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RESEARCH REPORT

Comparisons among *Cupressus arizonica* Greene, *C. benthamii* Endl., *C. lindleyi* Klotz. ex Endl. and *C. lusitanica* Mill. using Leaf Essential Oils and DNA Fingerprinting

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Abstract

The chemical composition of the leaf oils are reported for *Cupressus arizonica*, *C. benthamii* and *C. lindleyi* from Mexico and compared to the leaf oil of *C. lusitanica*, introduced to Portugal in ca. 1634. The oil of *C. arizonica* has moderate amounts of butyl methyl ether (8.6%), α-pinene (7.6%), β-phellandrene (5.7%), umbellulone (5.4%), isophyllocladene (5.8%), phyllocladene (4.1%), nezukol (8.5%) and phyllocladanol (14.2%). The oil of *C. benthamii* has a large amount of abietadiene (26%) and trans-totarol (19.3%), with moderate amounts of α-pinene (4.1%), cis-totarol (4.2%) and trans-ferruginol (5.0%). The oil of *C. lindleyi* has moderate amounts of butyl methyl ether (6.5%), limonene (14.0%), β-phellandrene (13.0%), umbellulone (8.1%), terpinen-4-ol (4.6%) and α-cadinol (10.0%). The oil of *C. lusitanica* has considerable abietadiene (11-24%), with moderate amounts of α-pinene (6.0-16.6%), sabinene (6.7-10.3%) and trans-totarol (5.1-6.5%). *C. lusitanica* was about as similar to *C. arizonica* as it was to *C. benthamii* or *C. lindleyi* in either terpenoids or Random Amplified Polymorphic DNAs (RAPDs). *C. benthamii* and *C. lindleyi* were quite distinct and continued specific recognition seems warranted. *C. lusitanica* appears to be a distinct taxon but it's origin is still not resolved.

Key Word Index

Cupressus lusitanica, *Cupressus benthamii*, *Cupressus lindleyi*, *Cupressus arizonica*, Cupressaceae, phyllocladanol, abietadiene, trans-totarol, limonene, β-phellandrene, α-pinene, sabinene, Random Amplified Polymorphic DNA, chemotaxonomy.

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Introduction

The origin of *Cupressus lusitanica* Mill. cultivated at Bussaco, Portugal has been one of the unsolved botanical mysteries of the Cupressaceae family. Farjon (1) recently reviewed the literature on this species. He agreed with Franco (2) that the taxon had been introduced to Portugal about 1634 from Mexico (or central America). In fact, there is a sign on the largest tree reading "Planted 1644" (Adams personal observation 1993). The oldest trees have been ring-counted as about 280 years old in 1914 (3) or about 360 years old in 1996. Previous workers have postulated the trees came from near Goa, India, but Farjon (1) says that there are no native *Cupressus* near Goa, and that none of the *Cupressus* from Asia are similar to *C. lusitanica*. Farjon (1) concluded that *C. lusitanica* was introduced from Mexico and is conspecific with *C. lindleyi* Klotz. ex Endl. Thus, he reduced *C. lindleyi* to *C. lusitanica* var. *lusitanica* and *C. benthamii* Endl. to *C. lusitanica* var. *benthamii* (Endl.) Carriere (1).

Carman and Sutherland (4) reported on the diterpenes from leaves of *C. arizonica* Greene. There are no reports on the whole leaf oil of *C. arizonica*. Similarly, there are no reports on the leaf oils of *C. benthamii* or *C. lindleyi*. Floreani et al. (5) report on the leaf oil of *C. lusitanica* cultivated in Argentina. However, in their plant materials section, they say their sample is "*C. lusitanica* Mill, also known as *C. glauca* Lamarck, *C. pendula* L'Heritier, *C. lindleyi* Klostzchiana, *C. sinensis* Hort., common name: Mexican cypress, cypress of Portugal...". So it is not clear if their cultivated materials actually came from Portugal or Mexico. They reported that the oil contained α-pinene (11.2%), β-pinene (16.5%) and δ-3-carene (19.4%), with 27 other components but no diterpenes (5). A more recent analysis of *C. lusitanica* oil (obtained from cultivated plants at an unspecified location in Portugal) by Carmo and Frazao (6) reported that it contained α-pinene (18%), β-pinene and sabinene combined (13.2%) and δ-3-carene, and myrcene combined (8.2%), with 17 other components in smaller amounts. No diterpenes were determined in that analysis. No analysis has been reported on *C. lusitanica* using leaf material from the type locality (Bussaco, Portugal) or from the original trees (which are still living) that were planted in ca. 1634.

Random Amplified Polymorphic DNAs (RAPDs) have been recently used for analyses of evolution and taxonomy (7-9). This seemed an opportune time to report on the essential oil components and also utilize RAPDs to investigate the theory (1) that C. *lusitanica* was recently (ca. 1634) derived from what has been called *C. lindleyi* (or possibly from *C. benthamii*) from Mexico. A third species which also occurs in Northern Mexico, *C. arizonica* Greene, was included in this study to provide an outgroup species that is clearly morphologically differentiated for comparison to the putative varieties of *C. lusitanica*.

Experimental

Specimens were collected from *C. arizonica*, cultivated, Waco, TX, 150 m, Adams, 6906; *C. benthamii*, composite sample from 5 trees, approx. 8 km nw of Pachuca, State of Hidalgo, Mexico, 2920 m, Adams, 6879; *C. lindleyi*, composite sample from 5 trees, Creel, State of Chihuahua, Mexico, 2250 m, Adams, 6821; *C. lusitanica*, the type locality, Busaco (=Bussaco), Portugal, 500 m, Adams 7071-7074; Adams 7071 was taken from one of the few remaining giant, original trees, ca. 30 m tall and 1 m dbh, ca. 349 years old. Trees 7072 and 7073 were from young (ca. 20-40 years old). Voucher specimens have been deposited at the BAYLU herbarium.

The oils were isolated by steam distillation (200 g of foliage, FW) using a circulatory Clevenger-type apparatus (10) for 2 h. The oil samples were concentrated (ether trap removed) with Nitrogen and stored at −20°C until analyzed. Mass spectra were recorded with a Finnegan Ion Trap (ITD) mass spectrometer, model 800, directly coupled to a Varian 6500 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 μm film thickness, fused silica capillary column (see reference 11 for operating details). Identifications were made using combined MS and retention times of reference compounds (11).

RAPDs analyses followed the procedure reported by Demeke et al. (9). Fresh or silica gel preserved

leaves (0.2 g) were ground in liquid nitrogen and DNA extracted by the SDS protocol (12). PVP (1% w/v) was added to the extraction buffer. PCR was performed in a volume of 25 µL containing 50 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton X-100, 0.2 mM of each DNTPs, 6 µM primers, 0.5 ng genomic DNA, and 1.0 unit of Taq DNA polymerase. A control PCR tube containing all components, without genomic DNA, was run with each primer to check for contamination. Ten-mer primers that gave several bright bands and did not have any false bands (in the controls) were used. The primers (123-261) were obtained from the University of British Columbia. Primers used (number, 5'-3' sequence): 123 GTC TTT CAG G; 131 GAA ACA GCG T; 143 TCG CAG AAC G,153 GAG TCA CGA G; 184 CAA ACG GCA C; 204 TTC GGG CCG T; 212 GCT GCG TGA C; 218 CTC AGC CCA G; 227 CTA GAG GTC C; 234 TCC ACG GAC G; 237 CGA CCA GAG C; 239 CTG AAG CGG A; 244 CAG CCA ACC G; 249 GCA TCT ACC G; 250 CGA CAG TCC C; 261 CTG GCG TGA C.

DNA amplification was performed in an MJ Programmable Thermal Cycler (MJ Research, Inc.). The thermal cycle used was 93°C (1.5 min) for initial strand separation, then 40 cycles of 37°C (2 min), 68°C (2 min), 90°C (1 min). Two additional steps were used: 37°C (2 min) and 68°C (5 min) for final extension. Amplification products were analyzed by electrophoresis in 1.5% agarose gels and detected by staining with ethidium bromide. Gels were photographed under UV light with Polaroid film 667. pGEM DNA (Promega) was used as molecular size markers. The size of the bands and their intensity was scored as: 0 = no band; 4 = faint ; 5 = medium; 6 = bright. Similarity measures were computed using absolute character state differences (Manhattan metric), divided by the maximum value for that character over all taxa (= Gower metric, 13,14). Principal coordinate analysis (PCO) follows Gower(15). The software package for 3-D ordination of RAPDs, PCO3D, is available from R.P. Adams for PC compatible computers.

Results and Discussion

Oil yields were: *C. arizonica* (0.42%), *C. benthamii* (0.25%), *C. lindleyi* (0.41%) and *C. lusitanica* (0.34%). Examination of the individual terpenoids (Table I), reveals that the *C. lusitanica* individuals are very similar to *C. benthamii*, although *C. lusitanica* appears to have two unique compounds: cis- and trans-sabinene hydrate. However, analyses (Cool, unpublished data) of the oils of several trees of *C. lindleyi* and *C. arizonica* trees from a different region revealed up to 1% of these compounds . Several of the unknown diterpenes (KI 2015, 2105, 2146, 2215, and 2295) seem to be restricted to *C. lusitanica* and *C. benthamii*. This supports the pattern obtained in the principle coordinate analysis (Figure 1) that *C. lusitanica* is most similar to *C. benthamii* in its terpenoids.

The pattern based on seventy two terpenoids (Figure 1) shows *C. lusitanica* individuals are most similar to *C. benthamii*. The terpenoids indicate that *C. lindleyi* is quite distinct from *C. benthamii*, with *C. arizonica* being somewhat intermediate. Note that *C. lusitanica* individuals L1 (7072) and L2 (7072) are most similar (Figure 1), with L3 (7073) being a little less similar.

The 16 primers resulted in a total of 187 RAPD bands. Analysis of these bands by principal coordinate analysis, revealed four groups: *C. arizonica*, *C. benthamii*, *C. lindleyi* and *C. lusitanica*. The *C. lusitanica* individuals did not cluster with either *C. benthamii* or *C. lindleyi* (Figure 2). *C. arizonica* was quite distinct, as expected, but the fact that C. *lusitanica* was slightly more similar to *C. arizonica* (0.621) than to *C. benthamii* (0.615) or *C. lindleyi* (0.615) was rather unexpected. Another unexpected result was the diversity among the three individuals of *C. lusitanica* (Figure 2). Note that individuals L1 (7071) and L2 (7072) are much more similar in their RAPDs than either is to L3 (7073). One might expect that the seed collection(s) used to establish *C. lusitanica* in Portugal might have a very narrow genetic base. This does not appear to be the case. Tree L1 (7071) was one of the original trees planted (dbh 1 m), whereas trees L2 (7072) and L3 (7073) were younger (dbh 12 cm) trees.

Neither the terpenoids nor the RAPDs analyses give strong support for the theory of the origin of *C. lusitanica* from either *C. benthamii* or *C. lindleyi*. These analyses do support the continued

Table I. Comparisons (percentage) of volatile leaf oils of *Cupressus lusitanica*, *C. benthamii*, *C. lindleyi* and *C. arizonica*

		C. lusitanica					
RI	Compound	7071	7072	7073	*C. benthamii*	*C. lindleyi*	*C. arizonica*
816	butyl methyl ether	-	-	-	1.4	6.5	8.6
851	(E)-2-hexenal	0.2	0.4	0.2	-	-	-
926	tricyclene	t	t	t	t	0.5	0.2
931	α-thujene	0.3	0.3	0.6	0.4	1.1	0.6
939	α-pinene	16.6	6.4	6.0	4.1	3.6	7.6
953	α-fenchene	0.1	0.2	0.1	-	-	-
953	camphene	t	0.1	0.1	0.1	0.6	0.3
976	sabinene	6.7	10.3	7.6	1.6	0.9	0.8
980	β-pinene	0.7	0.4	0.4	0.2	0.1	0.3
991	myrcene	2.8	1.8	3.5	1.0	2.5	3.2
1005	α-phellandrene	0.1	t	0.1	0.1	0.2	0.1
1011	δ-3-carene	1.2	8.1	3.6	0.5	t	-
1018	α-terpinene	0.7	0.6	0.8	0.7	1.9	0.8
1026	p-cymene	0.2	0.1	0.4	0.1	0.4	0.6
1031	limonene	1.4	1.0	1.8	1.0	14.0	2.5
1031	β-phellandrene	1.4	1.0	1.8	1.0	13.0	5.7
1033	1,8-cineole	0.9	2.4	0.3	-	-	-
1042	heptyl acetate	t	0.8	0.3	-	-	-
1062	γ-terpinene	1.3	1.1	1.2	1.0	2.3	1.2
1068	cis-sabinene hydrate	0.4	0.5	0.7	-	-	-
1088	terpinolene	1.0	1.1	1.6	0.8	2.3	1.0
1091	2-nonanone	t	0.6	0.2	0.1	-	-
1097	trans-sabinene hydrate	0.3	0.5	0.4	-	-	-
1098	linalool	-	-	-	0.1	0.3	0.1
1098	2-nonanol	0.3	1.0	0.7	-	-	-
1121	cis-p-menth-2-en-1-ol	0.3	0.2	0.5	0.1	0.3	-
1121	cis-pinene hydrate	-	-	-	-	-	0.1
1125	α-campholenal	0.2	0.1	t	-	-	-
1140	trans-p-menth-2-en-1-ol	0.3	0.2	0.3	0.1	0.2	0.2
1143	camphor	-	-	-	t	1.2	1.1
1144	trans-verbenol	0.2	0.1	0.7	-	-	-
1148	camphene hydrate	0.5	0.1	0.2	-	0.3	t
1166	p-mentha-1,5-dien-8-ol	0.1	0.3	0.1	-	-	-
1171	umbellulone	0.2	0.3	6.4	3.2	8.1	5.4
1177	terpinen-4-ol	3.9	3.0	4.7	2.3	4.6	2.5
1179	naphthalene	0.1	0.2	0.2	-	-	-
1183	p-cymen-8-ol	0.1	0.2	0.2	-	0.2	0.2
1189	α-terpineol	0.4	0.3	1.2	0.4	0.6	0.4
1193	cis-piperitol	0.1	t	0.3	t	t	-
1195	(Z)-4-decenal	-	-	-	-	-	0.1
1205	trans-piperitol	0.1	0.2	0.2	t	0.2	0.1
1228	citronellol	0.1	t	0.2	-	-	0.1
1244	methyl carvacrol	-	-	-	-	-	0.1
1285	bornyl acetate	t	t	t	t	1.0	t
1290	thymol	0.1	-	-	-	-	-
1350	α-terpinyl acetate	1.3	t	0.3	0.1	2.3	0.3
1402	longifolene	t	-	0.6	-	-	-
1418	β-caryophyllene	0.5	0.2	0.4	0.3	0.1	t
1446	cis-muurola-3,5-diene	0.6	0.7	0.1	0.6	t	1.5
1454	α-humulene	0.8	0.2	0.2	0.2	t	0.1

Table I. Continued

RI	Compound	*C. lusitanica* 7071	*C. lusitanica* 7072	*C. lusitanica* 7073	*C. benthamii*	*C. lindleyi*	*C. arizonica*
1460	cis-muurola-4(14),5-diene	1.5	2.1	0.2	1.2	-	3.9
1497	epi-zonarene	0.3	0.3	t	0.9	-	1.7
1499	α-muurolene	0.3	0.3	0.1	t	0.3	-
1513	γ-cadinene	t	t	t	t	0.5	-
1521	cis-calamenene	-	-	-	-	-	0.3
1524	δ-cadinene	0.2	0.2	0.1	0.3	1.7	0.4
1549	cis-muurol-5-en-4β-ol	0.2	0.4	t	-	-	0.2
1549	elemol	-	-	-	-	0.3	-
1554	cis-muurol-5-en-4α-ol	0.2	0.5	t	-	-	0.3
1581	caryophyllene oxide	0.4	0.2	0.6	0.1	-	-
1596	cedrol	t	0.7	0.2	-	0.2	0.1
1606	β-oplopenone	-	-	-	-	0.2	-
1606	humulene epoxide II	0.2	t	0.1	0.1	-	0.1
1614	1,10-di-epi-cubenol	-	-	-	0.1	-	0.1
1627	1-epi-cubenol	-	-	-	0.1	-	-
1630	α-acorenol	0.1	3.2	1.2	-	1.7	0.4
1634	β-acorenol	0.1	0.6	0.2	-	t	t
1640	epi-α-cadinol (=T-cadinol)	-	-	-	-	3.0	0.4
1641	epi-α-muurolol (=T-muurolol)	t	t	t	-	3.0	-
1645	α-muurolol (=torreyol)	-	-	-	-	1.3	-
1653	α-cadinol	0.8	0.4	0.2	0.5	10.0	0.6
1682	cis-14-normuurol-5-en-4-one	-	-	-	-	-	0.7
1881	oplopanonyl acetate	-	-	-	-	0.7	-
1898	isopimara-9(11),15-diene	-	-	-	-	-	0.1
1923	isohibaene	-	-	-	-	-	0.6
1963	isophyllocladene	-	-	-	-	-	5.8
1989	manoyl oxide	t	0.6	5.7	-	0.2	t
2010	epi-13-manoyl oxide	0.5	0.5	1.8	0.2	-	-
2011	phyllocladene	-	-	-	-	-	4.1
2015	(abieta-8,12-diene)	0.5	0.6	0.4	0.8	-	-
2054	abietatriene	2.5	1.6	1.4	3.4	0.2	0.2
2080	abietadiene	24.4	21.5	11.3	26.0	0.3	2.2
2105	diterpene	2.7	0.3	0.6	0.2	t	-
2126	nezukol	t	t	13.1	1.4	1.0	8.5
2146	(abieta-8(14),13(15)-diene)	0.9	0.8	0.4	1.0	-	-
2179	diterpene	0.7	0.8	0.5	0.8	t	-
2200	phyllocladanol	-	-	-	1.4	-	14.2
2215	diterpene	1.2	0.6	0.5	0.7	t	-
2230	diterpene	-	-	-	-	-	1.4
2265	diterpene	-	-	-	1.4	t	-
2278	cis-totarol	2.8	3.4	2.1	4.2	1.7	0.5
2295	diterpene	1.6	2.6	0.9	2.1	-	-
2303	trans-totarol	6.0	6.5	5.1	19.3	1.3	1.5
2325	trans-ferruginol	1.3	2.0	1.1	5.0	0.5	0.3
2391	abietol	-	-	-	0.7	-	0.1

Compounds in bold face are components that show major differences between the taxa.
RI = Retention indices on DB-5 (=SE54) column, temperature programmed 60° to 240°C at 3°C/min.
Compounds in parenthesis are tentatively identified.
Compositional values less than 0.1% are denoted as traces (t).
Unidentified components less than 0.5% are not reported.

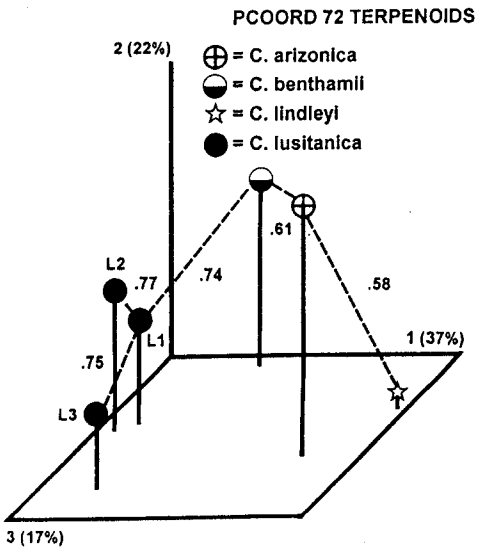

Figure 1. Principal coordinate ordination of *Cupressus arizonica*, *C. benthamii*, *C. lindleyi* and *C. lusitanica* based on 72 terpenoids. The percent of total variance accounted for is shown on each axis. The dotted lines and numbers indicate the nearest neighbor and similarity. Although each taxon appears to be distinct, *C. arizonica* is somewhat intermediate between C. *benthamii* and *C. lindleyi*.

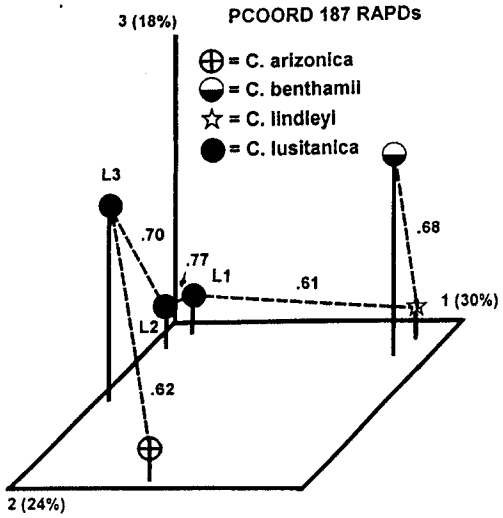

Figure 2. Principal coordinate ordination of *Cupressus arizonica*, *C. benthamii*, *C. lindleyi* and *C. lusitanica* based on 187 RAPD bands. The percent of total variance accounted for is shown on each axis. The dotted lines and numbers indicate the nearest neighbor and similarity. Each taxon appears to be distinct, but note the diversity among the *C. lusitanica* individuals. The pattern of diversity among the *C. lusitanica* individuals is the same as with the terpenes (Figure 1).

recognition of two taxa in Mexico, *C. benthamii* and *C. lindleyi*, comparable to the specific status of *C. arizonica*.

Mass spectra for the unidentified constituents: [ITMS, m/z (rel. int.): KI 2105, 41 (100), 55(41), 67(40), 81(41), 95(33), 109(20), 121(17), 135(10), 149(8), 163(4), 177(14), 191(48), 257(1), M^+272, diterpene; KI 2179, 41(100), 55(49), 67(44), 79(57), 91(67), 107(44), 123(35), 133(38), 147(18), 161(16), 173(7), 187(11), 201(4), 243(5), 257(20), 271(27), M^+286(9), diterpene alcohol; KI 2215, 41(100), 55(37), 69(11), 79(28), 91(23), 105(12), 117(11), 129(13), 141(47), 155(10), 162(13), 173(9), 183(42), 197(4), 211(49), 243(10), 253(47), 269(2), M^+286(9), diterpene alcohol; KI 2230, 41(100), 55(45), 67(44), 81(35), 91(32), 108(50), 123(35), 134(48), 147(30), 161(63), 175(8), 187(7), 247(6),257(20), 271(8), M^+286(3), diterpene alcohol; KI 2265, 41(100), 55(54), 67(44), 81(55), 91(70), 107(52), 121(36), 133(37), 147(30), 161(33), 175(18), 185(10), 199(13), 213(8), 227(16), 257(75), M^+? 284(17), diterpene ketone?; KI 2295, 41(100), 55(32), 69(31), 79(14), 91(20), 107(67), 121(16), 129(16), 143(10), 157(11), 173(6), 187(17), 199(13), 227(12), 245(75), 269(47), 285(5), M^+ 284?, diterpene ketone?

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