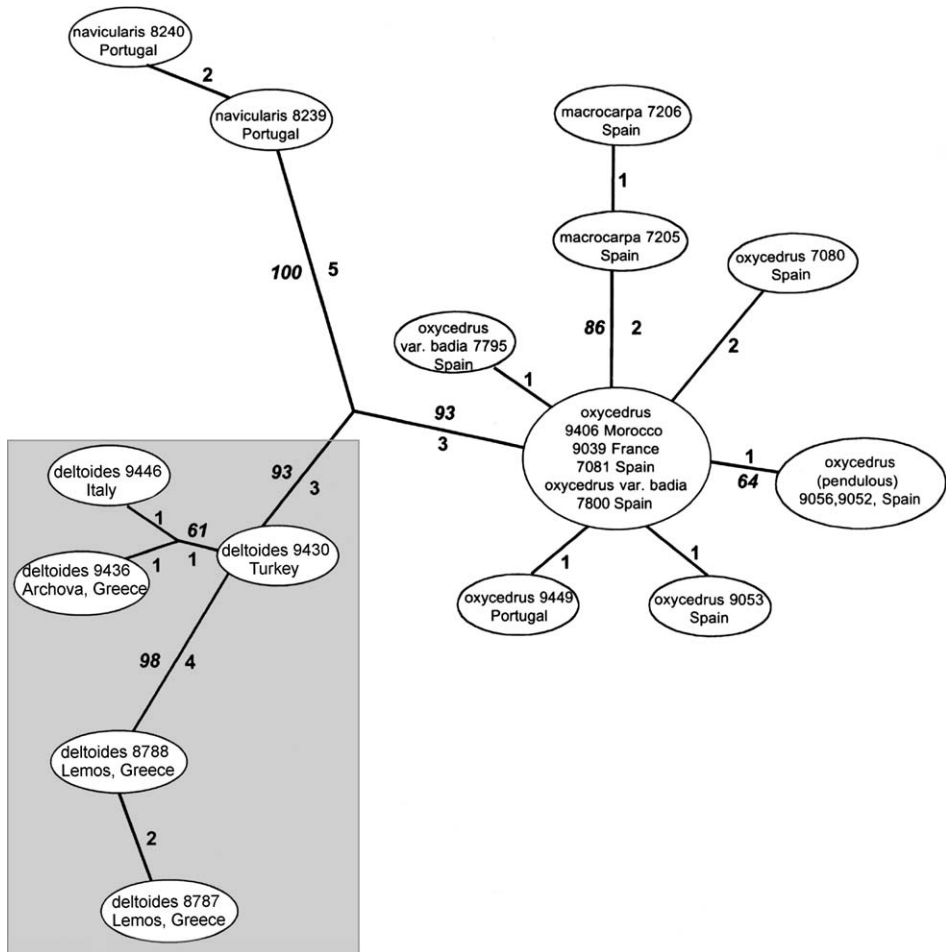


Fig. 6. Single most parsimonious haplotype tree based on ITS sequences. The branch lengths are the number of nucleotide differences between taxa. Notice the lower left is the eastern collections of *J. deltooides* (marked with a gray box) and the western collections are in the middle, right. Bootstrap values are shown as numbers in italics.

Table 3

Leaf length, width, leaf base shape and stomatal bands for *J. oxycedrus*

Character	MO	PO	SP	FR	IT	nGR	sGR	TK
Leaf length (mm)	15.0	14.7	14.4	14.5	13.0	11.5	12.0	11.7
Leaf width (mm)	1.70	1.43	1.28	1.70	2.10	1.80	1.57	1.80
<i>Leaf base shape:</i>								
Narrow (n), broad (b)	n	n	n	n	b	b	b	b
<i>Stomatal bands:</i>								
Flat(f) or sunken (s)	s	s	s	s	f	f	f	f

MO = Morocco, PO = Portugal, SP = Spain, FR = France, IT = Italy, sGR = southern Greece, nGR = northern Greece, TK = Turkey.

populations and shorter and wider in the eastern populations, there were no significant differences among the populations (Table 3). However, close examination of leaves revealed that the leaf base in the western populations (*J. oxycedrus*) are narrower than in the eastern populations, *J. deltooides* (Fig. 7). In addition, the stomatal bands in the western, *J. oxycedrus*, populations are sunken, giving the midrib a raised appearance. In contrast, in the eastern, *J. deltooides*, populations, most of the leaves have stomatal bands that are not sunken (Fig. 7), giving the leaf a flat surface appearance (actually concave as illustrated in Fig. 7).

J. deltooides and *J. oxycedrus* are as differentiated as *J. navicularis* and *J. macrocarpa*. At this point, they appear to be mostly allopatric, with the sharp line of differentiation being between France/w. Italy and central Italy. Examination of specimens at K, revealed that specimens from France and Spain had mostly narrow, long leaves and compared to wide, short in Italy and Greece. But many countries had specimens with various kinds of leaves (Table 4). So it is not sufficient

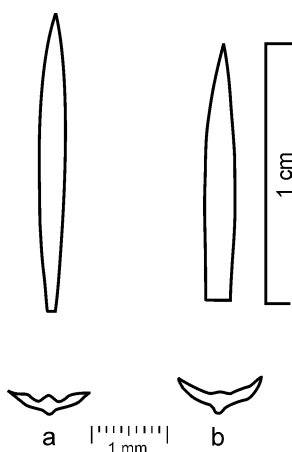


Fig. 7. Leaves of *J. oxycedrus* L. (a) and *J. deltooides* (b). The stomatal bands in *J. oxycedrus* are sunken in the leaves (a), whereas the bands are on the surface of leaves of *J. deltooides* (b).

Table 4

Observations on leaf length and width from specimens at K

Morocco: 3(W,L),2(mW, mL), 1(W,S); Algeria: 1(W,L); Tunisia: 1(W,L); Portugal: 2(W,L), 4(W,S), 1(N,S), 1(mN,L); Spain: n. Atlantic coast: 1(W,L); se Spain: 11(N,L); Balearic Islands: 4(N,L), 1(vN,L); France: 5(N,L), 2(W,L), 1(W,S), 1(vN,S); Italy: n. near Florence: 1(N,L); from Rome south: 3(W,S), 1(W,L), 1(N,L); Sicily: 1(N,L); Yugoslavia: 3(W,L), 2(W,S); Bulgaria: 1(mW,S), 1(mW,L); Greece: 4(W,L), 4(W,S); Turkey: 4(W,S), 3(W,L), 1(N,S); Crete: 1(W,S); Cyprus: 2(W,L), 1(N,L), 1(N,S), 1(mW,L), 1(W,mS); Lebanon: 2(W,L); Syria: 1(mW,mS), 1(mN,S), 1(W,L); Iraq: 4(W,L), 4(W,S); Iran: 1(mW, mL).

Codes: Leaf length: L (long) = 15–22 mm, S (short) = 9–14 mm; leaf width: N (narrow) = 1.0–1.5 mm, W (wide) = 1.6–2.2 mm.

to say that *J. oxycedrus* has narrow, long leaves and *J. deltoides* has wide, short leaves. *J. deltoides* occurs from Italy eastward into Greece and Turkey thence into Armenia, Iraq and Iran.

In summary, the evidence based on leaf essential oil composition, RAPDs fingerprinting, and ITS sequence data are concurrent in showing the differentiation of *J. deltoides* from *J. oxycedrus* at a level that is consistent with the divergence of *J. navicularis* and *J. macrocarpa* from *J. oxycedrus*. This speciation event shows divergence in the terpenoids, RAPDs fingerprints and ITS sequences, but very little differentiation in morphology. It seems likely that there are numerous cryptic plant species that will only be discovered when detailed populational analysis is performed using DNA data as in the present case that led to recognition of *J. deltoides* (Adams, 2004).

Acknowledgements

Thanks to Joaquin Altarejos, Spain, K.H.C. Baser, Turkey, and Alifriqui Mohamed, Morocco for field trip assistance. This work was supported in part with funds from NSF grant DEB-0316685 (to A.E.S. and R.P.A.) and from Baylor University and Kent State University.

References

- Adams, R.P., 1975. Statistical character weighting and similarity stability. *Brittonia* 27, 305–316.
- Adams, R.P., 1991. Cedarwood oil – analysis and properties. In: Linskens, H.F., Jackson, J.F. (Eds.), *Modern Methods of Plant Analysis: Oil and Waxes*. Springer, Berlin, pp. 159–173.
- Adams, R.P., 1999. Systematics of the multi-seeded eastern hemisphere *Juniperus* based on leaf essential oils and RAPD DNA fingerprinting. *Biochem. Syst. Ecol.* 27, 709–725.
- Adams, R.P., 2000a. Systematics of the one seeded *Juniperus* of the eastern hemisphere based on leaf essential oils and RAPD DNA fingerprinting. *Biochem. Syst. Ecol.* 28, 529–543.
- Adams, R.P., 2000b. Systematics of the smooth leaf margin *Juniperus* of the western hemisphere based on leaf essential oils and RAPD DNA fingerprinting. *Biochem. Syst. Ecol.* 28, 149–162.
- Adams, R.P., 2000c. Systematics of *Juniperus* section *Juniperus* based on leaf essential oils and RAPD DNA fingerprinting. *Biochem. Syst. Ecol.* 28, 515–528.
- Adams, R.P., 2001. Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectroscopy. Allured Publ., Carol Stream, IL.

- Adams, R.P., 2004. *Juniperus deltoides*, a new species, and nomenclatural notes on *Juniperus polycarpus* and *J. turcomanica* (Cupressaceae). *Phytologia* 86, 49–53.
- Adams, R.P., Demeke, T., 1993. Systematic relationships in *Juniperus* based on random amplified polymorphic DNAs (RAPDs). *Taxon* 42, 553–572.
- Adams, R.P., Hsieh, C.-F., Murata, J., Pandey, R.N., 2002. Systematics of *Juniperus* from eastern Asia based on leaf essential oils and RAPD DNA fingerprinting. *Biochem. Syst. Ecol.* 30, 231–241.
- Blattner, F.R., 1999. Direct amplification of the entire ITS region from poorly preserved plant material using recombinant PCR. *BioTechniques* 27, 1180–1186.
- Bradshaw Jr., H.D., Otto, K.G., Frewen, B.E., McKay, J.K., Schemske, D.W., 1998. Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (*Mimulus*). *Genetics* 149, 367–382.
- Charles, D.R., Goodwin, R.H., 1953. An estimate of the minimum number of genes differentiating two species of golden-rod with respect to their morphological characters. *Am. Nat.* 77, 53–69.
- Farjon, A., 1998. *World Checklist and Bibliography of Conifers*. The Royal Botanic Gardens, Kew, UK.
- Fiedorow, P., Odrzykoski, I., Szweykowski, J., Szweykowska-Kulinska, Z., 2001. Phylogeny of the European species of the genus *Pella* (Hepaticae; Metzgeriales) based on the molecular data from nuclear tRNA (CAA) (Leu) intergenic sequences. *Gene* 262, 309–315.
- Gleeson, D.M., Howitt, R.L.J., Ling, N., 1999. Cryptic species of mudfish in New Zealand. *Mol. Ecol.* 8, 47–57.
- 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.
- Hoot, S.B., Taylor, W.C., 2001. The utility of nuclear ITS, a LEAFY homolog intron and chloroplast *atpB*–*rbcL* spacer region data in phylogenetic analyses and species delimitation in *Isoetes*. *Am. Fern J.* 91, 166–177.
- Kearsey, M.J., Farquhar, A.G.L., 1998. QTL analysis in plants; where are we now? *Heredity* 80, 137–142.
- Kim, S.-C., Rieseberg, L.H., 1999. Genetic architecture of species differences in annual sunflowers: implications for adaptive trait introgression. *Genetics* 153, 965–977.
- Odrzykoski, L.J., 1999. Genetic differentiation and cryptic speciation in the *Conocephalum conicum* species group. Abstract. XVI International Botanical Congress, St. Louis, MO, USA.
- Omland, K.E., Tarr, C.L., Boorman, W.I., Marzluff, J.M., Fleisher, R.C., 2000. Cryptic genetic variation and paraphyly in ravens. *Proc. R. Soc. Lond. B* 267, 2475–2482.
- Para-Olea, G., Wake, D.B., 2001. Extreme morphological and ecological homoplasy in tropical salamanders. *Proc. Natl. Acad. Sci.* 98, 7888–7891.
- Perkins, S.L., 2000. Species concepts and malaria parasites: detecting a cryptic species of *Plasmodium*. *Proc. Royal Soc. Lond. B* 267, 2345–2350.
- Peterson, S.W., 2000. Bio-network news. *Diversity* 15, 31.
- Roca, A.L., Georgiadis, N., Pecon-Slattey, J., O'Brien, S.J., 2001. Genetic evidence for two species of elephant in Africa. *Science* 293, 1473–1477.
- Roy, B.A., Vogler, D.R., Bruns, T.D., Szaro, T.M., 1998. Cryptic species in the *Puccinia monoica* complex. *Mycologia* 90, 847–854.
- Rubinoff, D., Powell, J.A., 2001. Cryptic species and associations with relictual hostplants in the moth genus *Syndemis* (Tortricidae: Lepidoptera). Abstract, Ecol. Soc., Am. Ann. Mtg. San Diego, CA, USA.
- Scheffer, S.J., Lewis, M.L., 2001. Two nuclear genes confirm mitochondrial evidence of cryptic species within *Liriomyza huidobrensis* (Diptera: Agromyzidae). *Ann. Entomol. Soc. Am.* 94, 648–653.
- Shaw, A.J., 2001. Biogeographic patterns and cryptic speciation in bryophytes. *J. Biogeogr.* 28, 253–261.
- Somerville, C., Somerville, S., 1999. Plant functional genomics. *Science* 285, 380–383.
- Yatabe, Y., Masuyama, S., Darnaedi, D., Murkami, N., 2001. Molecular systematics of the *Asplenium nidus* complex from Mt. Halimam National Park, Indonesia: evidence of reproductive isolation among three sympatric *rbcL* sequence types. *Am. J. Bot.* 88, 1517–1522.