HYBRIDIZATION BETWEEN *JUNIPERUS BERMUDIANA* AND *J. VIRGINIANA* IN BERMUDA

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ABSTRACT

In 1942 two scale insects were accidentally introduced into Bermuda with devastating effects on *Juniperus bermudiana*, endemic to Bermuda. In an effort to repopulate junipers on Bermuda, two cultivated junipers from Florida were introduced by J. D. C. Darrell in 1940s (Darrell's cedar) and Reeve Smith in the 1950s (Smith's cedar). Analysis of SNPs of nrDNA and trnC-trnD cp DNA determined that Darrell's cedar is *Juniperus virginiana* var. *silicicola* and Smith's cedar is *Juniperus v*. var. *virginiana*. SNPs analysis reveal what appears to be F_1 hybrids between Darrell's cedar (*J. v.* var. *silicicola*) and *J. bermudiana*. In addition, two individuals were found that contained nucleotides from Darrell's cedar, Smith's cedar and *J. bermudiana*, suggestive of hybridization with backcrossing to the third taxon.

KEY WORDS: *Juniperus bermudiana*, Bermuda, nrDNA, trnC-trnD cp DNA, SNPs, Cupressaceae

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The early British sailors often stopped in Bermuda to make repairs on their ships from Bermuda cedar (*J. bermudiana)* because the island was completely covered with Bermuda cedar (Groves, 1955).

Unfortunately, *Juniperus bermudiana*, endemic to Bermuda, has been subject to attack by two scale insects, *Lepidosaphes newsteadi* and *Carulaspis minima*, these apparently introduced from the U.S. mainland prior to 1942 (Bennett and Hughes, 1959; Groves, 1955). The two insects cause defoliation and death. Groves (1955) estimated that 90% of the trees were dead by 1955. In 1978 William E. Sterrer,

Bermuda Biological Station (pers. comm.), estimated that perhaps 99% of the original trees were exterminated.

Adding to the problem, J. D. C. Darrell obtained a juniper species from the Royal Palms Nursery in Florida in the 1940s and brought it back to Bermuda (Whitney, 1955). It flourished and has been widely planted as an ornamental, or as a replacement for dying Bermuda cedar. It has become known as "Darrell's cedar". The latter is characterized by finer needles and denser foliage than Bermuda cedar. Both *J. bermudiana* and Darrell's cedar shed their pollen in February and March. Darrell's cedar sets seed and appears to be fertile; it also appears to hybridize with Bermuda cedar, given time, and this will likely lead to the complete loss of the Bermuda cedar germplasm. If "Darrell's cedar" came from Florida, it is certainly very closely related to the Bermuda cedar (Adams, 2004) and it is likely interfertile with the Bermuda cedar.

In a second effort to revive cedars, Reeve Smith brought male juniper(s) into Bermuda in the 1950s (Whitney, 1955). Smith's cedar was thought to be 'Barbados cedars' (*J. barbadensis*). Because Smith's cedar(s) were all males, they had to be reproduced by cuttings.

The present study was designed to utilize (Single Nucleotide Polymorphisms, SNPs) of nr DNA and trnC-trnD, cp DNA to determine the affinities of Darrell's Smith's cedar and Smith's cedar and to assess hybridization with *J. bermudiana*.

MATERIALS AND METHODS

Specimens collected: Taxon, acronym, collector number, location: *Juniperus barbadensis* (BA), *Adams 5367-5371;* Petit Piton, St. Lucia, BWI; *Juniperus bermudiana* (BM), *Adams 11080-11082*, Bermuda; *J. gracilior* var. *ekmanii* (EK), *Adams 7653-7654*, 3-4 km ne Mare Rouge, Pic la Selle, Haiti; *J. g.* var. *gracilior* (GR), *Adams 7664-7667*, w of Constanza, Dominican Republic; *J. g.* var. *urbaniana* (UR) *Adams 7656-7658*, 4-5 km ne Mare Rouge, Pic la Selle, Haiti; *J. lucayana* (LU): *Adams 5259-5280*, Havana Botanical Garden (seed from Sierra de Nipe), Cuba; *Adams 5281-5282*, Havana Botanical

Garden (seed from Isle de Pinos), Cuba; *J. saxicola* (SX) *Adams 5284-5285*, w slope of Pico Turquino, Prov. Granma/Santiago de Cuba boundary, Cuba; *J. virginiana* var. *virginiana* (VG) *Adams 6753-6755*; on I35, Hewitt, TX; *J. v.* var. *silicicola* (SI) *Adams 9186-9188*, Ft. Desoto Park, Mullet Key, Florida. Darrell's Cedar (DC): *Adams 11111-11114*, Bermuda; Smith's Cedar (SC): *Adams 11088-11090*, Bermuda; Putative hybrids: *Adams 11093*, *11094*, *11101*, *11102*, *11106*, *11107*, Bermuda. Herbarium vouchers for all of the aforementioned collections are deposited at 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 -20^{o} C until the DNA was extracted. DNA was extracted using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia CA).

SNPs obtained from DNA sequencing

ITS (nrDNA) and trnC-trnD amplifications were performed in 50 µl reactions using 10 ng of genomic DNA, 3 units Qiagen Taq polymerase, 5 µl 10x buffer [final concentration: 50 mM KCl, 10 mM Tris-HCl (pH 9), 0.01% gelatin and 0.1% Triton X-100), 1.75 mM $MgCl_2$, 20 µl Q solution (2X final), 400 µM each dNTP, 1.8 µM each primer and 4% (by vol.) DMSO].

Primers (5'-3'):

ITS: ITSA = GGA AGG AGA AGT CGT AAC AAG G;
ITSB = CTT TTC CTC CGC TTA TTG ATA TG.

ITSA and ITSB primers from Blattner (1999).

trnC-trnD: CDFor: CCA GTT CAA ATC TGG GTG TC
CDRev: GGG ATT GTA GTT CAA TTG GT

CDFor, CDRev primers from Demesure et al. (1995).

CD10F: AAA GAG AGG GAT TCG TAT GGA
CD3R: AAC GAA GCG AAA ATC AAT CA

CD10F and CD3R primers from Andrea Schwarzbach (pers. comm.).

The following PCR conditions were used: MJ Research Programmable Thermal Cycler, 30 cycles, 94^{o}C (1 min.), 50^{o}C (2 min.), 72^{o}C (2 min.), with a final step of 72^{o}C (5 min.). The PCR reaction was subjected to purification by agarose gel electrophoresis (1.5% agarose, 70 v, 55 min.). The nrDNA primers (ITSA, ITSB) produced a

band of approx. 1120 bp. The internal trnC-trnD primers, CD10F-CD3R produced a band of approx. 800 bp. 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. for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.). Alignments were made using Clustal W and then manually corrected. Indels were coded with a "-" for the first nucleotide and "I" for succeeding nucleotides such that an indel was treated as a single mutation event. Overall sequences have been deposited in GenBank (Schwarzbach et al., in prep.).

SNPs analyses

Aligned data sets (nrDNA and trnC-trnD) were analyzed by CLEANDNA (Fortran, R. P. Adams) to remove invariant data. Mutational differences were computed by comparing all SNPs, divided by the number of comparisons over all taxa (= Gower metric, Gower, 1971; Adams, 1975). Principal coordinate analysis was performed by factoring the associational matrix using the formulation of Gower (1966) and Veldman (1967). A minimum spanning network was constructed by selecting the nearest neighbor for each taxon from the pair-wise similarity matrix, then connecting those nearest neighbors as nodes in the network (Adams, 2004).

RESULTS AND DISCUSSION

Analyses of 1119 bp of nrDNA (ITS) sequences revealed 24 SNPs among the taxa, including a 3 bp deletion in both samples of *J. g.* var. *ekmanii* and a 1 bp insertion in all six samples of *J. v.* var. *virginiana* and *J. v.* var. *silicicola*. PCO of the SNPs resulted in 5 eigenroots that were larger than the average diagonal value. These 5 eigenroots accounted for 42.49, 20.48, 12.98, 8.54 and 5.80% of the variation among the OTUs or a total of 90.21%. From this factor analysis there appear to be 4 majors groups and 2 minor groups. Ordination (Fig. 1) shows four major groups: (*J. virginiana, J. v.* var. *silicicola* and Darrell's cedar), all the Caribbean junipers, *J. bermudiana*, and Smith cedar. The identical vertical bars imply that

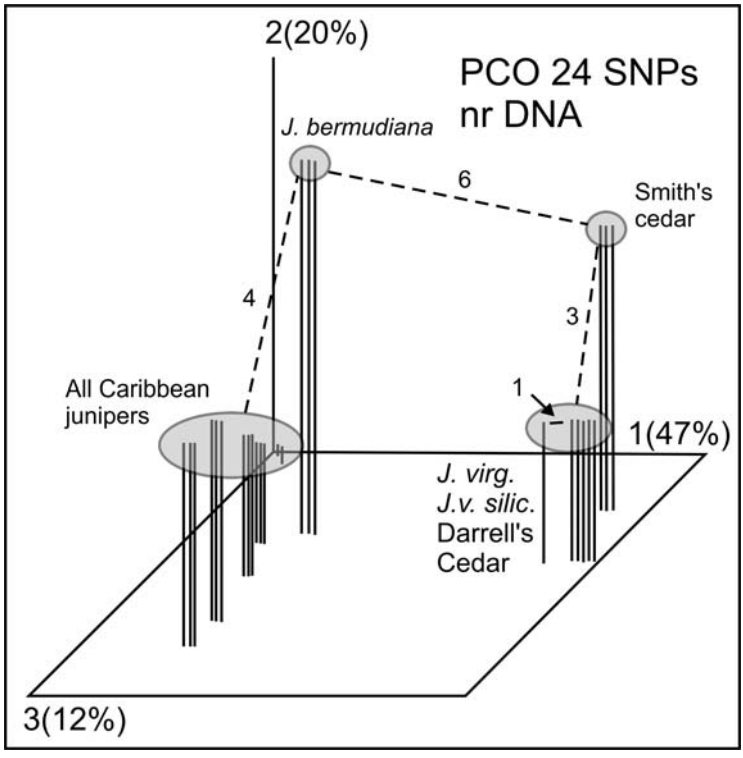

Figure 1. PCO of Caribbean taxa and *J. bermudiana*, Darrell's Cedar and Smith's Cedar. Number next to dashed line is the number of bp differences between groups.

(Fig. 1) there was no variation among these samples. One of the *J. v.* var. *silicicola* samples had a 1 bp difference from other samples of *J. v.* var. *silicicola* and *J. virginiana*. Smith's cedar appears to be a form of *J. virginiana* var. *virginiana* or *J. v.* var. *silicicola* based on ITS SNPs.

Analysis of trnC-trnD, cpDNA revealed 6 SNPs and PCO of these data yielded two eigenroots of 81 and 16%. Figure 2 shows that all the individuals in the analysis had a 553 bp sequence, in contrast to *J. virginiana* and Smith's cedar that both had 798 bp of sequence data.

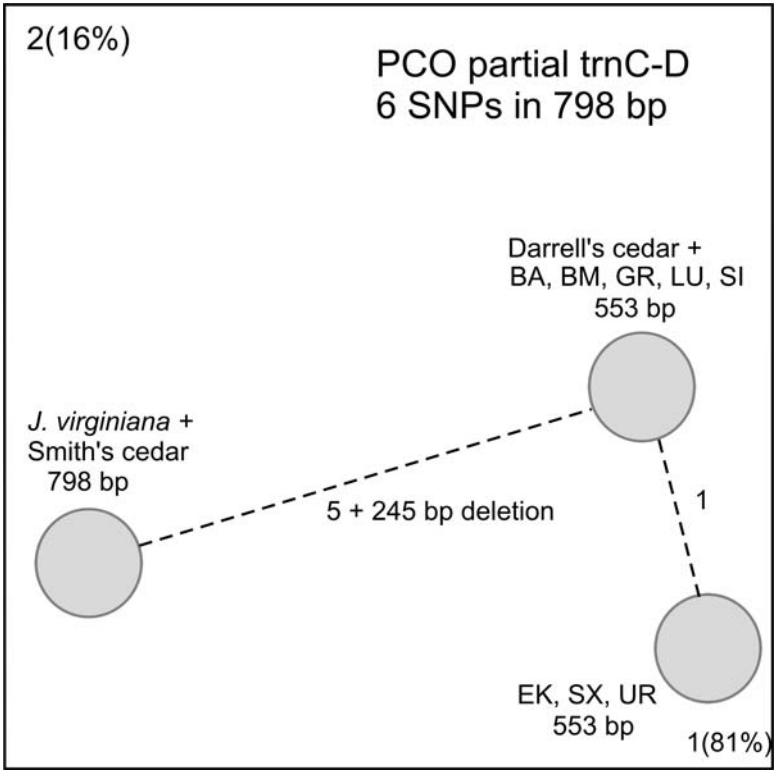

Figure 2. PCO of trnC-trnD SNPs. Numbers next to the dashed lines of the number of SNPs.

Clearly, Smith's cedar is allied with *J. virginiana* and Darrell's cedar is allied with the Caribbean junipers (including *J. v.* var. *silicicola*).

Figure 3 shows a summary of the ITS and trnC-trnD data. It is clear that Smith's cedar is a form of *J. virginiana* and Darrell's cedar is a form of *J. v.* var. *silicicola*.

Both Darrell's and Smith's cedars are resistant to the introduced scale leaf insects. This is readily understandable as they

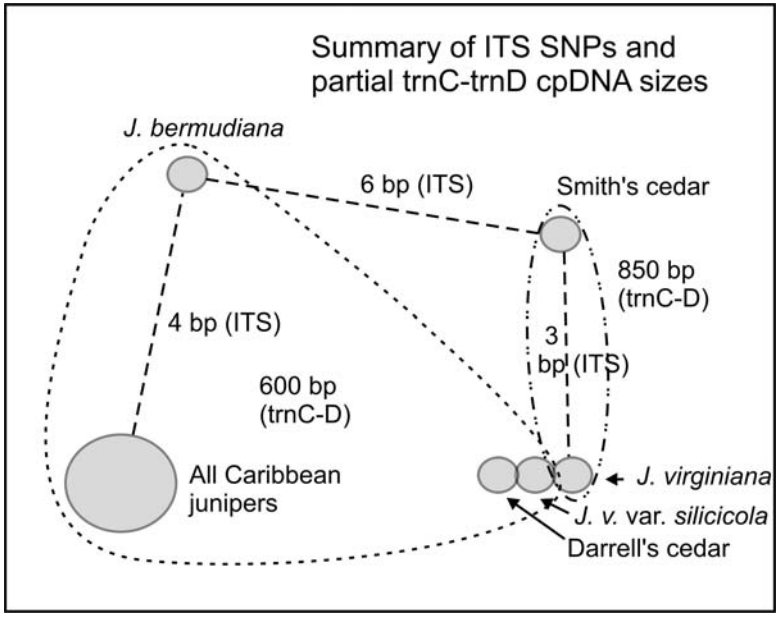

Figure 3. Summary of ITS and trnC-trnD data.

were introduced from the mainland and, as elements of *J. virginiana*, have co-evolved resistance to these scale insects. However, this presents a problem in Bermuda, as Darrell's and Smith's cedars are often planted instead of *J. bermudiana* because they are resistant to scale insects and thus, grow well on Bermuda.

The second problem is that hybridization between Darrell's, Smith's and Bermuda cedars seems likely. If hybrids are fertile, introgression of *J. virginiana* genes into *J. bermudiana* could occur.

HYBRIDIZATION

Darrell's cedar has finer foliage, leaves in threes, and is more bushy (less of a central axis) than *J. bermudiana*. Several trees were sampled that appeared to be morphologically intermediate between Darrell's cedar and *J. bermudiana.* Table 1 shows that plants 11101,

11102 and 11107 (Tivoli North) appear to be hybrids between *J. bermudiana* and Darrell's cedar. Plants 11093 and 11094 that were growing about 100 m from a row of male, Smith's cedars, each have the cpDNA (trnC-trnD, 850 bp) of Smith's cedars, but they also have nuclear DNA markers from *J. bermudiana* (79 C; 332 deletion; 589 A; 699 T; 944 A and 1059 T) and from Darrell's cedar (121 G; 441 T). Darrell's cedar has been planted in the vicinity, so it appears that the plants may be hybrids backcrossed to Darrell's cedar.

Plant 11106 (Aileen Morrison's house) is nearly pure *J. bermudiana*, but it appears that it may have some DNA markers of Darrell's cedar (note the small amounts of several nucleotides typical of Darrell's cedar, Table 1). Because nrDNA is composed of thousands of copies, it might take many generations of concerted evolution to remove these polymorphisms.

Table 1. Comparisons of SNPs from ITS and trnC-trnD fragment lengths for putative hybrids with *J. bermudiana*, Darrell's cedar (DC), Smith's cedar (SC), *J. virginiana*, and *J. v.* var. *silicicola*.

	ITS position									
taxon/sample	79	121	332	441	589	699	944	1059	trnC-D(bp)	Classification
J. bermudiana	C	C	-	C	A	T	A	T	600	*J. bermudiana*
Darrell's Cedar	T	G	G	T	C	C	G	A	600	*J. v.* var. *silicicola*
Smith's Cedar	T	C	G	C	C	C	G	A	850	*J. v.* var. *virginiana*
J. virginiana	T	G	G	G	C	C	G	A	850	*J. virginiana*
J. v. silicicola	T	G	G	G/C	C	C/T	G	A	600	*J. v.* var. *silicicola*
11093	C/T	C/G	G/-	C/T	A/C	C/T	A/G	A/T	850	J. *berm*. x DC x SC?
11094	C/T	C/G	G/-	C/T	A/C	C/T	A/G	A/T	850	J. *berm*. x DC x SC?
11101	C/T	C/G	G/-	C/T	A/C	C/T	A/G	A/T	600	J. *berm*. x Darrell's
11102	C/T	C/G	G/-	C/T	A/C	C/T	A/G	A/T	600	J. *berm*. x Darrell's
11107	C/T	C/G	G/-	C/T	A/C	C/T	A/G	A/T	600	J. *berm*. x Darrell's
11106	C	C	-	C(T)*	A	T(C)	A(T)	T(A)	600	*J. berm* backcrossed to DC?

*base in () was a small peak, less than 20% the height of the parent peak.

Old *J. bermudiana* tree at Devonshire Church cemetery, Bermuda, *Adams 11080*

Darrell's cedar (*J. v.* var. *silicicola*) planted along Cedar Ave., Hamilton, Bermuda. cf *Adams 11112-11114.*

Smith's cedar, *J. virginiana*. at Ms. Nea Smith's house. cf *Adams 11088-11092.*

Putative hybrid of *J. bermudiana* x Darrell's cedar *(J. v.* var. *silicicola*), cf. *Adams 11101*.

Trees at Aileen Morrison's house.
Left: *J. bermudiana*, putative backcross with Darrell's cedar. *Adams 11106*

Right: young *J. bermudiana* tree. This tree appears to be showing disease symptoms. Note loss of foliage at the top.

Planted trees along Cedar Ave., Hamilton, Bermuda.

Foreground: diseased *J. bermudiana* tree. Note the loss of foliage on the upper branches.

Background: healthy Darrell's cedar (*J. v.* var. *silicicola*)

As old diseased *J. bermudiana* trees died on Cedar Ave., Hamilton, Bermuda, they have often been replaced with Darrell's cedar. Darrell's cedar grows very well as it is resistant scale insects that it co-evolved with on the United States mainland.

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