TAXONOMIC AFFINTITY OF RUSHFORTH'S BHUTAN JUNIPER AND *JUNIPERUS INDICA* USING SNPs FROM nrDNA AND cp trnC-trnD, TERPENOIDS AND RAPD DATA

Robert P. Adams
Biology Department, Baylor University, Box 727
Gruver, TX 79040, USA
Robert_Adams@baylor.edu

Julie A. Morris
Department of Biological Sciences, University of Texas at Brownsville
Brownsville, TX, 78520, USA.

Andrea E. Schwarzbach
Department of Biological Sciences, University of Texas at Brownsville
Brownsville, TX, 78520, USA.

ABSTRACT

SNPs from nrDNA and cp trnC-trnD were analyzed from *J. indica*, *J. recurva* and Rushforth's juniper from Bhutan and compared with previous terpene and RAPD data. These data, taken together, show that Rushforth's juniper is allied, but distinct from *J. indica* and a new variety is named: ***J. indica*** var. ***rushforthiana*** R. P. Adams from Bhutan.

KEY WORDS: *Juniperus indica, J. indica.* var. *rushforthiana, J. recurva, J. wallichiana*, Cupressaceae, nrDNA, trnC-trnD, SNPs, essential oils, terpenes, DNA fingerprinting, systematics.

The taxonomy of *J. indica* Bertol. and *J. wallichiana* Hook f. & Thomson ex Brandis has been confusing. Farjon (2005, p. 311) cleared up this confusion, stating "This species (*J. indica*, my addition) has long been known as *Juniperus wallichiana* Hook. f. & Thomson but that name was not validly published until it was taken up by Brandis (1874) by which time Bertoloni (1862) had validly published *Juniperus indica* based on the *same collections* (italics mine) made by Hooker & Thomson in Sikkim". So it appears that Bertoloni and

Brandis used the same collections to name *J. indica* and *J. wallichiana*, respectively!

Farjon (2005) designated the illustration of Bertoloni (1862) as the lectotype of *J. indica* and then designated *J. D. Hooker s.n.*, India, Sikkim, Lachen River, Lachen, K as the lectotype for *J. wallichiana*. Farjon (p 313, 2005) concludes that "There is no doubt that this (*illustration of Bertoloni,* my addition) represents the same species and that *J. wallichiana* by its delayed validation had become superfluous." If, as Farjon indicates, both *J. indica* and *J. wallichiana* were based on the same specimens, then they are indeed the same taxon.

In 1997, Keith Rushforth allowed me (RPA) to collect leaf samples from two trees he cultivated at Abbotsmarsh Arboretum, Devon, England. Adams 8140 (= ex seed from Rushforth 0802, Soe, Bhutan) and Adams 8141 (= ex seed from Rushforth 0899, Lingshi, Bhutan) became the source of putative "*J. wallichiana*, Bhutan" for analyses of terpenes (Adams, 1999) and RAPDs (Adams, 2000). However, in view of Farjon's historical research (2005), these samples should be merely labeled "Rushforth's Bhutan juniper". However, an examination of the leaf oil compositions and DNA fingerprinting showed some differences (Adams, 2000) and led to the recognition of both *J. indica* and *J. wallichiana* (Rushforth's juniper) as separate species in the monograph of *Juniperus* (Adams, 2004). In the key, Adams (2004) keyed *J. indica* as "monecious, shrubs and shrubby trees" versus *J. wallichiana* (Rushforth's juniper) as "dioecious, trees with a strong central axis".

Examination of two Hooker f. *n.s.* specimens from Sikkim at Kew revealed an annotation of "syn type". One of these Hooker f. *s. n.* specimens is apparently the lectotype of *J. wallichiana* designated by Farjon (2005) These Hooker f. specimens match the morphology of *J. indica Adams 7625-7627* from Nepal. In addition, my *J. indica* specimens from Nepal were large shrubs and small trees (to 4 m) which agree with the description of *J. indica* (Farjon, 2005). However, the Rushforth junipers from Bhutan, although very similar in morphology, were monecious and were large trees with a strong central axis. This does not fit any known variety of *J. indica*. Farjon (2005) recognized a new variety, *J. indica* var. *caespitosa* Farjon as a

decumbent or ascending shrub 50-100 cm tall. Foliage branches (nearly) erect, very dense with short branchlets. Seed cones when mature (sub) globose to broadly ovoid, (4.5-) 5-8 x 4 - 6.5 mm, blue-black. Its distribution is in NW Nepal, S Xizang (Tibet), and Bhutan. However, my both my *J. indica*, Nepal specimens and Rushforth's Bhutan juniper specimens have seed cones that are 9-12 mm long and turbinate, not (sub)globose and they are shrubs-small trees or large trees, respectively. So neither my *J. indica,* Nepal nor the Rushforth Bhutan collections are *J. indica* var. *caespitosa*.

Recent DNA sequence phylogenetic research of *Juniperus* (Schwarzbach et al., in prep.) has revealed (Fig. 1) that *J. indica* and

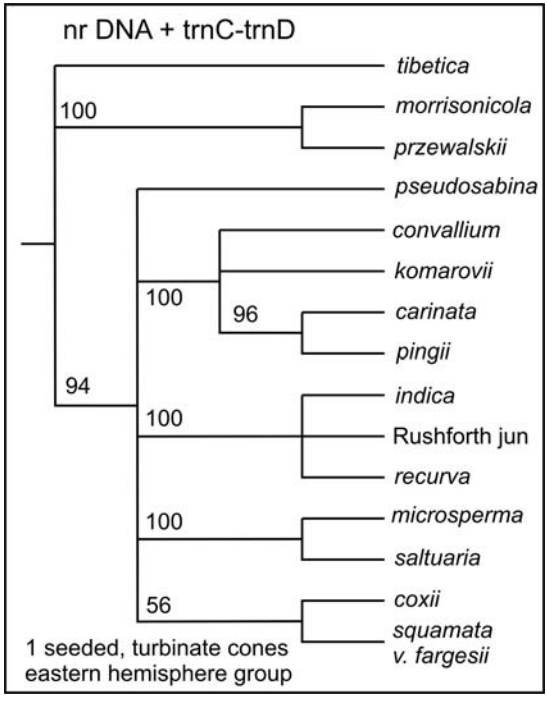

Figure 1. Bayesian tree based on combined nrDNA and cp trnC-trnD sequences from Schwarzbach et al. (in prep.). Notice that *J. indica* and Rushforth's juniper are in a clade with *J. recurva*. Numbers above the branch points are posterior probabilities on a percent basis.

Rushforth's juniper is in a clade with *J. recurva*. The large clade (Fig. 1) contains all the 1-seeded, turbinate-cone *Juniperus* of the eastern hemisphere. Based on this limited sampling, it appears uncertain if Rushforth's juniper is a species distinct from both *J. indica* and *J. recurva*. This result prompted us to broaden the scope of sequencing to look for SNPs in nr DNA and trnC-trnD regions for these three taxa.

Previous work on analyses of the volatile leaf oil compositions of *J. indica* and Rushforth's juniper revealed that their oils are very similar (Adams, 2000). Their oils differ primarily in (*indica*, Rushforth's juniper): α-pinene (2.8%, 9.4%); trans-thujone (16.0, 0.1); trans-sabinyl acetate (15.7, 0.1); trans-murrola-4(14),5-diene (0.9, 3.9); γ-cadinene (0.7, 3.8); 1-epi-cubenol (0.3, 2.4); 8-α-acetoxyelemol (0.0, 0.8); and nezukol (0.0, 4.0). *Juniperus indica* and Rushforth's juniper are very similar in their terpenes (Fig. 2). In fact, most of these differences are in trace components. *Juniperus recurva*,

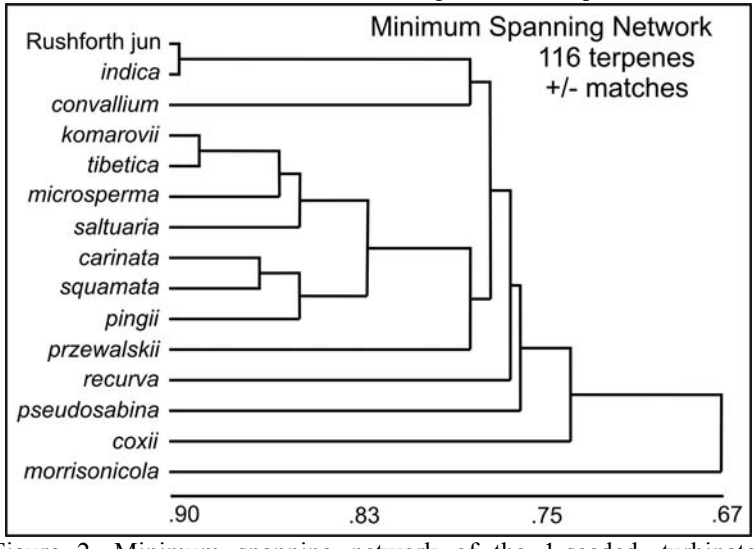

Figure 2. Minimum spanning network of the 1-seeded, turbinate junipers of the eastern hemisphere based on terpenes. Notice that the oils of *J. indica* and Rushforth's juniper are the most similar in this group. From Adams (2000).

although similar in its sequence data (Fig. 1) is quite different in its oils (Fig. 2). Based solely on terpene data, one could treat *J. indica* and Rushforth's juniper as conspecific.

A minimum spanning network based on RAPDs (data from Adams, 2000) shows (Fig. 3) *J. indica* and Rushforth's juniper link loosely, about at the level of other distinct species such as *J. tibetica* and *J. saltuaria*. *Juniperus recurva* is not closely linked to *J. indica* and Rushforth's juniper, in contrast to the sequence data (Fig. 1).

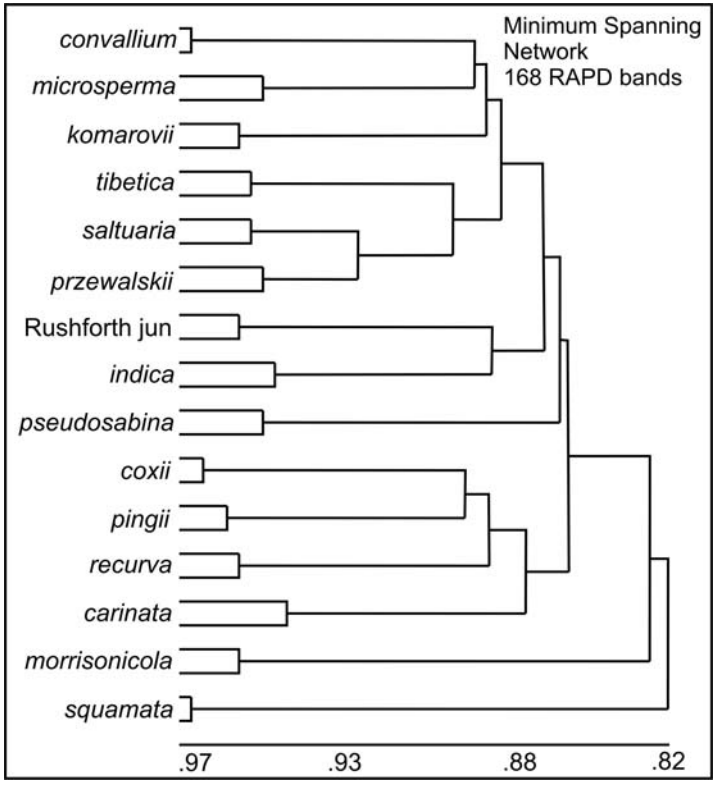

Figure 3. Minimum spanning network based on 168 RAPDs for the 1 seeded, turbinate junipers of the eastern hemisphere. From Adams (2000).

Based on limited morphological (including flowering dates), terpenoid and RAPDs data, Adams (2000, 2004) recognized both *J. indica* and *J. wallichiana* (i.e., *J. wallichiana* for Rushforth's juniper). Of course, unknown to Adams at the time, the name *J. wallichiana* could not be used.

The aforementioned sequence analysis of Schwarzbach et al. (in prep) was based on one accession per species for *J. indica*, *J. recurva* and Rushforth's juniper. As a result, the relationships of the species were established in a basic framework. However, the sampling in this previous study did not allow any assessments on intraspecific variation or the monophyly of the taxa involved. The purpose of this present paper is to re-examine the taxonomy of *J. indica* and Rushforth's juniper using SNPs from sequence data (nrDNA and cpDNA trnC-trnD) by the addition of multiple accessions for each taxon as well as with comparison with the morphologically quite distinct *J. recurva*.

MATERIALS AND METHODS

Specimens used in this study: *J. indica*, *Adams/Chaudary, 7625-7627*, between Yangjin Gompa and Langtang Glacier, 4000 m, Nepal; *J. recurva*, *Adams 7215, 7217-7219*, Sing Gompa, 3570 m, Nepal; Rushforth's juniper, *Rushforth/Adams 8140-8141* ex Bhutan, 11400 ft (*8140*, Rushforth 0802) and 13,250 ft (*8141*, Rushforth 0899), seed germinated and plants cultivated UK. Voucher specimens for all collections are deposited at BAYLU, except *Rushforth 0802, 0899* that are deposited at E.

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 from juniper leaves by use of a Qiagen mini-plant kit (Qiagen DNeasy, Qiagen, Valencia, CA) as per manufacturer's instructions.

SNPs obtained from DNA sequencing

ITS (nrDNA) and trnC-trnD 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 or K (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. All 12 (A-L) of the Epi-Centre's buffers were screened and buffer K gave the cleanest, most abundant amplification for both ITSA/ITSB and buffer E was best for trnC-trnD (CD10F/CD3R) primers. However, buffers D, F, G, H, and J were nearly as good as buffer E or K.

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.).

Amplification and sequencing of *J. indica* proved to be difficult using ITSA/ITSB. So two additional primers were designed based on aligned conifer sequences from GenBank:

ITSA-42F GAT TGA ATG ATC CGG TGA AGT Tm 56° C
42 bp upstream from ITSA into the 18S region.

ITSB+57R ATT TTC ATG CTG GGC TCT Tm 52° C
57 bp downstream from ITSB into the 26S region.

The nrDNA primers (ITSA-42F, ITSB+57R) produced a band of approximately 1210 bp. The internal (partial) trnC-trnD primers, CD10F-CD3R produced a band of: 776 bp for Rushforth's juniper, 775 bp for 2 individuals of *J. indica* (1 deletion); and 770 for one individual (the aforementioned deletion, plus a string of 5bp unique deletion), 3 individuals of *J. recurva* contained 775 bp with no variation in their sequences. In contrast, the sample of *J. indica* 7218 had several insertions not found in any sample in this study: a 57 bp, 21 bp, and a 1 bp. In addition, 7218 had a single SNP shared by all plants in this study. Due to these factors, 7218 was omitted from the partial trnC-trnD data analysis.

The following PCR conditions were used: MJ Research Programmable Thermal Cycler, 30 cycles, 94°C (1 min.), 50°C (2 min.), 72°C (2 min.), with a final step of 72°C (5 min.). 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. 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

Sequencing nrDNA (ITS region) resulted in: 1210 bp of data for each *J. indica* sample; 1209 bp for each Rushforth's juniper sample (1 bp deletion); and 1179 bp for each *J. recurva* sample (29 bp deletion only found in *J. recurva*, coded as a single SNP; 1 bp deletion only found in *J. indica*, and 1 bp deletion shared in *J. indica* and Rushforth's juniper samples). Aligning sequences of *J. indica* (3 individuals), *J. recurva* (4 individuals) and Rushforth's juniper (2 individuals) revealed 10 SNPs among these individuals (the 29 bp indel was treated as a single SNP). PCO of these individuals gave three eigenroots accounting for 64.9%, 23.4% and 6.0% of the variance among

individuals. PCO ordination shows (Fig. 4) three groups: *J. indica* (no variation among individuals), *J. recurva* and Rushforth's juniper. *Juniperus indica* and Rushforth's juniper are separated by 3 SNPs, whereas *J. recurva* is separated from *J. indica* by 5 SNPs. One SNP was found between 2 individuals of *J. recurva* (Fig. 4) and one SNP was found between the 2 Rushforth's juniper individuals (Fig. 4). These differences are comparable to those found between Caribbean junipers (Adams et al., 2008) of 3-7 SNPs; the nw US junipers (Adams, 2007) of 4-5 SNPs; and the Mediterranean junipers (Adams et al., 2005) of 6-8 SNPs.

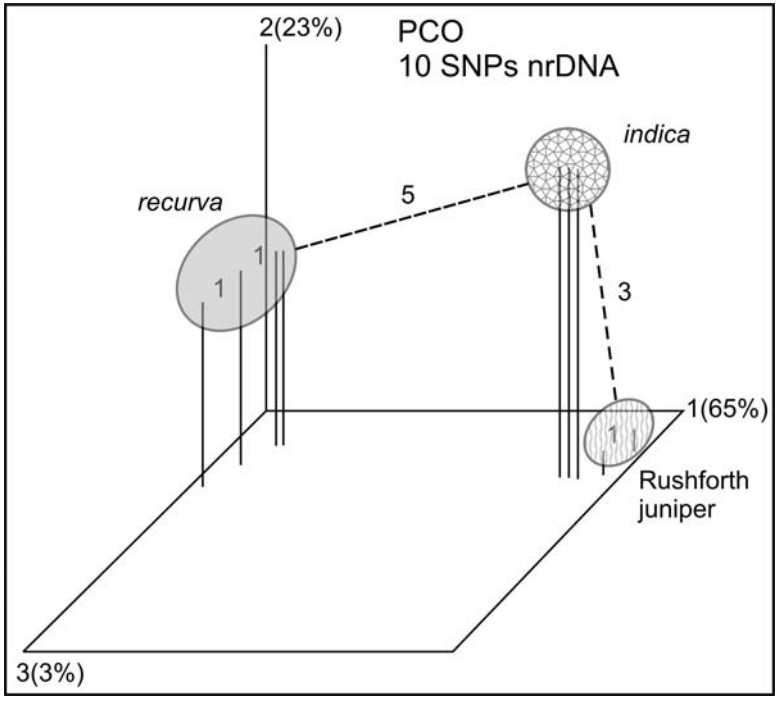

Figure 4. PCO based on 10 nrDNA SNPs. Dashed lines show the minimum linkage between groups. Numbers above the dashed lines are the number of SNP events separating the groups. Closely spaced lines denote identical DNA sequences.

Sequencing and aligning the partial trnC-trnD sequences revealed 5 SNPs. Factoring the association matrix resulted in 3 eigenroots that accounted for 67.1%, 24.7% and 6.0% of the variance among these individuals in their partial trnC-trnD SNPs. Ordination of these individuals revealed (Fig. 5) the three taxa to be equally separated but by only 2 SNPs. There was no variation within *J. recurva* and Rushforth's juniper, but one SNP was present within *J. indica* (Fig. 5).

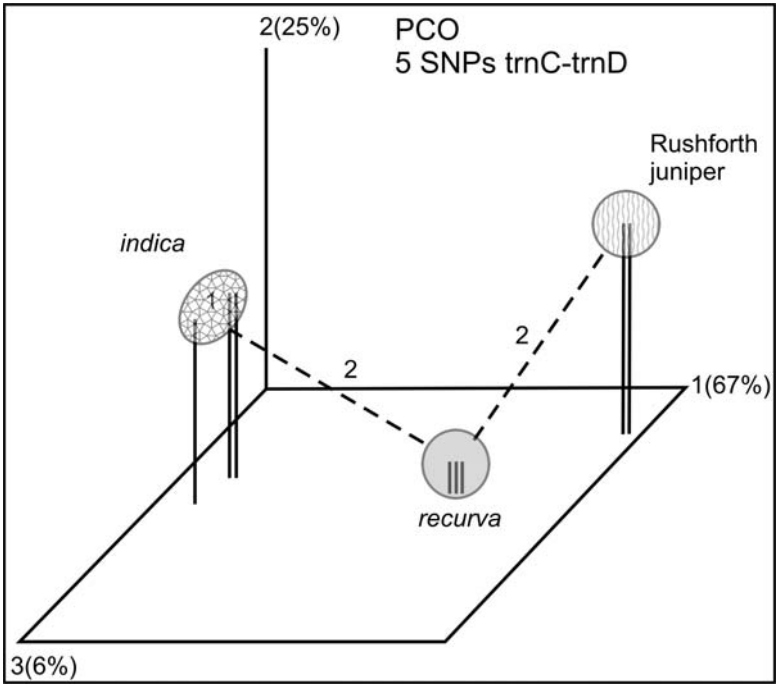

Figure 5. PCO ordination based on 5 SNPs from trnC-trnD sequences. Dashed lines show the minimum linkage between groups. Numbers above the dashed lines are the number of SNP events separating the groups. Closely spaced lines denote identical DNA sequences.

These trnC-trnD SNPs differences are similar to those found between Caribbean junipers (Adams et al., 2008) of 0 - 1 and 5 SNPs.

The trnC-trnD SNPs failed to separate *J. maritima* and *J. virginiana* in the nw US junipers (Adams, 2007), but *J. scopulorum* and *J. virginiana* were separated by 7-9 SNPs.

Combining the 10 nrDNA SNPs and 5 trnC-trnD SNPs for a PCO analysis resulted in 3 eigenroots of 51.2%, 40.6% and 3.0%. Ordination (Fig. 6) shows that *J. indica* and Rushforth's juniper are

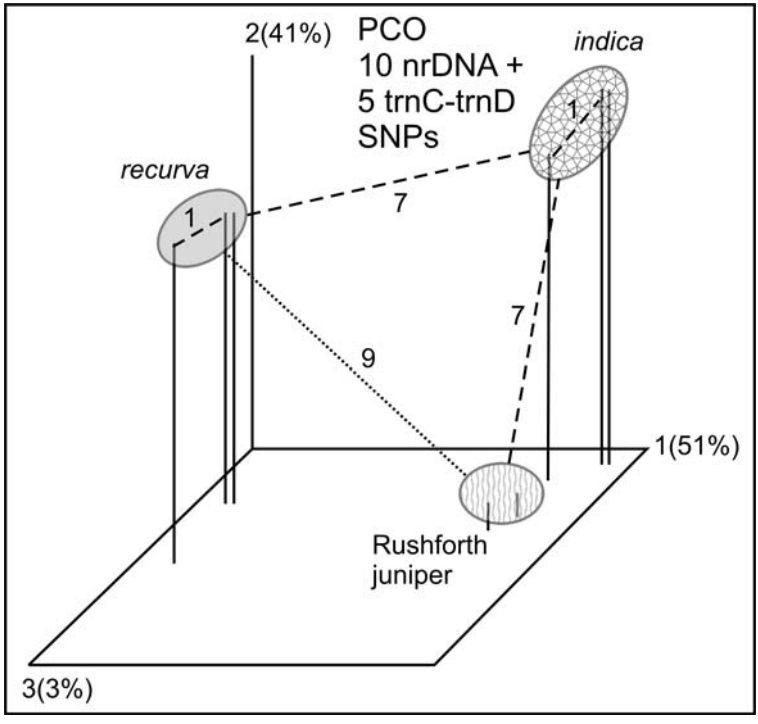

Figure 6. PCO using SNPs from both nrDNA and trnC-trnD. Dashed lines show the minimum linkage between groups. The dotted line shows the linkage between *J. recurva* and Rushforth's juniper is 9 SNPs. Numbers above the dashed lines are the number of SNP events separating the groups. The closely spaced lines denote identical DNA sequences.

separated by 7 SNPs as are *J. indica* and *J. recurva*. In contrast, *J. recurva* and Rushforth's juniper are separated by 9 SNPs. These differences are about the same as found (Adams et al., 2008) between *J. excelsa* and *J. polycarpos* varieties (7 - 9 SNPs).

To summarize the data bearing on the taxonomic status of *J. indica* and Rushforth's juniper: Terpenes - favor a very close relationship (Fig. 2) perhaps at the infraspecific level; RAPDs - indicate more differentiation at the variety or specific level (Fig. 3); combined SNPs from nr DNA plus partial trnC-trnD support *J. indica* and Rushforth's juniper as distinct species (Fig. 6). The morphology supports *J. indica* and Rushforth's juniper being conspecific.

Based on the composite of all these data to date, I propose a new varietal name for Rushforth's Bhutan juniper:

Juniperus indica Bertol. **var. *rushforthiana*** R. Adams, **var. nov.** Rushforth's juniper, Type: Bhutan, Soe, at Soe Tajitang campsite, tree 15 m, 11,400 ft., *Rushforth 0802 (= Adams 8140)* (HOLOTYPE: BAYLU).

Junipero indicae similis sed sexu dioecio; arbores axe centrali valido.

Similar to *J. indica*, except this variety is dioecious, trees with a strong central axis.

Distribution: Bhutan, also, likely to occur in neighboring Xizang (Tibet) at 11,000 ft to timberline.

Other specimens: *Rushforth 0899* (*=Adams 8141*), Bhutan, Lingshi, on path to Yale La, 13,250 ft, coppiced 2 m.

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