SPECIATION OF *JUNIPERUS CEDRUS* AND *J. MADERENSIS* IN THE ARCHIPELAGOS OF CANARIES AND MADEIRA BASED ON TERPENOIDS AND nrDNA AND petN-psbM SEQUENCES.

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ABSTRACT

Analyses of nrDNA and petN-PsbM sequence data, combined with leaf terpenes data, revealed that putative *J. cedrus* from Madeira is as distinct from *J. cedrus* of the Canary Islands as it is from *J. oxycedrus*. These data support the recognition of the Madeira juniper at the specific level, *J. maderensis* (Menezes) R. P. Adams **comb. et stat. nov.** *Phytologia 92(1): 44-55 (April, 2010).*

KEY WORDS: *Juniperus cedrus, J. maderensis, J. oxycedrus, J. macrocarpa, J. brevifolia,* Cupressaceae, Madeira Island, Canary Islands, nrDNA, cp petN-psbM, taxonomy.

In a previous paper of this issue, we reported on the leaf oils of *Juniperus cedrus* Webb & Berthol. from Madeira and the Canary Islands (Adams et al. 2010). The leaf oils were found to differ greatly from putative *J. cedrus* in Madeira to those of *J. cedrus* of the Canary Islands (Fig. 1). In fact, the oils from *J. cedrus* of the Canary Islands were more similar to the *J. oxycedrus* oil (0.620, Fig. 1) than to putative *J. cedrus* oil from Madeira (0.495, Fig. 1).

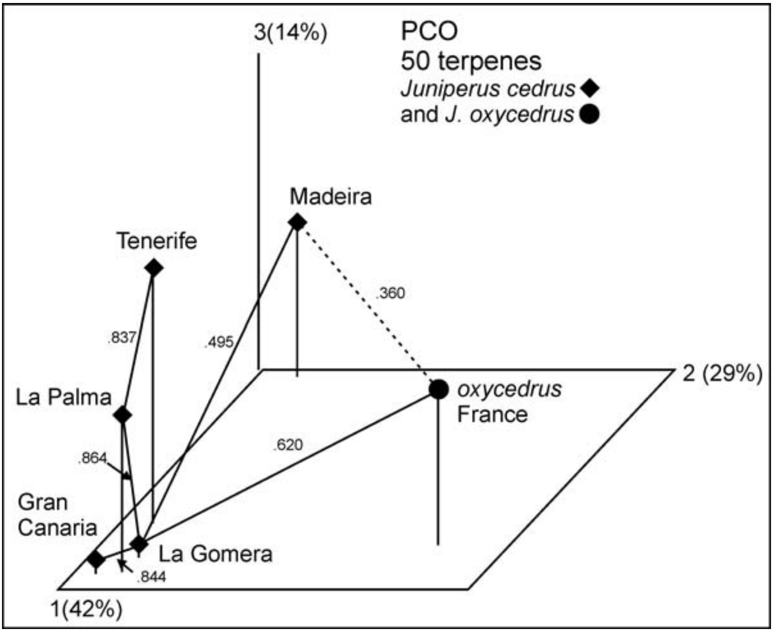

Figure 1. PCO based on 50 terpenes. Note the differentiation of *J. cedrus*, Madeira from the Canary Island populations. Numbers next to lines are similarities. Adapted from Adams et al. (2010).

The Madeira juniper was described as *J. oxycedrus* L. subsp. *maderensis* Menezes (Bull. Acad. Int. Georg. Bot. 18: xii. 1908). Recently the taxon was transferred to *Juniperus cedrus* subsp. *maderensis* (Menezes) Rivas Mart. et al. [Itinera Geobot. 15(2): 703. 2002]. Farjon (2005) noted that leaf size and shape vary considerably in *J. cedrus* and concluded that plants from Madeira were allied with *J. cedrus* and not *J. brevifolia*.

Adams (2008) reported that *J. cedrus* of the Canary Islands, is in a clade with *J. oxycedrus* and *J. macrocarpa*, not in the distinct clade of *J. brevifolia*, *J. navicularis* and *J. deltoides* (Fig. 2).

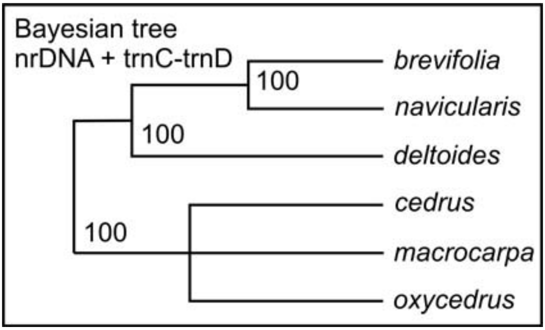

Figure 2. Partial Bayesian tree based on nrDNA + trnC-trnD sequences. Numbers at the nodes are posterior probabilities on a percent basis.

The purpose of this paper is to examine DNA sequence data of the nrDNA and petN-spacer-psbM cp regions to resolve the taxonomic affinities of *J. cedrus* from the Canary Islands and putative *J. cedrus* from Madeira.

MATERIALS AND METHODS

Plant material (GenBank accessions: nrDNA, petN-psbM) - *J. cedrus* (*11497*, GU139568, GU139573; *11519*, GU139569, GU139574). Madeira Island: cultivated at Agriculture Dept., Camacha, 32º 40.374'N, 16º 50.834'W, 650 m, (= tree analyzed by Pino et al. 2003), *Adams 11496*; 32º 41.871'N, 16º 52.986'W, 1143 m, (= trees 1-5 analyzed by Cavaleiro et al. 2002), *Adams 11497-11501*; Canary Islands: Gran Canaria Island, Montaña del Cedro, 27º 57'N, 15º 44'W, 850 m, collected by Beatriz Rumeu, *Adams 11505-11507*; La Palma Island, Piedra Llana and La Caldera de Taburiente, 28º 45.069'N, 17º 50.150'W, 2160 m, *Adams 11509-11513*; Tenerife Island, Riscos de La Fortaleza, 28º 18.868'N, 16º 35.975'W, 2150 m, *Adams 11518-11522*; La Gomera Island, Garajonay National Park, 28º 6.544'N, 17º 13.533'W, 1339 m, *Adams 11523-11527*. *J. brevifolia*, (*8152*, GU139571, GU139576), Pico Verde lookout, San Miguel Island, Azores, Portugal, 800m, *Adams 8152-8153*; *J. macrocarpa*, (*7205*, GU139570, GU139575)15 km w of Tarifa on sand dunes, Spain, 36º 04.996'N, 5º 42.104' E, 30 m, *Adams 7205-7206*; *J. oxycedrus* (*9449*, GU139567, GU139572) 4 km e of Forcalquier, France, 44º 04.06'N, 5º 59.19' E, 490 m, *Adams 9039*,1 km sw of Vila Nova de Foz Coa, Portugal, 41º 04.125'N, 7º 07.651' E, 360 m, *Adams 9449*. Voucher specimens are deposited at the Herbarium, Baylor University (BAYLU).

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20º 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). Principal coordinate analysis was performed by factoring the associational matrix based on the formulation of Gower (1966) and Veldman (1967).

RESULTS AND DISCUSSION

Phylogenetic trees Figure 3 shows a NJ tree based on nrDNA sequence data. A prominent feature of the tree is the clade of *J. cedrus, J.*

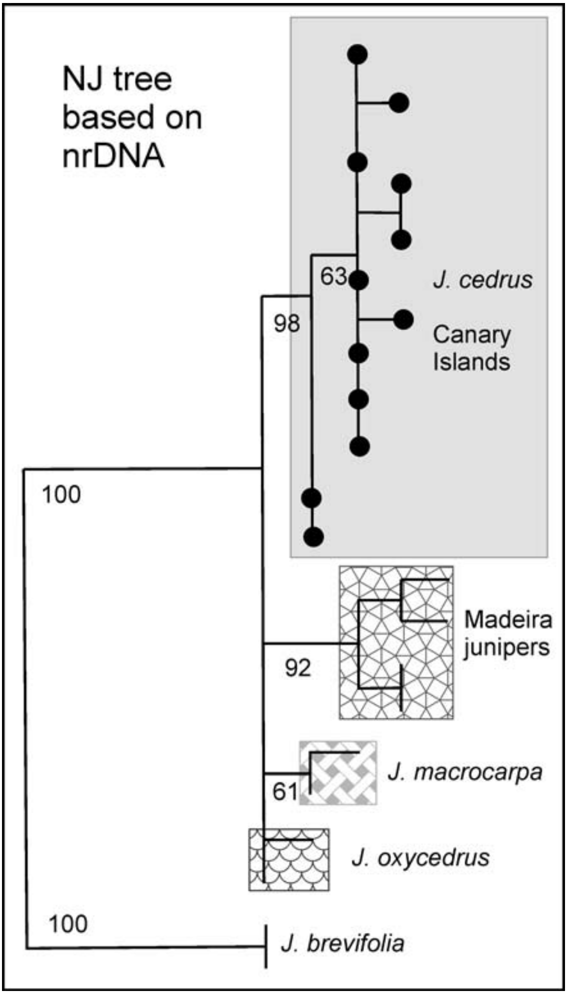

Figure 3. NJ tree based on nrDNA sequences. Numbers at the nodes are bootstrap probabilities (1000 reps.).

macrocarpa and *J. oxycedrus* (Fig. 3). This is similar to the Bayesian tree (Fig. 2). *Juniperus brevifolia* is quite distinct compared to the *J. cedrus* - *macrocarpa* - *oxycedrus* clade. There is strong support (92%) for the clade of the Madeira junipers (putative *J. cedrus*).

The NT tree based on petN-psbM sequences has a congruent topology (Fig. 4), but the Madeira and Canary Island junipers are not resolved into clades as seen in the nrDNA data (Fig. 3).

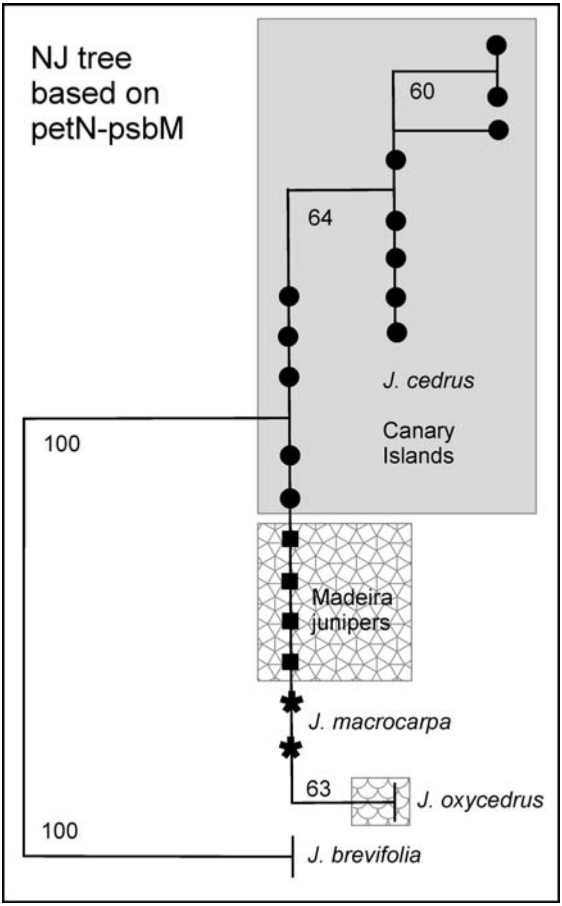

Figure 4. NJ tree based on petN-psbM sequences.

Combining the nrDNA and petN-psbM data, resulted in a NT tree (Fig. 5) that gives stronger support for the *J. cedrus*, Canary Islands and Madeira juniper clades. It should be noted that there is stronger support for the Madeira juniper clade than for the *J. macrocarpa* and *J. oxycedrus* clades. The evolution of the Madeira junipers is quite significant and correlates perfectly with leaf essential oil differentiation (Fig. 1).

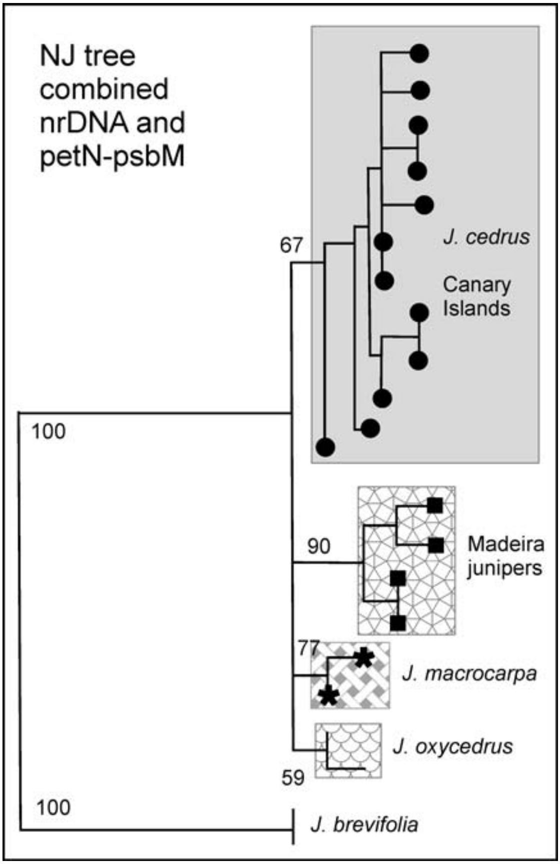

Figure 5. NJ tree based on combined nrDNA and petN-psbM data.

SNPs analyses Figure 6 shows minimum spanning networks for nrDNA (left) and petN-psbM (right). The Madeira junipers are quite diverse in their nrDNA, but uniform in petN-psbM (Fig. 6). *Juniperus cedrus* from the Canary Islands are very uniform in both nrDNA and petN-psbM DNA.

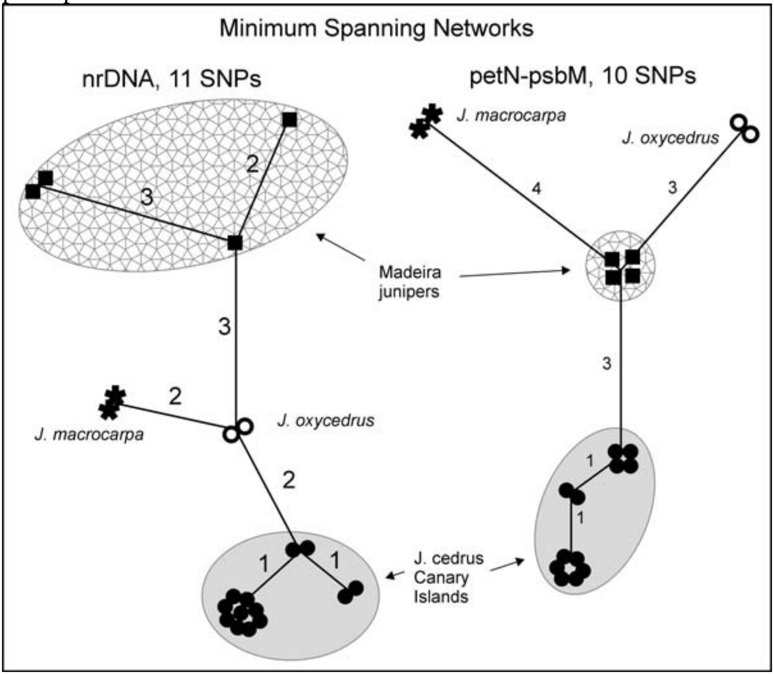

Figure 6. Minimum spanning networks for nrDNA and petN-psbM. Numbers next to the lines are the number of SNPs differences between nodes. Multiple symbols at nodes connote no differences among those individuals.

Combining SNPs from nrDNA and petN-psbM presents a clearer picture of the relationships (Fig. 7). Each of the groups differ by 6 or 7 SNPs. There seems to be considerable diversity in the Madeira junipers, but recall that this diversity is just in nrDNA, so it is

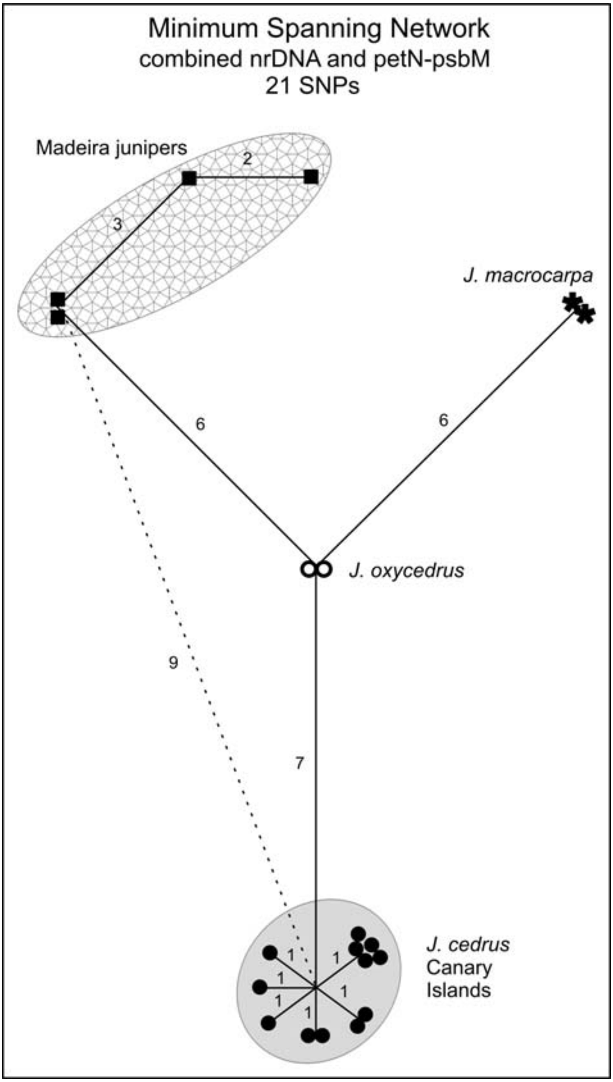

Figure 7. Minimum spanning network based on 21 SNPs from nrDNA and petN-psbM. The dotted line is the shortest link (9 SNPs) connecting the Madeira junipers with *J. cedrus*, Canary Islands.

possible that additional genetic data will not be as diverse. Notice (Fig. 7) that the nearest link between the Madeira junipers and the Canary Island *J. cedrus* is 9 SNPs.

Juniperus cedrus from Madeira has been treated as a subspecies (*J. cedrus* subsp. *maderensis* (Menezes) Rivas Mart. et al.). However, both the volatile leaf oil composition and the DNA sequence data support recognition of the Madeira juniper at the specific level:

Juniperus maderensis (Menezes) R. P Adams, **comb. et stat. nov.**

Basionym: *Juniperus oxycedrus* L. subsp. *maderensis* Menezes, Bull. Acad. Int. Geogr. Bot. 18 (No. 227-228): xii. 1908. Type: Madeira, Serra do Faial, Curral das Freiras, Portugal.

Distribution: Endemic to Madeira on the highest peaks in rocky areas. Nobrega collected it from Pico Ferreiro in 1988 and found 16 juniper plants on Pico das Torres in 1990. It is extremely rare and endangered in nature, but the seeds have been collected and it is now widely cultivated on Madeira.

Synonyms: *Juniperus grandifolius* Link, in Buch, Phys. Beschr. Canar. Ins.: 159. 1825. Farjon (2005) noted that this is a *nom. inval*. under Art. 34.1.

Juniperus cedrus Webb & Berthel. subsp. *maderensis* (Menezes) Rivas Mart. et al. Itinera Geobot. 15(2): 703. 2002.

Conservation: Although the species is rare and endangered in its native habitat, it is widely cultivated on Madeira Island. The greatest threat may be the introduction of related species (*J. cedrus* and *J. oxycedrus*) and possible hybridization with germplasm dilution effects. It would seem wise to prohibit the introduction of *J. cedrus* and *J. oxycedru*s into cultivation in Madeira and to continue to collect seeds and establish plants in cultivation on Madeira.

Because *Juniperus cedrus* does not grow on Madeira, it appears to be endemic to the Canary Islands. There, *J. cedrus* is composed of small, isolated populations. The diversity in its oils and DNA suggest that it is a mostly uniform taxon. Hence, due to the delicate status of conservation, it is urgent to restore old natural areas where *J. maderensis* previously grew on Madeira. It would seem prudent to prohibit the introduction of *J. maderensis* from Madeira, or *J. oxycedrus* from Morocco, into cultivation in the Canary Islands due to possible hybridization with subsequent germplasm contamination of *J. cedrus.*

Table 1 shows some distinguishing features between *J. cedrus*, J. *maderensis* and *J. oxycedrus*.

Table 1. Distinguishing characteristics to separate *J. cedrus*, J. *maderensis* and *J. oxycedrus*.

	J. cedrus	J. maderensis	J. oxycedrus
Leaf tips	blunt to acute rarely mucronate tipped	usu. with a mucronate tip but not an elongated tip	elongated, mucronate tips
Stomatal bands	wider than midrib.	approx. as wide as midrib	approx. as wide as midrib
Leaf midrib	indistinct, usu. covered with bloom	distinct, green not covered with bloom	distinct, green not covered with bloom
Seed Cones (mature)	bright red, little or no bloom	brown-red, with bloom	brown-red to bright red, little bloom
Seed cones (mature)	larger than leaf length	~same as leaf length	smaller than leaf length

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