

Analysis of decurrent-leaved Himalayan junipers: Discordance between leaf morphology and DNA**Robert P. Adams**Biology Department, Baylor University, Box 97388, Waco, TX 76798, USA email
Robert_Adams@baylor.edu**Andrea E. Schwarzbach**

Department of Biomedicine, University of Texas at Brownsville, Brownsville, TX 78520, USA.

Keith Rushforth

The Shippen, Ashill, Cullompton, Devon EX15 3NL UK

and

Ram P. ChaudharyExecutive Director, Research Centre for Applied Science and Technology (RECAST), Tribhuvan
University and Professor, Central Department of Botany, TU, Kathmandu, Nepal**ABSTRACT**

Several accessions of decurrent-leaved junipers of the turbinate seed cone taxa of *Juniperus* section were DNA bar-coded using data from nrDNA, and 4 cp regions in an attempt to identify unusual specimens of *J. coxii*, *J. fargesii*, *J. squamata*, and *J. recurva* from both wild and cultivated sources. The variation in the size, shape and branching angles of decurrent leaves was incongruent with the DNA bar-coding data. Specimens from these taxa differ chiefly in leaf morphology. The plasticity of decurrent leaves, neoteny and hybridization appear to make the use of only leaf type data unreliable for identification of these questionable or difficult specimens. Published on-line: www.phytologia.org *Phytologia* 95(2):125-131 (May 1, 2013).

KEY WORDS: *Juniperus*, Cupressaceae, DNA bar-coding, decurrent leaves.

The turbinate seed cone *Juniperus*, section *Sabina* contain a group of closely related taxa with decurrent leaves (Adams and Schwarzbach, 2012, 2013). *Juniperus* has at least three types of leaves: acicular (as found in spruce, fir, etc.) where the entire leaf drops from the stem (Fig. 1, left); decurrent (whip) leaves with a sheath that clasps the stem and a blade that extends outward from the blade at various angles (Fig. 1, center); and scale leaves with tips that are usually appressed to the next scale leaf on the stem (Fig. 1, right). In section *Sabina*, decurrent leaves are always present on a seedling and the plant continues to make these juvenile (decurrent) leaves for several years. Most junipers in sect. *Sabina* begin the production of scale-like leaves (adult leaves) after a few years and then decurrent (whip) leaves are only found at the tips of rapidly growing branches.

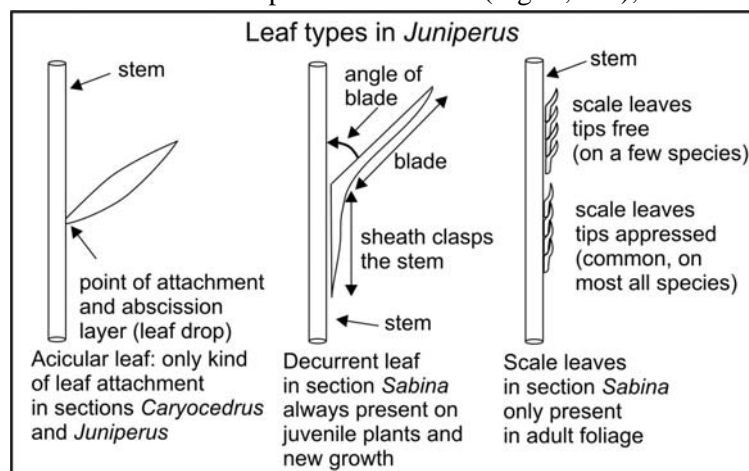


Figure 1. Leaf types in *Juniperus*.

However, for nearly every species of sect. *Sabina*, the senior author has observed a few mature (adult) trees in a population that are frozen in neoteny that have only juvenile (decurent or whip) leaves. *Juniperus chinensis* is very unusual in that it has both decurrent and scale-like leaves interspersed on branches in mature (adult) trees. *Juniperus saxicola* (now recognized as *J. gracilior* var. *saxicola* Britt. & Wils.) is a taxon that is frozen in neoteny and has only juvenile (decurent) leaves. Another Caribbean juniper (*J. barbadensis* var. *barbadensis*) has several mature (adult) trees in the little population at the summit of Petit Piton, St. Lucia, BWI that have only decurrent (juvenile) leaves (Adams, 2011).

It appears that several taxa in the turbinate group also exhibit neoteny (Fig. 2, *J. squamata*, *J. s. var. wilsonii*, *J. fargesii*, *J. morrisonicola*, *J. recurva* and *J. coxii*). Although these taxa appear distinct in the photos in Figure 2, in nature and cultivation, there is tremendous variation in size, shape and blade angle of their leaves. This has presented considerable difficulty in the identification of specimens from these taxa.

The purpose of this paper is to report on DNA bar-code analysis of 'difficult' specimens that have been tentatively identified to determine if DNA bar-coding could aid in classifying these 'difficult' specimens.

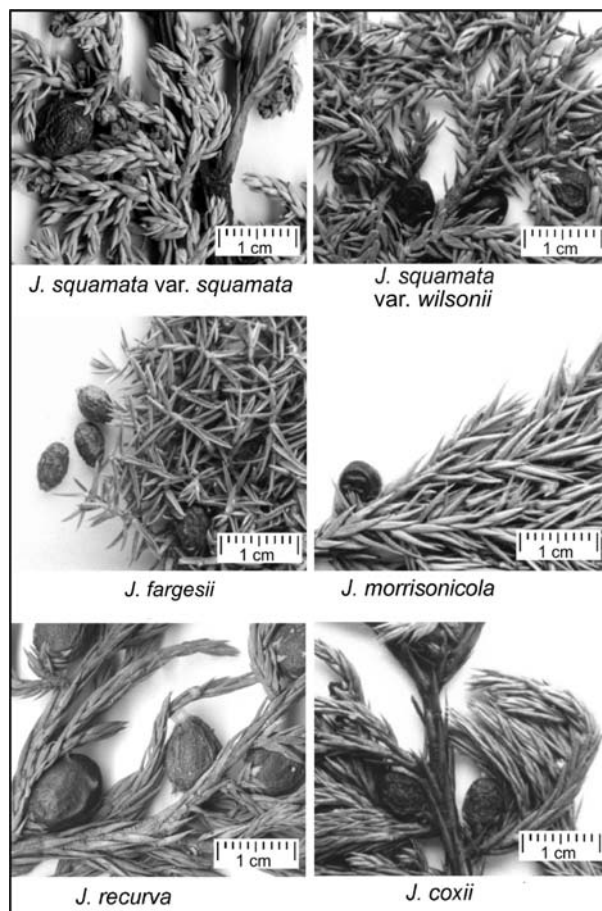


Figure 2. Leaves and seed cones of selected *Juniperus* species.

MATERIALS AND METHODS

Specimens used in this study: *J. carinata*, Adams 8497-99, near White Horse Mtn., Deqin County, Yunnan, China, *J. convallium*, Adams 6781-83, 17 km e Tewo, Gansu, China, *J. convallium* f. *pendula*, Adams 6779, Langmusi, Gansu, China, *J. communis* L. var. *communis*, Adams 7846, 7847, Stockholm, Sweden (outgroup), *J. coxii*, Adams 8137-38, clone from Type tree, Abbotsmarsh Arboretum, UK, ex Burma, Chimili valley, *J. fargesii*, Adams 6769-6770, Lian Wha Mtn., s of Kangle, Gansu, China, *J. indica*, Adams 8775, Dumpa, Jomson, Nepal, *L N Sharma J2, J16*, Dhikur Pohkari, Manang, Nepal, *J. indica* var. *caespitosa*, Adams 7625-27, near Yangjin Gompa, Nepal, *J. komarovii*, Adams 8518-20, near Zhe Gu Mtn., Maerkan County, Sichuan, China, *J. microsperma*, Adams 8522-24, near Zhe Gu Mtn., Maerkan County, Sichuan, China, *J. morrisonicola*, Adams 8681-2, Younger Bot. Gard., Scotland, ex Taiwan, *J. pingii*, Adams 8506-7, near White Horse Mtn., Deqin County, Yunnan, China, *J. pingii* var. *miehei*, Boufford, Kelley, Ree & Wu 29745, (acc. 13598) Baxoi Xian, Anjiu La (pass), Xizang (Tibet), China, *J. pseudosabina*, Adams 7808-10, 30 km n Jarkent (Paniflor), Kazakhstan, *J. przewalskii*, Adams 6775-77, 25 km w Jone, Gansu, China, *J. recurva*, Adams 7215, 7219, Cholan Pati lodge, Nepal, *L N Sharma GJ14, GJ15*, (acc. 12140, 12141), Mobro Madi, Nepal, *J. rushforthiana*, Adams 8140-41, Abbotsmarsh Arboretum, UK, ex Lingshi, Bhutan *J. saltuaria*, Adams 6789-91, on Duoer River, 23 km e Forestry Station, Gansu, China, *J. squamata*, Adams 6796, Xian Bot. Garden, Shaanxi, China, *J. squamata* cv. *Meyeri*, Adams 13547, Arnold Arboretum acc. 10316*A, *J. tibetica*, Adams 8512-16, on

Maerkan River, near Zhe Gu Mtn., Maerkan County, Sichuan, China, *J. uncinata*, Adams 7212-14, Lauri Binayak, Nepal.

Specimens of uncertain affinity:

F3, F4, 'fargesii', Adams 8491-93, near White Horse Mtn., Zhongdian County, Yunnan, China,

F6, 'fargesii', *K. Rushforth 3704*, (acc. 13505) Nyima La, descent into Rong Valley at Tumbatse, Tibet, cult. in UK,

F7, 'fargesii', *Chadwell 6137*, (acc. 13506) Nyima, Annapurna Himalayas, Nepal, cult. in UK,

F8, 'fargesii', *K. Rushforth 8258*, (acc. 13507) on s side of Jira La, Arunachal Pradesh state, India, cult. in UK,

P2, 'pingii', *D. Boufford 37031*, (acc. 11767), seeds, Sichuan, China,

S3, 'squamata', *K. Rushforth 5405A*, (acc. 8287), Nyima La, near 47 campsite, Xizang (Tibet), China,

S4, S5, 'squamata', *L N Sharma J2GJ10, GJ11*, (acc. 12936, 12937), Nyarku, Nepal,

S6, 'squamata', Adams 7012, Kunming Botanic Garden, Kunming, China,

S7, 'squamata', *K. Rushforth 907*, (acc. 13504), on route from Linghsi Dzong to Yale La, Bhutan, cult. in UK,

U3, 'uncinata', *K. Rushforth 9512*, (acc. 13508), near Jang Gomba campsite, India, cult. in UK,

W1, '*J. squamata* f. *wilsonii*', Adams 5521, Accession 1010-64A, cultivated from seeds from *E. H. Wilson* 985 (Holotype) collection, Arnold Arboretum, USA, ex. China,

W4, 'wilsonii or 'pingii'', *K. Rushforth 3853*, (acc. 12912), n of Lhasa, Xizang (Tibet), China, cult. in UK, Voucher specimens are deposited in the herbarium, BAYLU, Baylor University.

DNA extraction, PCR amplification, sequencing and data analyses

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

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, trnD-T, trnL-F, trnS-G) 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 MgCl₂ 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 primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010).

The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing 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.) or Sequencher v. 5 (genecodes.com). Sequence datasets were analyzed using Geneious v. 5.4 (Drummond et al. 2011), the MAFFT alignment program and the PAUP* program, version 4.0b10 (Swofford 2003) for neighbor joining, parsimony, and maximum likelihood tree searches. Further analyses utilized the Bayesian analysis software Mr. Bayes v.3.1 (Ronquist and Huelsenbeck 2003). For phylogenetic analyses, appropriate nucleotide substitution models were selected using Modeltest v3.7 (Posada and Crandall 1998) and Akaike's information criterion. Minimum spanning networks were constructed from mutational events (ME) data using PCODNA software (Adams et al., 2009; Adams, 1975).

RESULTS AND DISCUSSION

The overall pattern of variation (Fig. 3) is the same as shown by Adams and Schwarzbach, 2012, 2013). Our points of interest are in the groupings that contain the ‘difficult’ specimens. Specimens collected as ‘fargesii’, with leaf blades diverging from the stem by 45° - 90° are scattered in: Group I (F3,

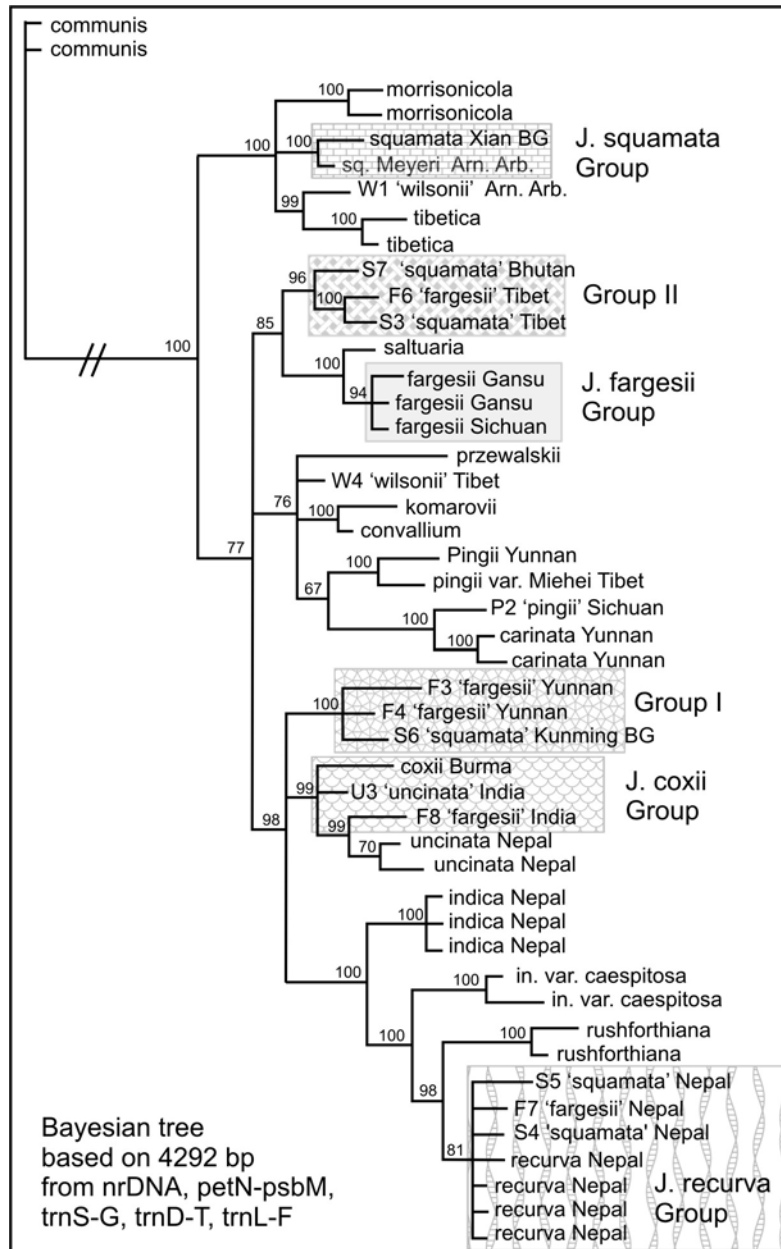


Figure 3. Bayesian tree showing the ‘difficult’ specimens in relation to other members of the turbinate seed-cone taxa of sect. *Sabina*. The divergent species, *J. pseudosabina* is not shown. Numbers at the branch points are posterior probabilities (as percent). Groups are discussed in the text.

F4), Group II (F6) *J. coxii* Group (F8) and *J. recurva* Group (F7). Specimens referred to as ‘squamata’, with leaf blades mostly appressed are found in: Group I (S6), Group II (S3, S7) and *J. recurva* Group (S4, S5). The two specimens of ‘wilsonii’ are in a clade with *J. tibetica* (W1) and in a clade with *J. pingii*, *J. komarovii*, etc. (W4).

Utilizing the large amount of indel information as well as substitutions gives a minimum spanning network with a slightly different perspective (Fig. 4).

***Juniperus fargesii* group**

Three of the *J. fargesii* individuals (*Adams 6769, 6770, Gansu; Adams 8521, Sichuan*) displayed no variation in their DNA bar-codes.

Notice the variation in blade angle from nearly 90° in the Gansu specimens to about 45° in the Sichuan specimen (Fig. 5).

Several other ‘difficult’ specimens collected as ‘fargesii’, are scattered about the network: F3, F4 is in Group I; F8 ‘fargesii’ India is in the *J. coxii* group; F6 ‘fargesii’ Tibet is in Group II (Fig. 4); and F7 ‘fargesii’ Nepal is in the *J. recurva* group.

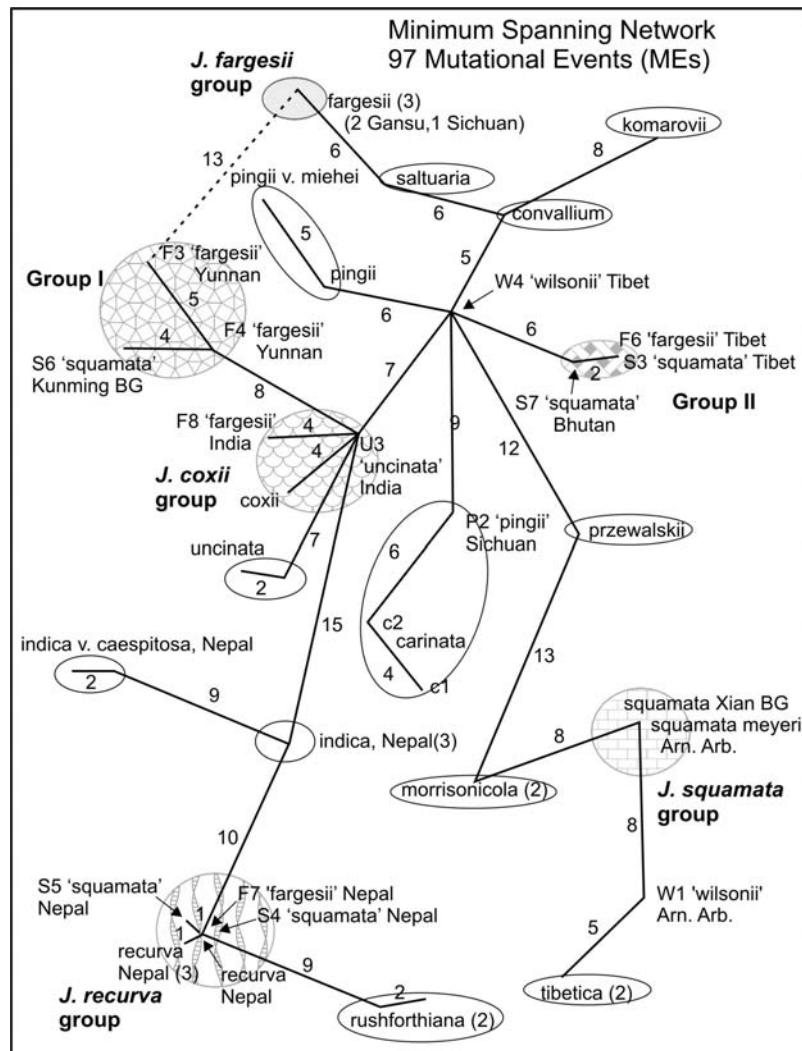


Figure 4. Minimum spanning network based on 97 MEs. Numbers on lines are the number of MEs. Open ovals enclose currently recognized species and varieties.

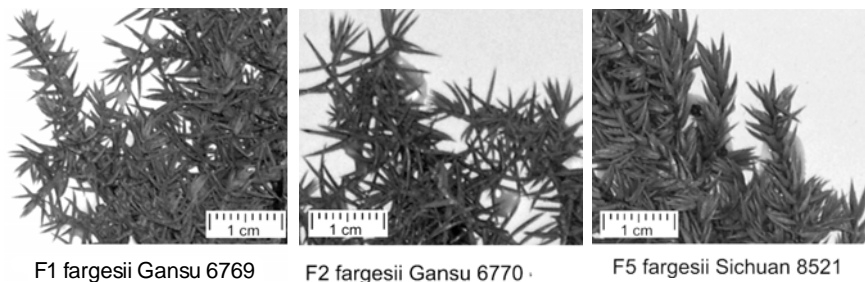


Figure 5. *J. fargesii* group: Gansu, 6769 and 6770; Sichuan 8521.

***Juniperus coxii* group: *J. coxii*, Yunnan, 8508, F8 ‘fargesii’ India, KR8258, and U3 ‘uncinata’ India, KR9512,**

This group is linked to *J. coxii*, Yunnan by ‘uncinata’ KR9512, India (4 MEs, Fig. 3) and ‘fargesii’ KR8258, India (4 MEs, Fig. 3.). *Juniperus coxii* has only decurrent leaves with the blades tightly appressed to the stem (Fig. 6, left). The photos of both ‘fargesii’ KR8258 and ‘uncinata’ KR9512 are from plants cultivated in the UK. Their foliage may be still juvenile.



Figure 6. *J. coxii* group: *J. coxii*, Yunnan; F8 ‘fargesii’ India, KR8258, cultivated in UK; and U3 ‘uncinata’ India, KR9512, cultivated in UK.

***Juniperus recurva* group: *J. recurva*, Nepal 7219, S5 ‘squamata’ Nepal 12937; and F7 ‘fargesii’ Nepal, Chadwell 6137.**

Juniperus recurva has decurrent leaves with the blades tightly appressed. However, ‘squamata’ Nepal Sharma 12937; and ‘fargesii’ Chadwell 6137, Nepal differed by only 1 or 2 MEs from the typical *J. recurva*. Yet, their leaves are quite different from *J. recurva* (Fig. 7). The photo of ‘fargesii’ Chadwell 6137, Nepal (Fig. 7) is from cultivated material and the foliage may be juvenile.



Figure 7. *J. recurva* Group: *J. recurva*, Nepal 7219, S5 ‘squamata’ Nepal 12937; F7 ‘fargesii’ Nepal, Chadwell 6137, cultivated in UK.

***Juniperus squamata* Group: *J. squamata* Xian Bot. Gard. 6795 and *J. squamata* cv. *Meyeri*, Arn. Arb, 13547.**

This group contains two specimens that had no DNA differences (*J. squamata*, Xian Bot. Gard., 6795, *J. squamata* var. *Meyeri*, Arn. Arb., 13547). These specimens are very similar morphologically (Fig. 8) and are similar to the type. These *J. squamata* specimens are linked by a difference of 8 ME to *J. morrisonicola* and W1 ‘wilsonii’. W1 ‘wilsonii’ from Arnold Arboretum looks very much like the type specimen, however, it differs by only 5 MEs from *J. tibetica* (shown with adult leaves in Fig. 8, right-most). It is surprising that none of the putative *J. squamata* in this study had DNA like *J. squamata* from Xian Botanic Garden.



Figure 8. *J. squamata* Group: *J. squamata*, Xian Bot. Gard.; *s. cv. Meyeri*, Arn. Arb.; plus W1 '*wilsonii*', Arn. Arb. 5521 and *J. tibetica* that differs by only 5 MEs from W1 (see Fig. 4).

Group I: F3 'fargesii' Yunnan, 8491, F4 'fargesii' Yunnan, 8492 and S6 'squamata' cultivated at Kunming Botanic Garden, 7012.

Group I is not very uniform in its DNA (Fig. 3), nor in its morphology. The two 'fargesii' from Yunnan have large blade angles (Fig. 9) that would seem typical of *J. fargesii* (Fig. 4), but note that 8491 is separated by 13 MEs from typical *J. fargesii* (6769, Fig. 3). The cultivated 'squamata' 7012 from KGB is only 4 MEs from 'fargesii' 8492 (Fig. 3), and its blade tips are quite appressed (Fig. 9).

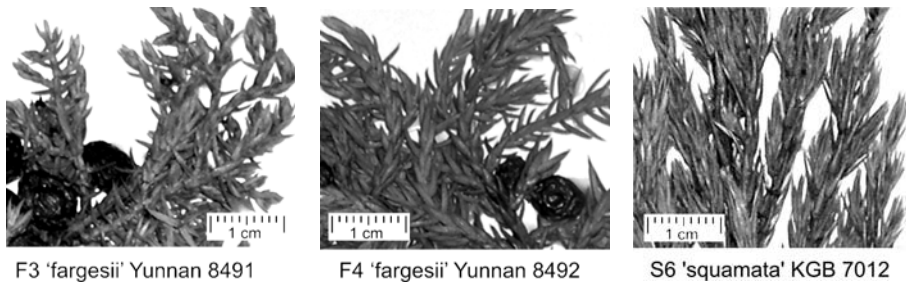


Figure 9. Group I: F3 'fargesii' Yunnan 8491, F4 'fargesii', Yunnan 8492 and S6 '*squamata*', cultivated at Kunming Botanic Garden, 7012

Group II. F6 'fargesii', Tibet, KR3704, S7 'squamata', Bhutan, KR907 and S3 'squamata', Tibet, KR5405A.

The DNA of this group is most like that of *J. convallium* (Fig. 3). Group II is diverse in both DNA and morphology (Fig. 10). However, photos of specimens of F6 'fargesii', KR3704, Tibet and S7 'squamata', Bhutan, KR907 are from materials cultivated in the UK and may have juvenile leaves.



Figure 10. **Group II:** F6 'fargesii', Tibet KR3704, cultivated in UK, S7 'squamata', Bhutan, KR907, cultivated in UK and S3 'squamata', Tibet KR5405A.

The presence of Groups I and II, not clearly allied with any known species suggests that these may be cryptic species that have not previously been recognized, or problems of classifying these 'difficult' specimens may be a result of incomplete lineage sorting. Degnan and Rosenberg (2009) defined incomplete lineage sorting as "failure of two or more lineages in a population to coalesce, leading to the possibility that at least one of the lineages first coalesces with a lineage from a less closely related population". Yu et al. (2011, 2012) recently addressed the problems of extensive hybridization in nature and incomplete lineage sorting, and their effects on phylogenetic trees and networks.

Adams et al. (2009) found hybridization between *J. recurva* and *J. uncinata* in Nepal. It also seems very likely that some of the 'difficult' specimens analyzed in this study are hybrids or backcrosses. This could be a major limitation in applying DNA bar-codes to identify 'difficult' specimens with our current numerical methods. This is particularly true in this (and many DNA studies) where most of the data is from cp DNA that is uni-parentally inherited.

Overall, this exercise in DNA bar-coding these taxa left much to be desired. The discordance of bar-codes and morphology renders the study of little practical use. Field identification was poorly correlated with assignment to species by DNA bar-coding. The plasticity of decurrent leaves, neoteny and hybridization appear to make the use of only leaf type data as unreliable for identification of these taxa.

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