

Taxonomic reassessment of some Central Asian and Himalayan scale-leaved taxa of *Juniperus* (*Cupressaceae*) supported by random amplification of polymorphic DNA

Robert P. Adams¹ & Yerlan Turuspekov²

Summary

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Analysis of central Asian *Juniperus* using RAPD revealed that *J. centrasiatica*, *J. turkestanica*, and *J. pseudosabina* appear to belong to a single species, to be named *J. pseudosabina*. This conclusion is also supported by previous work on terpenoids. Putative *J. indica* from Nepal (shrub form) was found to be distinct from *J. pseudosabina*. It appears that the common scale-leaved shrub or tree juniper of the Himalayas should be called *J. indica* not *J. pseudosabina*.

Introduction

The taxonomy of *Juniperus* of Central Asia has recently been re-examined (Farjon, 1992; Silba, 1986, 1990), all of the revisions being based on morphological data from herbarium specimens. The present study is focused on three central Asian junipers: *J. centrasiatica* Kom. (type locality southeast of Kasgar, Xinjiang, China, close to CX, Fig. 1), *J. pseudosabina* Fisch. & al. (type locality from the Tarbagatai Mts, north of PK in Fig. 1), and *J. turkestanica* Kom. (restricted to this central Asian region). A fourth taxon, *J. indica* Bertol., which was treated as a synonym of *J. pseudosabina* in the flora of Bhutan (Grierson & Long, 1983), was collected from Nepal and included in this study. Finally, in order to better judge the relative similarities of the aforementioned taxa, *J. sabina* L., a widely distributed, distinct species ranging from Spain into China and Mongolia, was included in the analysis.

A previous RAPD analysis of *Juniperus pseudosabina*, *J. sabina*, and *J. turkestanica* (Adams & Demeke, 1993) utilised plants collected from the Almaty Botanic Garden. Subsequent work has revealed that the plants named "*Juniperus pseudosabina*" were actually a cultivar of *J. sabina*. In addition, the cultivated *J. sabina* plants analysed were not typical wild *J. sabina* of central Asia. In this study, we used only materials collected from wild, natural populations, with the aim of resolving the relationships between *J. centrasiatica*, *J. turkestanica*, and *J. pseudosabina*, and their relationship to *J. indica* (shrub form, Nepal).

Materials and methods

Specimens were collected as follows: *Juniperus centrasiatica*, CX: China, Xinjiang, Oetak, 125 km S. of Kasgar, 2980 m, 28 Jul 1996, Adams 7820-7825;

¹ Plant Biotechnology Center, Baylor University, Box 669, Gruver, TX 79040, U.S.A.

² Plant Genetics Department, Institute of Plant Physiology, Genetics & Bioengineering, 45 Timirjazev Str., 480070 Almaty, Kazakstan.

J. turkestanica, TK: Kazakstan, 35 km E. of Turgen, 2400 m, 19 Jul 1996, Adams 7817-7819; TX: same location as CX, 28 Jul 1996, Adams 7829-7831; *J. pseudo-sabina*, PK: Kazakstan, 30 km N. of Jarkent (Panifilov), 2000 m, 18 Jul 1996, Adams 7808-7810; PM: Mongolia, Altai Mts, 2550 m, 19 Aug 1995, Adams 7592-7595; PX: China, Xinjiang, Tian Shan, 40 km S. of Fukan City, above Heaven Lake, 2120 m, 1 Aug 1996, Adams 7833-7835; *J. sabina*, SX: same location as PX, 1 Aug 1996, Adams 7836-7838. Voucher specimens are deposited at SRCG.

The identifications of *Juniperus centrasiatica*, *J. turkestanica*, and *J. pseudo-sabina* were based on comparison with type and isotype materials examined and photographed during a visit by R.P.A. to LE, viz.: *J. centrasiatica*: 1899, Roborovski (syntype); *J. turkestanica*: 22 Apr 1905, lectotype, Pačoveova; *J. pseudo-sabina*: specimen 1. coll./annotated by Komarov as *J. pseudosabina*, from Tarbajatai Mts, 1841. The identification of *J. indica* was based on comparison with a photographic image of the type: 1839, Bertoloni (BOLO). The identification of *J. sabina* is based on comparison with the Linnean type (LINN).

Leaves were desiccated in silica gel (Adams & al., 1992) in the field. DNA was extracted using the hot CTAB protocol (Doyle & Doyle, 1987) with the addition of 1% (w/v) PVP and Pronase E (150 µg). PCR was performed in a volume of 15 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 2.0 mM MgCl₂, 0.01% gelatine and 0.1% Triton X-100, 0.2 mM of each dNTP, 0.36 µM primers, 0.3 ng genomic DNA, 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.

Ten-mer primers (Table 1) were used that gave several bright bands, did not have any false bands (in the controls), and yielded reproducible results in replicated analyses. 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 for final extension: 38°C (2 min) and 72°C (5 min). Amplification products were analysed by electrophoresis on 1.5% agarose gels and detected by staining with ethidium bromide. The gels were photographed under UV light with Polaroid film 667. pGEM DNA (Promega) was used as a molecular weight marker. The RAPD bands were scored by molecular weight and assigned a code based on primer number prefix and molecular weight category. In addition, the RAPD band intensity was scored as: 0 = no band; 4 = faint; 5 = medium; 6 = bright band, in reference to a grey tone standard. Previously (Adams & Demeke, 1993), we scored bands in 6 classes but changed to 4 classes for this study as this is easier and still preserves subtle information that we feel would be lost if just presence/absence scoring were utilised. Data were also analysed as presence/absence, but there seemed to be more resolution using quantitative scoring than merely using qualitative scoring.

The data were coded into a matrix of taxa by character values. 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). Division by the character state range was tried and found to be less informative than using the maximum observed character value (i.e., including zero in the range). Principal co-ordinate analysis (PCO) of the similarity matrix follows Gower (1966).

Table 1. List of the primers used in this study for the random amplification of polymorphic DNA (RAPD) by PCR.

| Code | Sequence (5'-3') | Code | Sequence (5'-3') |
|------|------------------|------|------------------|
| 131 | GAA ACA GCG T | 249 | GCA TCT ACC G |
| 134 | AAC ACA CGA G | 250 | CGA CAG TCC C |
| 184 | CAA ACG GAC C | 268 | AGG CCG CTT A |
| 212 | GCT GCG TGA C | 327 | ATA CGG CGT C |
| 218 | CTC AGC CCA G | 338 | CTG TGG CGG T |
| 234 | TCC ACG GAC G | 346 | TAG GCG AAC G |
| 239 | CTG AAG CGG A | 347 | TTG CTT GGC G |

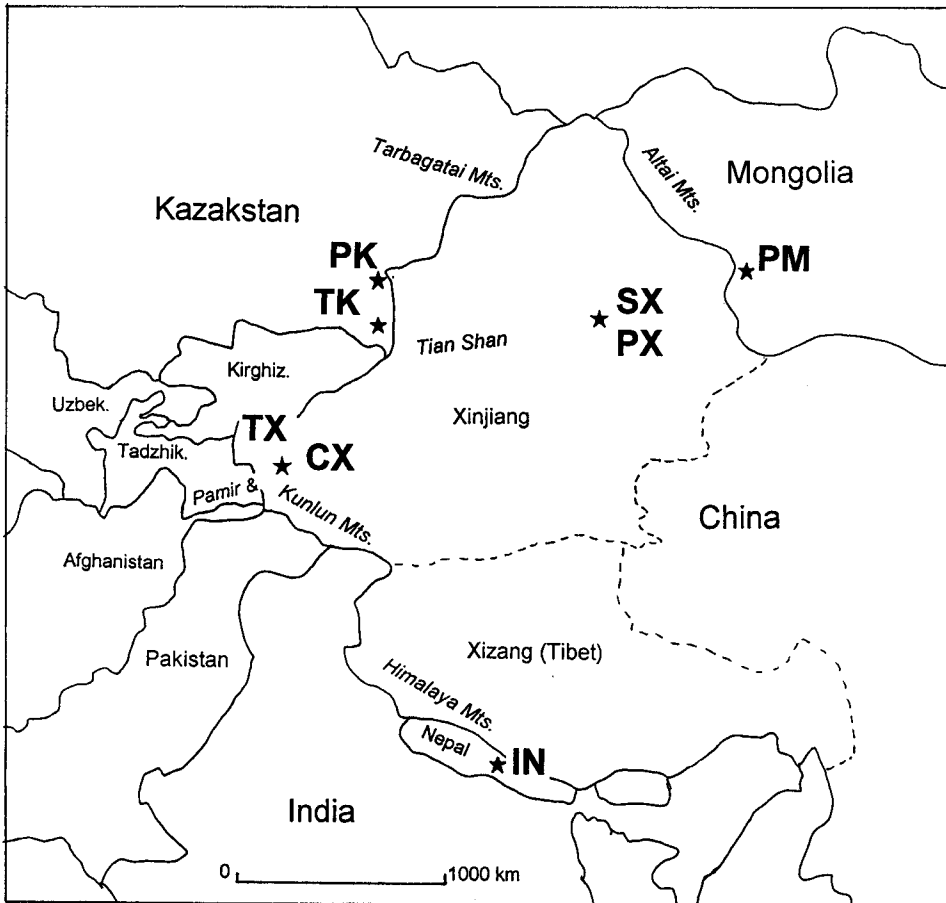


Fig. 1. Location of populations of *Juniperus* collected. – PK = *J. pseudosabina*, Kazakstan; PM = *J. pseudosabina*, Mongolia; PX = *J. pseudosabina*, Xinjiang, China; TK = *J. turkestanica*, Kazakstan; TX = *J. turkestanica*, Xinjiang, China; CX = *J. centrasiatca*, Xinjiang, China; SX = *J. sabina*, Xinjiang, China; IN = *J. indica*, Nepal.

Results

The first nine eigenroots extracted 35.7%, 15.2%, 9.1%, 6.3%, 5.6%, 4.7%, 4.6%, 3.9%, and 3.2% of the variance among the fifteen OTUs. The eigenroots appear to asymptote after the first four roots. Ordination using the first three co-ordinate axes reveals several patterns (Fig. 2). All of the samples of *Juniperus centrasiatica*, *J. pseudosabina*, and *J. turkestanica* cluster together very tightly (0.85-0.92). *J. indica* is distinct from the *J. pseudosabina* complex (Fig. 2; 0.77 similarity to the nearest neighbour). *J. sabina* was the most distinct, having only a 0.56 similarity to the nearest neighbour in the *J. pseudosabina* complex (Fig. 2). The fourth co-ordinate axis (not shown) tended to separate *J. pseudosabina* from all the other OTUs.

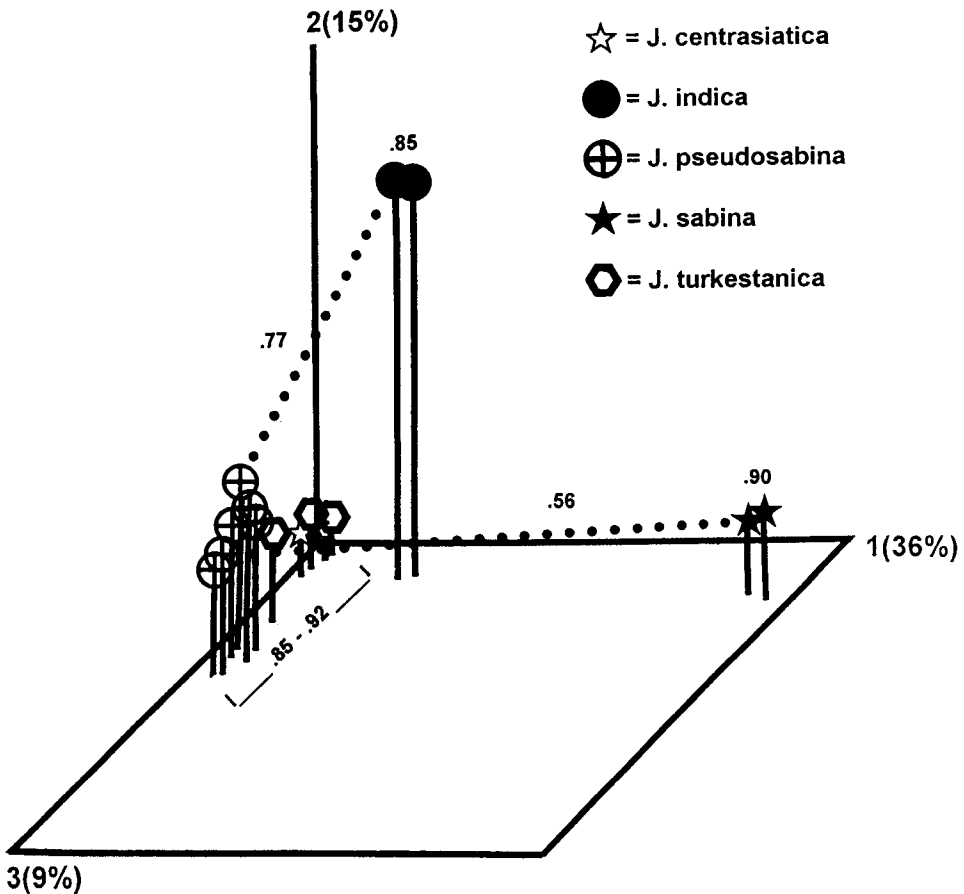


Fig. 2. Ordination of molecular data similarities (PCO, 255 RAPD bands) for 15 OTUs in *Juniperus*. The dotted line is the minimum spanning network. The number next to a dotted line is the similarity between the two connected OTUs. A range of similarities (0.85 to 0.92) is used to indicate similarity between the plants of *J. centrasiatica*, *J. pseudosabina*, and *J. turkestanica*.

To examine the *Juniperus pseudosabina* complex in greater detail, *J. indica* and *J. sabina* were removed from the data set and a new PCO was performed. The first five eigenroots accounted for 23.0%, 14.7%, 12.9%, 10.9%, and 9.5% of the variance among the eleven OTUs. These results are depicted in Fig. 3. Although there is some clustering of the *J. pseudosabina* OTUs, note that *J. turkestanica* from Kazakstan (TK1) is a little more similar (0.83) to *J. pseudosabina* from Mongolia (PM1) than it is to another *J. turkestanica* plant from the same Kazakstan population, TK2 (0.81 similarity). In addition, one of the *J. pseudosabina* individuals from Xinjiang (PX1) is most similar to an individual of *J. pseudosabina* from Kazakstan (PK2; 0.79) and the other plant from Xinjiang (PX2) is most similar to a plant from Mongolia (PM1; 0.80). Thus, it is apparent that regional differences are about level with individual differences. The samples of *J. centrasiatica*, *J. pseudosabina*, and *J. turkestanica* form a continuum of variation.

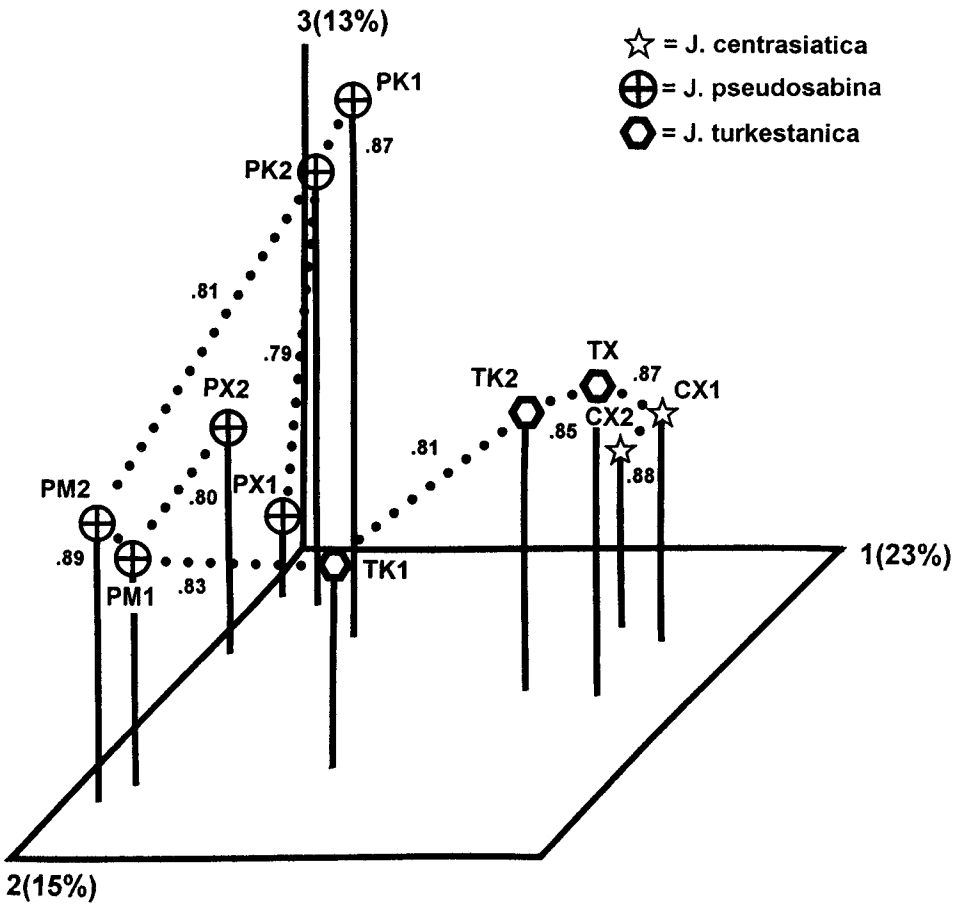


Fig. 3. Ordination of molecular data similarities (PCO, 255 RAPD bands) for 11 OTUs in the *Juniperus pseudosabina* complex. – PK1, 2 = *J. pseudosabina*, Kazakstan; PM1,2 = *J. pseudosabina*, Mongolia; PX1, 2 = *J. pseudosabina*, Xinjiang, China; TK1, 2 = *J. turkestanica*, Kazakstan; TX = *J. turkestanica*, Xinjiang, China; CX1, 2 = *J. centrasiatica*, Xinjiang, China.

In addition to these analyses of DNA data, the volatile leaf oils of these taxa have been analysed using populational averages (Adams & al., 1998). Fig. 4 (modified from Adams & al., 1998) shows that a similar pattern is found in the volatile oils as in the RAPD data. However, *Juniperus pseudosabina* from Mongolia (PM, Fig. 4) is very distinct in its oils and the oil of *J. sabina* is not very distinct from that of *J. pseudosabina*. Volatile oils are reported as percentages of the total oil and, occasionally, single gene mutations can lead to chemical polymorphism that may change the chemical profile rather drastically (by the large increase of one component such as cedrol and consequential decreases of other components in a competitive enzyme kinetics situation). The plants of *J. pseudosabina* from Mongolia were collected from a small population (c. 20-30 individuals) that appeared to be isolated from other *J. pseudosabina* populations. It is likely that a deviating chemical variant has become fixed in this small, isolated population. In any case, we do not see any patterns in the terpene data that would separate *J. centrasiatica* or *J. turkestanica* from *J. pseudosabina* from Kazakstan and Xinjiang, no more then in the RAPD data.

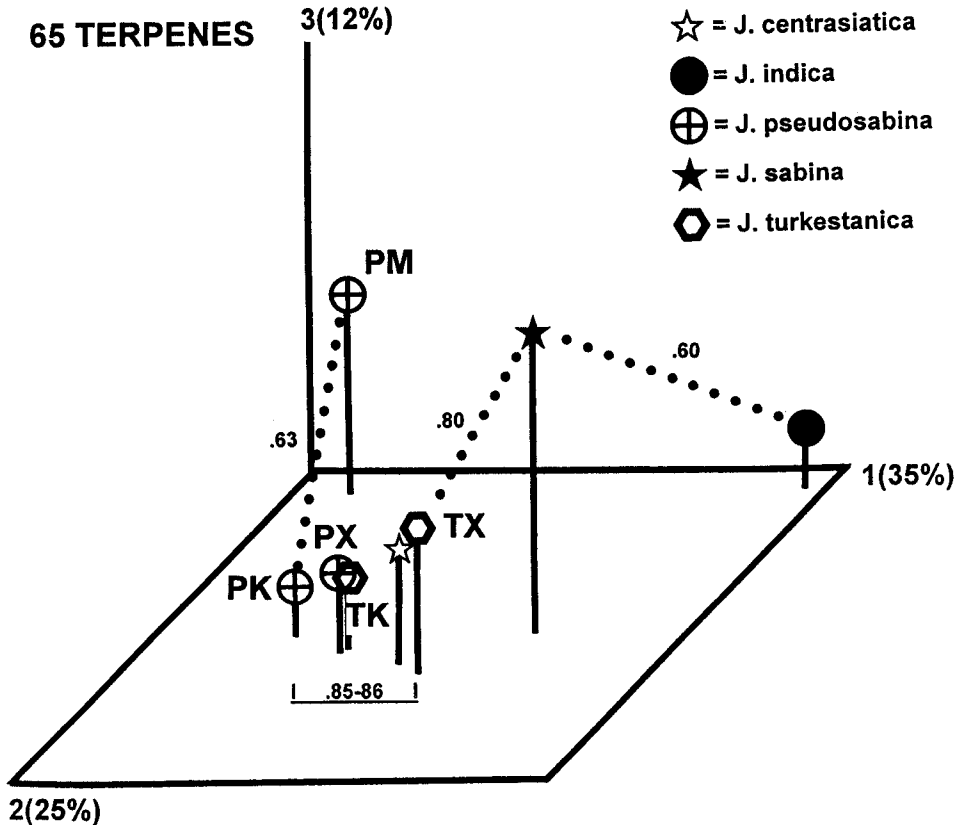


Fig. 4. Ordination of terpenoid similarities (PCO, 65 terpenoids) for 8 *Juniperus* OTUs. – Adapted from Adams & al. (1998).

Discussion

The similarity of *Juniperus centrasiatica* to *J. turkestanica* was apparent upon examination (R.P.A.) of a syntype of the former name and the lectotype of the latter. Morphologically, it appeared that the syntype of *J. centrasiatica* merely represented a small tree form of *J. turkestanica* (a shrub). The DNA data support these observations.

Komarov (1934) recognised both *Juniperus pseudosabina* and *J. turkestanica*. He keyed them out as: “tree or large shrub; fruits 6-10 mm long, seeds longitudinally grooved” (*J. turkestanica*) vs. “dwarf shrub; fruits 5-8 mm long; seeds smooth” (*J. pseudosabina*). However, we have seen *J. pseudosabina* as a large (and dwarf) shrub, and the fruit size values clearly overlap in nature. We were unable to separate two taxa on the basis of seed morphology. The recognition of *J. centrasiatica* was primarily on the basis of it being a tree (not a shrub as *J. turkestanica*; Komarov, 1924). Yet, Komarov (1934) later referred to *J. turkestanica* as a “tree or large shrub”. In the population of *J. centrasiatica* examined, on the north slopes of the Kunlun Mts, typical shrubs, shrubby trees, and trees with a strong central axis were seen. It appears that the growth form is variable. Recently (in 1997) we examined *J. turkestanica* in the far western portion of the Tian Shan in Kazakhstan and found some plants that had several ascending branches, just as seen in *J. centrasiatica* in Xinjiang. The situation appears similar to that observed by the senior author in *J. communis* L. in Switzerland, where various shrub and tree forms occur.

Comparisons of the lectotype of *Juniperus turkestanica* with representative materials of *J. pseudosabina* at LE did not reveal any apparent morphological differences. Our DNA data show (Fig. 3) that variation is continuous throughout the complex, with no sub-clustering into taxa that might correspond to *J. centrasiatica*, *J. pseudosabina*, or *J. turkestanica*. We have found no support for the contention of Silba (1984) that *J. turkestanica* should be treated as a distinct variety of *J. pseudosabina*, as *J. pseudosabina* var. *turkestanica* (Kom.) J. Silba, and there appears to be no justification for maintaining *J. centrasiatica*, *J. turkestanica*, and *J. pseudosabina* as distinct species. By nomenclatural priority, *J. pseudosabina* is the correct name for the combined taxon. *J. centrasiatica* appears to be merely a shrub or small tree form of *J. pseudosabina*.

This study also addresses the controversy concerning the distinctness of *Juniperus pseudosabina* and *J. indica*. Jain (1976) recognised *J. pseudosabina* (“native of Altai and Tarbagatay mountains”) as the only shrubby juniper with scale-like leaves in the Himalayas. In the flora of Bhutan (Grierson & Long, 1983), *J. indica* was treated as a synonym of *J. pseudosabina*. In contrast, Hara & al. (1978) list *J. indica* Bertol. as the scale-leaved, shrubby juniper in Nepal.

The DNA data support the recognition of *Juniperus indica* as a species distinct from *J. pseudosabina*. It appears that the common, scale-leaved juniper of the Himalayas is *J. indica* and not *J. pseudosabina*. Curiously, in the original description *J. indica* was stated to be arborescent (Bertoloni, 1853), yet most floras describe it as a shrub. Only the shrub form was analysed in this study. Collection and analyses of the tree form may shed additional light on this taxonomic problem.

It has been suggested that we should have isolated each band and analysed it by Southern blotting and restriction analysis. Rieseberg (1996) recently analysed the homology of 220 co-migrating RAPD bands generated among three sunflower species

by Southern hybridisation as well as restriction and gel analyses of the fragments, which revealed that 9% of the bands that co-migrated were not homologous. Williams & al. (1993) found that 10% of co-migrating bands in several species of *Glycine* were not homologous. Intergeneric analyses between six *Brassica* species and *Raphanus sativus* revealed that about 20% of the co-migrating bands were not homologous (Thormann & al., 1994). However, Thormann & al. (1994) also found that, within each of the seven species, all co-migrating bands were homologous. It is reasonable to expect that on the infraspecific level, co-migrating bands will be homologous, yet between different species and genera, homology will be less complete.

The same situation was faced by chemosystematists 30 years ago. Flavonoid work began by comparisons of spots and colours and eventually led to the identification of compounds. Terpene research originally utilised just retention times on gas chromatographic charts; with the advent of combined gas chromatography – mass spectroscopy – computer data systems, almost every peak can now be identified (Adams, 1995). Yet, no one identifies the components for every individual in a population which, obviously, only show quantitative differences.

In order for homology to present a problem in principal coordinate analysis, the “false homologies” would have to be non-random. If two co-migrating bands are not homologous this will be a random event. That is, any two co-migrating bands are as likely to be homologous (depending on how closely related the individuals are) as any other two co-migrating bands. Such random events contribute to the “error variance” in principal coordinate analysis and can be seen by the asymptoting of eigenvalues after the first few eigenroots are removed.

Adams (1972) discussed the case where two co-running (or more properly “co-eluting”) gas chromatographic peaks, assigned the same identity, might in fact be non-homologous (i.e., represent two different compounds). He found that even when such compounds accounted for 20% of the total character matching weight, this had but minimal effects on the Gower metric of similarity. In the present study we are specifically examining the question “are these taxa, which are essentially morphologically indistinguishable, conspecific?”. We are not dealing with distantly (or even distinct) species. A more general examination of the impact of “false homology” on principal coordinate analyses is beyond the scope of this paper but will be treated at a later time.

Also, in the present study, there is general agreement between the RAPD data and the terpenoid data. We see no need therefore, to test and confirm the homology of the co-migrating RAPD bands we detected.

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