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Variation among *Cupressus* species from the eastern hemisphere based on Random Amplified Polymorphic DNAs (RAPDs)

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Abstract

Random Amplified Polymorphic DNAs (RAPDs) data were analyzed from eighteen taxa of *Cupressus* from the eastern hemisphere. The following fourteen taxa were distinct in their RAPDs: *Cupressus assamica* (India), *C. atlantica* (Morocco), *C. austrotibetica* (Tibet), *C. cashmeriana* (Bhutan), *C. chengiana* (Sichuan), *C. darjeelingensis* (India), *C. duclouxiana* (Yunnan), *C. dupreziana* (Algeria), *C. funebris* (China), *C. gigantea* (Tibet), *C. jiangeensis* (Sichuan), *C. sempervirens* (Iran and cultivated), *C. tonkinensis* (Vietnam), and *C. torrulosa* (Nepal). Individuals of *C. chengiana* (Sichuan) were not very similar, but loosely allied with *C. cashmeriana*. Considerable diversity was found in the *C. austrotibetica–gigantea* complex as well as within *C. chengiana*. *Cupressus sempervirens* samples from Iran (native) and the cultivated strict form (Spain) were found to be nearly identical. Of all the *Cupressus* from eastern hemisphere, *C. lusitanica* (cult. Portugal) was most similar to *C. assamica* (India). Comparing *C. lusitanica* and *C. assamica* with three *Cupressus* species from Mexico and SW USA, *C. arizonica*, *C. benthamii*, and *C. lindleyi*, revealed that *C. lusitanica* was somewhat more similar to *C. benthamii* and *C. lindleyi*, from Mexico, than to *C. assamica*, from India. This strengthens the argument that *C. lusitanica* had its origin in the western hemisphere.

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1. Introduction

Farjon (1998) lists 17 species and 8 varieties in the genus *Cupressus*. Many of these taxa are found in very restricted areas in central Asia. Field collections have been very difficult. The genus is poorly understood. In addition, the taxa are very commonly cultivated and this has presented difficulties in defining their natural ranges. A case in point is the introduction of *Cupressus lusitanica* to a monastery in Bussaco, Portugal about AD 1634. *Cupressus lusitanica* was thought to have been introduced from near Goa, India but both Farjon (1993) and Franco (1945) believe it came from Mexico. Farjon (1993) considered it conspecific with *C. lindleyi* of Mexico. However, a study (Adams et al., 1997) of the leaf oils and Random Amplified Polymorphic DNAs (RAPDs) using samples from an original tree and a younger tree of *C. lusitanica* at Bussaco versus *C. arizonica* (SW USA) and the only two Mexican taxa, *C. benthamii*, and *C. lindleyi*, found that all four of these taxa to be about equally related, so the Mexican origin of *C. lusitanica* was left in doubt.

Recently, DNA sequencing of matK and rbcL for members of the Cupressaceae has further comfused the taxonomic limits of *Cupressus*. (Gadek et al., 2000) These sequences showed that *Chamaecyparis nootkatensis* is more closely related to *Cupressus* than to other *Chamaecyparis* species. So the status of several *Cupressus* species is not well established.

The purpose of this paper is to investigate the taxa of *Cupressus* in the eastern hemisphere using RAPDs as an initial attempt to shed some understanding on the limits of these taxa and their relationships.

2. Materials and methods

The following specimens were utilized *C. arizonica* Greene, Adams 9144, 9145, cultivated, Texas, USA; *C. assamica* Silba, Adams 9309 and 9310, Hillier Gardens and Arboretum, Rushforth s.n.; *C. atlantica* Gaussen, Adams 9422, 9423, near Tizin-Test, Morocco; *C. austrotibetica* Silba, Rushforth 5528, clones A and B, Tibet, and Rushforth 6015, clones A and B, Tibet; *C. benthamii* Endl., Adams 8710, 8711, Hidalgo, Mexico; *C. duclouxiana* Hickel, Adams 6691, ex. Kew 164-80.06264 and Rushforth 2603, both Yunnan, China; *C. cashmeriana* Royle ex. Carr., Grierson and Long 234, Hillier Gardens and Arboretum, ex. Bhutan; Hillier Gardens and Arboretum (19870475), clone of neotype at Kew; *C. chengiana* S.Y. Hu, Wang 0028, from Silba, source Sichuan, China and Rushforth s.n., source Kangding, Sichuan, China; *C. chengiana* var. *kansouensis* Silba, Rushforth s.n., ex. Wang 0027, clones A and B, source Silba from Gansu, China; *C. darjeelingensis* Silba, Rushforth s.n. Hillier Gardens and Arboretum 1995 and RGBK (1997-4900), ex Silba 19847,

Darjeeling, India; *C. dupreziana* A. Camus, Adams 6692, Kew (278-70.06118), Tassili-n-Ajjer, Algeria 1 of 40 'trees of Tamarit', Rushforth s.n. Hillier Gardens and Arboretum (19750308), origin unknown; *C. funebris* Endl., Rushforth s.n., cultivated at Kilmacurragh, Ireland believed to be from Wilson 798, ex. Hubei, China and Rushforth s.n., RBGE (19802479), ex E. China; *C. gigantea* W.C. Cheng and L.K. Fu, Rushforth 4792B and 5787A, Tibet; *C. jiangeensis* N. Zhao, Rushforth s.n., ex. Wang 0026, clones A and B, ex Sichuan; *C. lindleyi* Klotzsch ex. Endl., Adams 8706, 8707, Chihuahua, Mexico; *C. lusitanica* Mill., Adams 7071, 7072, cultivated Bussaco, Portugal, 349 year old tree and young tree, respectively; *C. sempervirens* L., Rushforth s.n., Hillier Gardens and Arboretum 19783627, ex. Elburz Mts., Iran and Adams 9216, cultivated at Madrid, Spain; *C. tonkinensis* Silba, Hudson s.n., cultivated at Tregrehan, UK, from Silba s.n. from Vietnam or southern China and C. Morgan, s.n., cultivated at Bedgebury National Pinetum, UK; *C. torulosa*, D. Don in Lambert, Rushforth s.n. from Schilling 2412, ex. Marpha, Nepal and Rushforth s.n. from Hillier Gardens and Arboretum 19760042, origin unknown. Adams specimens and Rushforth's numbered specimens are deposited at SRCG herbarium and E herbarium, Royal Botanic Gardens, Edinburgh, respectively.

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 the leaves by use of the Qiagen DNeasy Mini-plant extractors. Ten-mer primers were purchased from the University of British Colombia (5′–3′): 153: GAG TCA CGA G; 184: CAA ACG GAC C; 204: TTC GGG CCG T; 218: CTC AGC CCA G; 239: CTG AGG CGG A; 244: CAG CCA ACC G; 250: CGA CAG TCC C; 265: CAG CTG TTC A; 338: CTG TGG CGG T; 347 TTG CTT GGC G; 389: CGC CCG CAG T; 431: CTG CGG GTC A.

PCR was performed in a volume of 15 ml containing 50 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 mM 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), 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.

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). Principal coordinate analysis (PCO) of the similarity matrix follows Gower (1966).

3. Results and discussion

The removal of RAPD bands that only appeared in one sample and those bands that were not constant within taxa (e.g. bands that did not have fidelity within taxa or populations) resulted in 329 bands that displayed taxonomically useful information.

Clustering by a minimum spanning network is shown in Fig. 1. The taxa are well resolved into either single species or as a species complex. Fourteen taxa are clearly quite distinct in this analysis and these are arranged into five groups (Fig. 1).

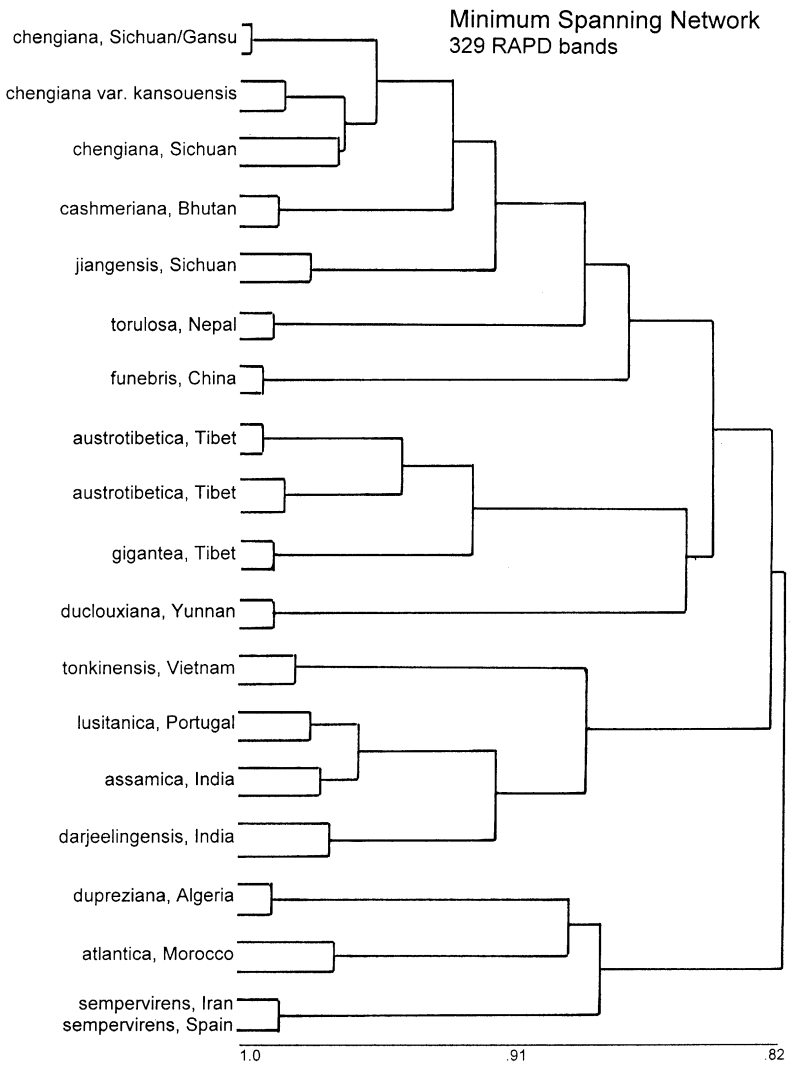

Fig. 1. Minimum spanning network based on 329 RAPD bands. Note the five major groups. See text for discussion.

3.1. Group 1: chengiana–cashmeriana–funebris–jiangeensis–torulosa (China–Bhutan–NW India–Nepal–Tibet)

Cupressus chengiana and *C. chengiana* var. *kansouensis* samples clustered (Fig. 1), but rather loosely and not by varieties. This suggests that var. *kansouensis* is not distinct or that there might be several cryptic varieties of *C. chengiana*. *Cupressus cashmeriana* appears distinct (Fig. 1), supporting Farjon (1998) and Rushforth (1987) recognition of *C. cashmeriana*. *Cupressus jiangeensis* has been recognized (Farjon, 1998) as a variety (*C. chengiana* var. *jiangeensis* (N. Zhao) Silba), so its association with the *C. chengiana* group (Fig. 1) was expected. However, it is so distinctive that support for specific recognition is strengthened by this analysis. *Cupressus torulosa*, from Nepal and NW India, and *C. funebris* (widely cultivated across central and southern China) were loosely linked with the *C. cashmeriana–C. chengiana* group (Fig. 1).

3.2. Group 2: austrotibetica–gigantea (Tibet)

Cupressus austrotibetica and *C. gigantea* from Tibet form a loose but very distinct group (Fig. 1). Individuals from the two sampled sites of *C. austrotibetica* population are almost as different as some of the recognized species in this study (Fig. 1). This is surprising. KR 5528 was collected from young trees growing on a rocky bank above the road at circa 2100 m beside the Yigrong tsangpo at 30.01′44N, 95.00′39E (although on the forested ridges above there are trees 40–60 m in height from 2100 m to circa 2800 m). KR 6015 was collected from two maturing trees of 15 m growing beside a steep cliff at the edge of a side chu to the Po tsangpo, circa 2200 m. The two sties are circa 15 km apart at opposite ends of what appears to be a single population along this river gorge. The town of Tangmai is located at the confluence of the Yigrong and Po Tsangpos (tsangpos is Tibetan for big river) between these two collection sites. Silba has named a taxon *C. tongmaiensis* and this data suggests there may be something in this taxon.

Although Farjon (1998) followed Franco (1969) in treating *C. austrotibetica* as a synonym of *C. duclouxiana*, the present data do not support that treatment. The KR 6015 entity is clearly distinguished from *C. duclouxiana* by the much smaller cones to a maximum of 1.2 cm which have 8 scales each with a projection and which only become rounded (as in *C. duclouxiana*) after the prickle has been eroded flat by the atmosphere which takes circa 5 years. *Cupressus austrotibetica* has also been confused with *C. torulosa*, from which it differs in the small cones with ultimate shoots of less than 1 mm, and as shown in this present DNA analysis.

Cupressus gigantea occurs from just west of Nang (29°03’11”N, 93°03’22”E, 3200m) in a band circa 50 km in length east along the Yigrong tsangpo. It is found on mainly serpentine rocks from 3000 m up to circa 3700 m. KR 4792 was from this area, from just west of Nang at 3200 m. There is also a population between Nyingshi and Bayi (at 29°37’19”N, 94°23’58”E, 3100 m). This occurs on a dry rocky site around a cemetery. The largest tree (which is probably an accretion of three trees) is approximately 30 m in height with a bole diameter of 5 m. However,

this population appears to be naturalized rather than indigenous. The data shows that these two populations are indistinguishable. Silba's *C. tangmaiensis* var. *ludlowii* was named from the Nang population and is a synonym of *C. gigantea*, not of *C. torulosa* as suggested by Farjon (1998).

3.3. Group 3: duclouxiana (Yunnan)

Cupressus duclouxiana is very distinct (Fig. 1) and not closely associated with other groups.

3.4. Group 4: assamica–darjeelingensis–tonkinensis (India–Vietnam)

The material available for the study of these three taxa was of somewhat uncertain origin, and clearly was neither wild origin material (e.g. as for the *C. austrotibetica* and *C. gigantea* samples) nor cultivated material with a good trail back to the original introduction. With this caveat, the data shows these three taxa as not related to other Asiatic cypresses but with some affinity to *C. lusitanica* (see following discussion). *Cupressus tonkinensis* has been treated as a synonym of *C. torrulosa* (Farjon, 1998) but these data show the plant analyzed here to be quite distinct in its RAPDs (Fig. 1). *Cupressus tonkinensis* is named from collections in Lang Son province, Vietnam where scattered old trees occur on the kharst limestone peaks. Material of local cultivated origin from this area differs from *C. torulosa* in the ultimate shoots being flattened and four angled, not terete, but was not available for this study. The material of *C. tonkinensis* used in this study was from seedlings which have not developed mature shoots and may not be the same plant. Both *C. assamica* and *C. darjeelingensis* have been treated as synonyms of *C. cashmeriana* (Farjon, 1998). However, in the RAPDs analysis, *C. assamica* and *C. darjeelingensis* are not similar to *C. cashmeriana*, but form a distinct group (Fig. 1) with *C. tonkinensis* and *C. lusitanica* (however, see discussion following). The material of *C. assamica* at the Hillier Gardens and Arboretum appeared to be variable in juvenile foliage characters. Accordingly, we sampled the range; Adams 9309 was selected as being visually similar to C. cashmeriana in its bluish foliage in flattened, pendent sprays, whereas Adams 9310 had green foliage in three-dimensional, erect sprays. Two additional, *C. assamica* plants from the same seed origin (although one grown at RBG Kew) were analyzed (data not shown) and found to give the same result.

The uncertainty over the origin of the best available material for the study precludes taking a definite view on the specific status of these three taxa. It does, however, highlight the need for further investigation and suggests that Farjon's (1998) treatment may not be satisfactory.

3.5. Group 5: atlantica–dupreziana–sempervirens (n. Africa–Iran)

Cupressus sempervirens from an Iranian seed origin is essentially identical to the strict form cultivated throughout the Mediterranean region (Fig. 1). *Cupressus sempervirens*, *C. atlantica* and *C. dupreziana* form a distinct group. *Cupressus atlantica*

and *C. dupreziana* have been treated as varieties of *C. sempervirens* (Silba, 1986), but this data favors the continued recognition of *C. atlantica* and *C. dupreziana* at the specific level (Farjon, 1998; Rushforth, 1987).

3.5.1. C. lusitanica and allies

In view of the strong clustering of *C. lusitanica* with *C. assamica* from India, we decided to perform an additional analysis comparing *C. lusitanica* and its most similar eastern hemisphere taxon, *C. assamica*, with the three *Cupressus* species from Mexico: *C. arizonica*, *C. benthamii* and *C. lindleyi*. The results (Fig. 2) show that *C. lusitanica* is more similar to *C. benthamii* and *C. lindleyi* than to *C. assamica*. Previous work (Adams et al., 1997) showed *C. benthamii* and *C. lindleyi* as most similar, then *C. lusitanica* linked to *C. lindleyi* and finally *C. arizonica* linked to *C. lusitanica*. The present study, utilizing more samples, gave the same order of clustering for *C. benthamii*, *C. lindleyi* and *C. lusitanica*. It appears that *C. lusitanica* is a little more likely to have come from Mexico (Guatemala or elsewhere in the western hemisphere?) than from India (or elsewhere in the eastern hemisphere). However, the data in support of that thesis is not very strong. Hopefully, when we complete DNA fingerprinting for all the *Cupressus* of the western hemisphere, we will find a closer match to *C. lusitanica*. Conversely, *C. lusitanica* seeds may have been collected from an unusual population in Mexico or central America (or from eastern Asia) that we have not analyzed in this report, and perhaps which no longer exists.

The *Cupressus* of the eastern hemisphere are generally represented by relictual populations or cultivated plants. Yet, the amount of genetic variation seen in the RAPD banding was large. Clearly there are five major groups: *chengiana–cashmeriana–funebris–jiangeensis–torulosa* (w. China–Bhutan–Nepal–Tibet); *austrotibetica–gigantea* (Tibet); *duclouxiana* (Yunnan); *assamica–darjeelingensis–tonkinensis*

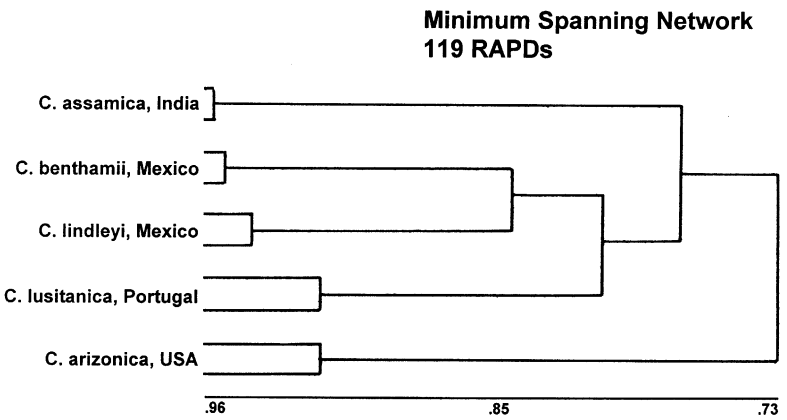

Fig. 2. Comparison of Mexican *Cupressus* with *C. lusitanica*, Portugal and *C. assamica*, India. *Cupressus lusitanica* is a little more similar to the *Cupressus* from Mexico and than the nearest link to an eastern hemisphere *Cupressus* (i.e. *C. assamica*).

(India–Vietnam); and *atlantica–dupreziana–sempervirens* (n. Africa–Iran) suggesting there might be as many as fourteen species.

Although additional sampling might yield better resolution of the taxa, it seems more likely that DNA sequencing (in progress) will be needed to produce a robust treatment and to resolve the more distant relationships among these taxa.

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