

**GEOGRAPHIC VARIATION IN *JUNIPERUS SABINA* L.,
J. SABINA VAR. *ARENARIA* (E. H. WILSON) FARJON,
J. SABINA VAR. *DAVURICA* (PALL.) FARJON AND
J. SABINA VAR. *MONGOLENSIS* R. P. ADAMS**

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ABSTRACT

Populations of *J. sabina* L., *Juniperus sabina* var. *arenaria* (E. H. Wilson) Farjon, *J. sabina* var. *davurica* (Pall.) Farjon, *J. sabina* var. *mongolensis* R. P. Adams, along with plants of *J. chinensis* L., *J. erectopatens* (Cheng & L. K. Fu) R. P. Adams and *J. procumbens* (Siebold) ex Endl. were examined by use of RAPD markers in concert with terpenoid and sequence data from the literature. *Juniperus chinensis* and *J. procumbens* were confirmed to be very distinct from *J. sabina*. In general RAPD data agreed with essential oils and sequence data from the literature and affirmed the more distant relationship of *J. chinensis* to the *J. sabina* varieties. *Juniperus sabina* var. *arenaria*, the sand-loving juniper, was very similar to *J. sabina* var. *davurica* from Mongolia. However, *J. sabina* var. *mongolensis*, on sand dunes in

Mongolia, was quite distinct in both its sequence data and RAPD markers. Farjon's move (2001) of *J. sabina* var. *arenaria* from *J. chinensis* to *J. sabina* is supported by essential oils, RAPD markers and sequence data. The major geographic pattern in *J. sabina* var. *sabina* was a west to east trend from the Sierra Nevada, Spain to the Pyrenees, Switzerland, and Tian Shan, Xinjiang. Pleistocene refugia and recolonization are discussed in relationship to the present pattern of genetic differentiation found in *J. sabina*.

KEY WORDS: *Juniperus sabina*, *J. s.* var. *arenaria*, *J. s.* var. *davurica*, geographic variation, RAPDs, nrDNA, trn C- trn D, terpenoids.

The genus *Juniperus* consists of approximately sixty-seven species (Adams, 2004), all of which grow in the northern hemisphere, although, *J. procera* Hochst. ex Endl. also grows southward along the rift mountains in East Africa into the southern hemisphere (Adams, 2004). The recent monograph of the genus (Adams, 2004) divides *Juniperus* into three sections: *Caryocedrus* (one species, *J. drupacea* Labill.); *Juniperus* (= *Oxycedrus*) with 11 species and *Sabina* (the remaining 55 species).

Section *Sabina* can be further divided into junipers with serrate and those with entire (smooth) leaf margins. The serrate leaf margined junipers are confined to the western hemisphere except for *J. phoenicea*, which may have a greater affinity to the smooth leaf margined junipers (Schwarzbach et al., 2008).

The *Juniperus* of section *Sabina*, of the eastern hemisphere can be further divided into two groups based on the number of seeds per female cone (often called berry) and female cone shape. The single seed/cone (single-seeded) *Juniperus* of the eastern hemisphere have cones that are ovoid with a noticeable pointed tip, whereas the multi-seeded *Juniperus* are generally globose and often have an irregular surface. *Juniperus sabina* L. is a smooth leaf margined, multi-seeded juniper of the eastern hemisphere. It is very widely distributed from Spain through Europe to Kazakhstan, western China, Mongolia and Siberia (Fig. 1). *Juniperus sabina* has a range that is discontinuous between Europe and central Asia. The species is generally a small shrub less than 1 m tall and ranges up to 1-2 m wide. But in the Sierra

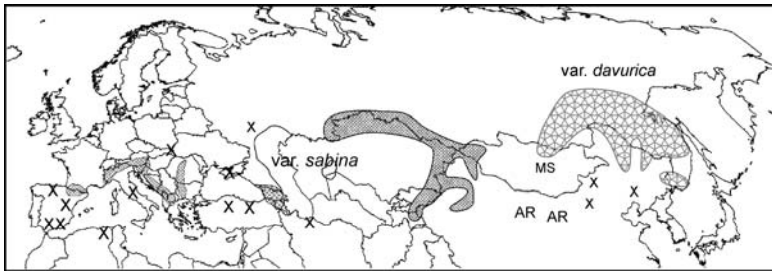


Fig. 1. Distribution of *J. sabina* and its varieties. AR = *J. s. var. arenaria*, MS = *J. s. var. mongolensis*. X = outlying populations of putative *J. sabina*.

Nevada of Spain, it forms a horizontal shrub and in Mongolia it occurs as a prostrate plant on sand dunes.

Wilson (1928) described a prostrate shrub found growing on sand dunes at Lake Qinghai, China as a new variety of *J. chinensis* (*J. chinensis* var. *arenaria* E. H. Wilson). Recently, Farjon (2001) moved the taxon to *J. sabina*, as *J. sabina* var. *arenaria* (E. H. Wilson) Farjon. In addition, Farjon (2001) moved *J. davurica* to *J. sabina* as a new variety, *J. sabina* var. *davurica* (Pall.) Farjon. Table 1 shows the classical and current classifications of the species that have been allied with *J. sabina*. It is apparent from table 1 that the taxonomy of these

Table 1. Comparison of the treatments of *J. sabina* allied taxa.

<u>Classical</u>	<u>Farjon (2005)</u>	<u>Adams (2004)</u>	<u>This study results</u>
<i>J. sabina</i>	<i>J. sabina</i>	<i>J. sabina</i>	<i>J. sabina</i>
<i>J. chinensis</i> var. <i>arenaria</i>	<i>J. sabina</i> var. <i>arenaria</i>	<i>J. sabina</i> var. <i>arenaria</i>	<i>J. sabina</i> var. <i>arenaria</i>
<i>J. sabina</i> var. <i>erectopatens</i>	<i>J. chinensis</i> var. <i>chinensis</i>	<i>J. erectopatens</i>	<i>J. erectopatens</i>
<i>J. davurica</i>	<i>J. sabina</i> var. <i>davurica</i>	<i>J. davurica</i>	<i>J. sabina</i> var. <i>davurica</i>

taxa is not well understood and additional characters are needed to resolve this problem.

DNA sequencing of nrDNA and trnC-trnD (Schwarzbach et al., 2008) based on new collections of *J. sabina* var. *arenaria* from Lake Qinghai and a river bank in Gansu, as well as additional samples from Mongolia, has led to a different picture of the relationships in the *chinensis-erectopatens-davurica-sabina* complex (Fig. 2). Notice that *J. erectopatens* was 100% supported as a distinct clade, as previously shown in both essential oils and RAPD data (Adams, 1999). There was no support for treating *J. erectopatens* as a synonym of *J. chinensis* (Farjon, 2005). *Juniperus erectopatens* is a cryptic species in its morphology, but it is quite distinct as an evolutionary unit in its terpenes, RAPD markers and DNA sequence data. *Juniperus chinensis* (and *J. procumbens*) were also well supported (100%) as being distinct

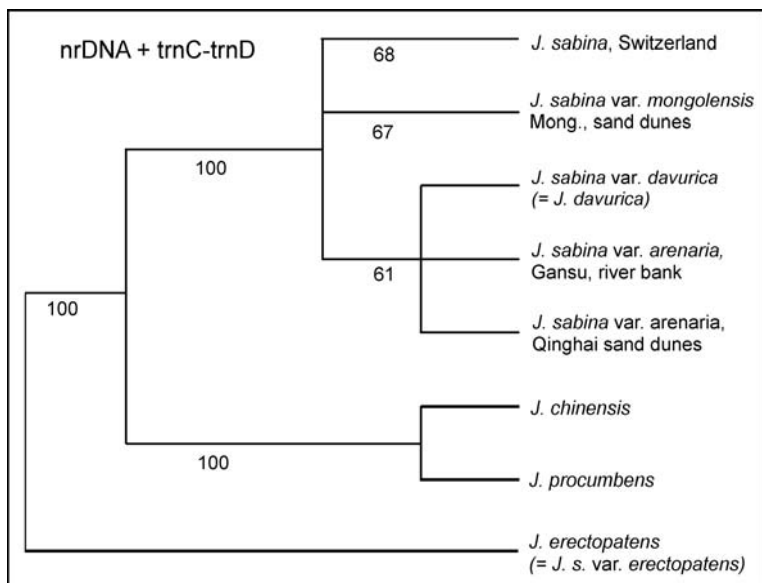


Fig. 2. Phylogenetic tree (from Schwarzbach et al. 2008). Numbers are bootstrap values. See text for discussion.

from *J. sabina* and *J. davurica* (Fig. 2), again as has been shown by their essential oils and RAPD data (Adams, 1999). Among the *J. sabina*, *J. s. var. arenaria* and *J. s. var. davurica* samples, there (Fig. 2) was some support (61-68%) for infraspecific taxa. The *J. sabina* plants from Mongolian sand dunes are distinctly separated (Fig. 2) and have recently been recognized as a new variety, *J. sabina var. mongolensis* R. P. Adams (2006).

Principal coordinate analyses (PCO) of the leaf essential oils (Adams et al., 2006) of these taxa confirmed (Fig. 3) that *J. chinensis* was distinct from the *J. sabina* - *davurica* complex. Adams et al.

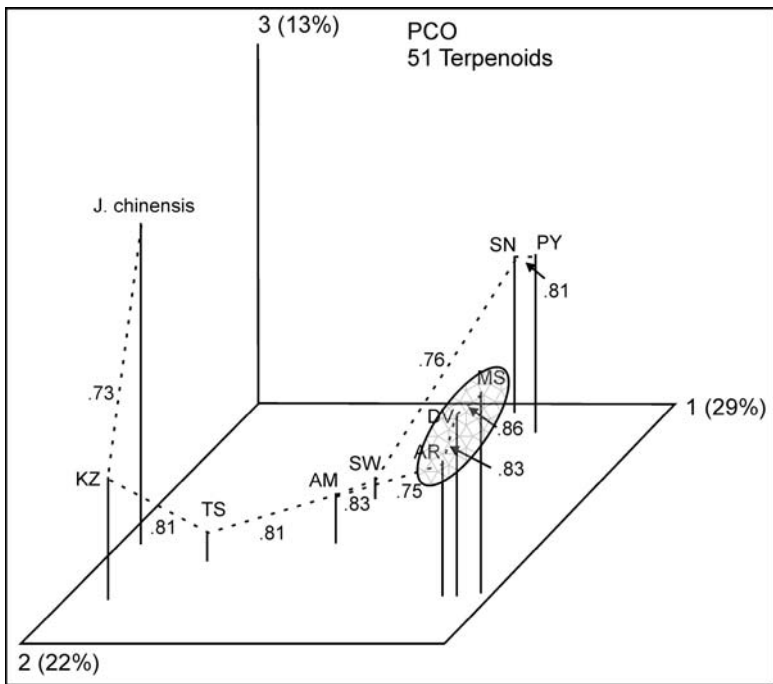


Fig. 3. Principal Coordinate Ordination (PCO) (from Adams et al., 2007) based on 51 terpenoids. Notice the distinct ordination of *J. chinensis* and the prostrate junipers of n China and Mongolia (AR = *J. s. var. arenaria*; DV = *J. s. var. davurica*; MS = *J. s. var. mongolensis*).

(2006) also found that *J. sabina* var. *davurica* (DV, Fig. 3) had a terpenoid composition very similar to that of *J. sabina* var. *arenaria* (AR, Qinghai sand dunes) and *J. sabina* var. *mongolensis* plants growing on Mongolian sand dunes southwest of Ulan Bator (MS). The Iberian *J. sabina* (SN, PY) plants' terpenoids were quite different from nearby Switzerland (SW) and central Asia (AM, TS, KZ).

The purpose of the present study is to examine geographic variation among populations of *J. sabina* from Spain to Mongolia by analyzing and integrating RAPD marker information with terpenoid (Adams et al., 2006) and nrDNA and trnC-trnD sequence data (Schwarzbach et al. 2008).

MATERIAL AND METHODS

Plant Material

Specimens used in this study (species, popn. id., location, collection numbers): *J. chinensis*, CH, Lanzhou, Gansu, China, Adams 6765-67; *J. sabina* var. *davurica*, DV, 15 km se Ulan Bator, Mongolia, Adams 7252, 7253, 7601; *J. procumbens*, Japan, Adams 8683, 8684, 9150; *J. sabina*, Sierra Nevada, Spain, Adams 7197, 7199, 7200; Pyrenees Mtns., Spain/ France border, Adams 7573-77; Switzerland, Adams 7611, 7612, 7614, 7615; Tian Shan Mtns., Xinjiang, China, Adams 7836-38; *J. sabina* var. *arenaria*, sand dunes, Lake Qinghai, Qinghai, China, Adams 10347-52; river bank, Gansu, J-Q. Liu and Adams 10354-56; *J. sabina* var. *mongolensis*, sand dunes, 80 km sw Ulan Bator, Mongolia, Adams 7254-56; Voucher specimens for all collections are deposited at Baylor University Herbarium (BAYLU).

Molecular

One gram (fresh weight) of 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 using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia CA). The RAPD analyses follow that of Adams and Demek (1993). Ten-mer primers were purchased from the University of British Columbia (5'-3'): 134, AAC ACA CGA G; 153, GAG TCA CGA G; 184, CAA ACG GCA C; 212, GCT GCG TGA C; 218, CTC AGC CCA G; 239, CTG AAG CGG A; 249, GCA TCT ACC G; 250, CGA CAG TCC C; 268, AGG CCG CTT

A; 327, ATA CGG CGT C; 338 CTC TGG CGG T; 346, TAG GCG AAC G; 347, TTG CTT GGC G; 413, GAG GCG GCG A; 478, CGA GCT GGT C.

PCR stock solutions (Taq, primer, buffer) were made in bulk so that all the PCR reaction tubes for a primer were prepared using the same bulk stock. This is a critical factor for minimizing variation in band intensities from sample to sample (see Adams et al. 1998, for protocols to minimize PCR band variation). PCR was performed in a volume of 15 μ l containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 2.0 mM MgCl₂, and 0.1% Triton X-100, 0.2 mM of each dNTPs, 0.36 μ M 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.). Samples were run in duplicate to insure reproducibility (Adams et al., 1998). A temperature profile was obtained for each well of the thermocycler to be sure that no variation occurred on the heating/ cooling block. The thermal cycle used was: 94°C (1.5 min) for initial strand separation, then 40 cycles of 40°C (2 min), 72°C (2 min), 91°C (1 min). Two additional steps were used: 40°C (2 min) and 72°C (5 min) for final extension. The temperature inside a PCR tube containing 15 μ l buffer was monitored with a temperature probe, quantitated and printed for each step for each of the 40 cycles for every PCR run (Adams et al. 1998) to insure that each cycle met temperature specifications and that each PCR run was exactly the same. Amplification products were analyzed by electrophoresis on 1.5 % agarose gels, 75V, 55 min, and detected by staining with ethidium bromide. The gels were photographed over UV light with Polaroid film 667 and scanned to digital images. The digital images were size normalized in reference to pGem® DNA size markers before band scoring. Bands were scored as present (1) and absent (0). Bands that were inconsistent in replicate analyses were not scored.

Associational 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 was performed by factoring the associational matrix based on the formulation of Gower

(1966) and Veldman (1967). It should be noted that problems of homology of RAPD DNA bands on agarose gels can be significant (Rieseberg, 1996), but these errors can be accounted for using multivariate statistical methods (PCO) (Adams and Rieseberg, 1998).

RESULTS AND DISCUSSION

Factoring the association matrix resulted in four eigenroots accounting for 23.9%, 14.8%, 9.0% and 7.0% before the eigenvalues began to asymptote into non-significant values (Cattell, 1966; Rummel, 1970). *Juniperus erectopatens* was not included in this study because both terpenoid (Adams 1999) and sequence data (Fig. 2, Schwarzbach et al. 2008) have previously shown that it is a very distinct species. The first three principal coordinates show three major groupings (Fig. 4). Notice that *J. procumbens* is well resolved.

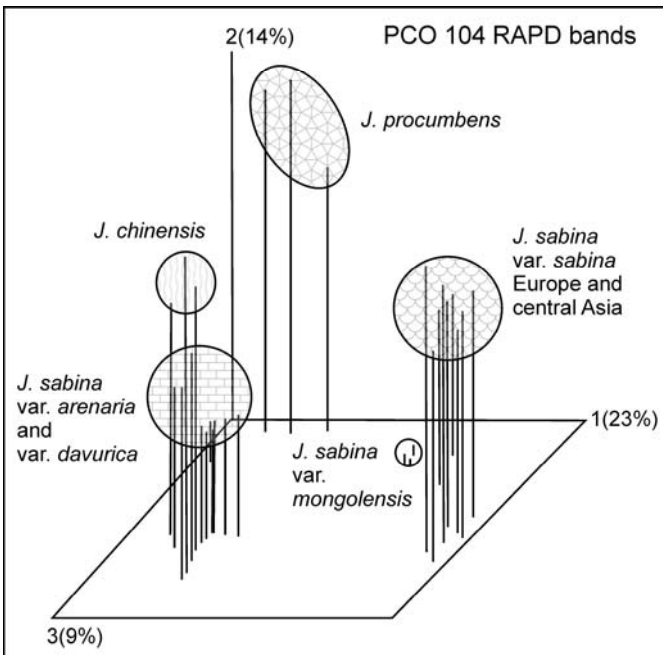


Fig. 4. PCO based on 104 RAPD bands showing the distinct nature of *J. procumbens*. See text for discussion.

Juniperus chinensis appears near *J. sabina* var. *davurica* and *J. sabina* var. *arenaria*, but after removing *J. procumbens* from the data matrix, a subsequent PCO analysis shows that *J. chinensis* is clearly resolved (Fig. 5).

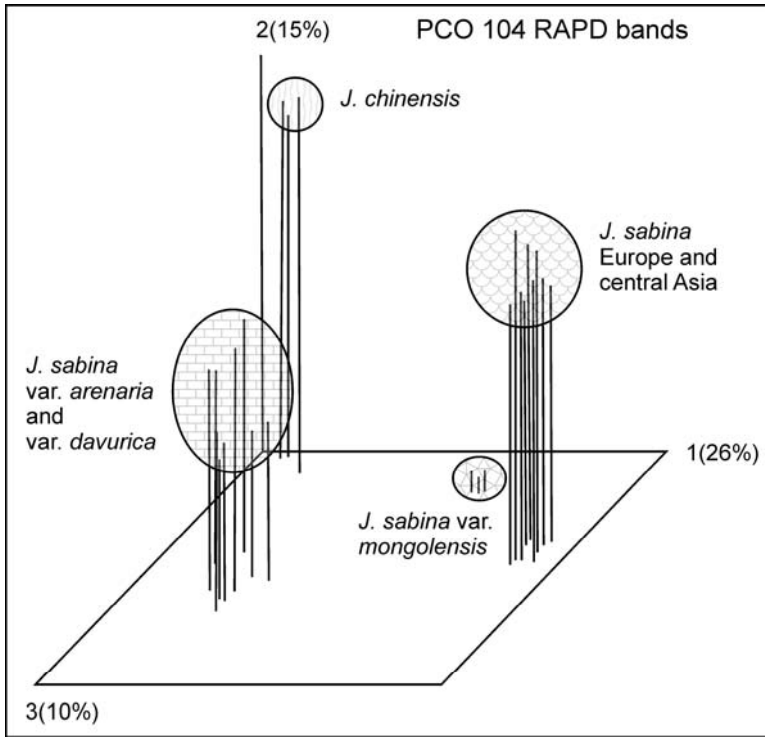


Fig. 5. PCO depicting four major groups, with *J. chinensis* well separated from *J. sabina*.

Because the sequence data clearly showed that *J. chinensis* and *J. procumbens* are not part of the *J. sabina* - *arenaria* - *davurica* - *mongolensis* complex, these two taxa were removed from the data set and a new PCO was performed. Factoring resulted in eigenroots accounting for 27.4%, 15.5%, 7.4% and 6.4%. The eigenroots began to asymptote after the fourth eigenroot. Ordination reveals four groups (Fig. 6) composed of *J. sabina* (Europe and Tian Shan, Xinjiang, China); *J. sabina* var. *mongolensis* and *J. sabina* var. *arenaria* - var. *davurica*. A close relationship between *J. sabina* var. *arenaria* and *J. sabina* var. *davurica* has also been shown in their DNA sequence data

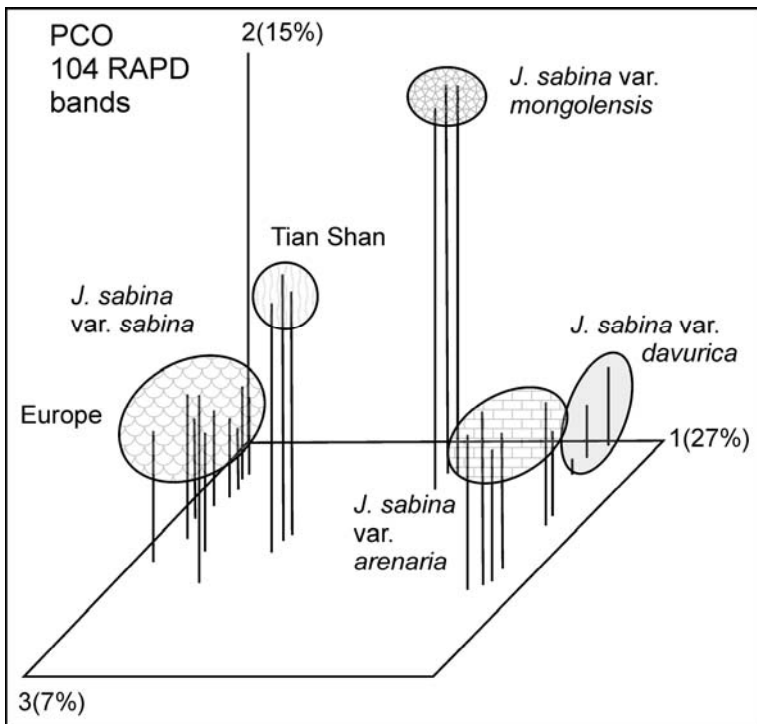


Fig. 6. PCO of *J. sabina* and its varieties. There are three major groups: European - central Asia (*J. s.* var. *sabina*); *J. s.* var. *mongolensis*; and *J. s.* var. *arenaria* - var. *davurica*.

(Fig. 2) and terpenoids (Fig. 3). However, the DNA sequence data (Fig. 2) clearly separates *J. sabina* var. *mongolensis*, but the terpenoids of *J. s.* var. *arenaria*, *J. s.* var. *davurica* and *J. s.* var. *mongolensis* are very similar (Fig. 3). The RAPDs data is more congruent with the DNA sequence data than with the terpenoid data. This same kind of association was reported by Adams et al. (2003) who examined five species of *Juniperus* (in section *Juniperus*), and found a 0.95 correlation between DNA sequence and RAPD data but only a 0.30 correlation between DNA sequence and terpenoid data sets. They concluded that the terpenoid data was more useful at and below the species level.

To examine variation in *J. sabina* from Spain to central Asia, *J. sabina* var. *arenaria* and *J. s.* var. *davurica* were removed from the data set and a new PCO was performed. Factoring this matrix resulted in three eigenroots (33.8%, 12.7%, 9.8%). PCO ordination (Fig. 7)

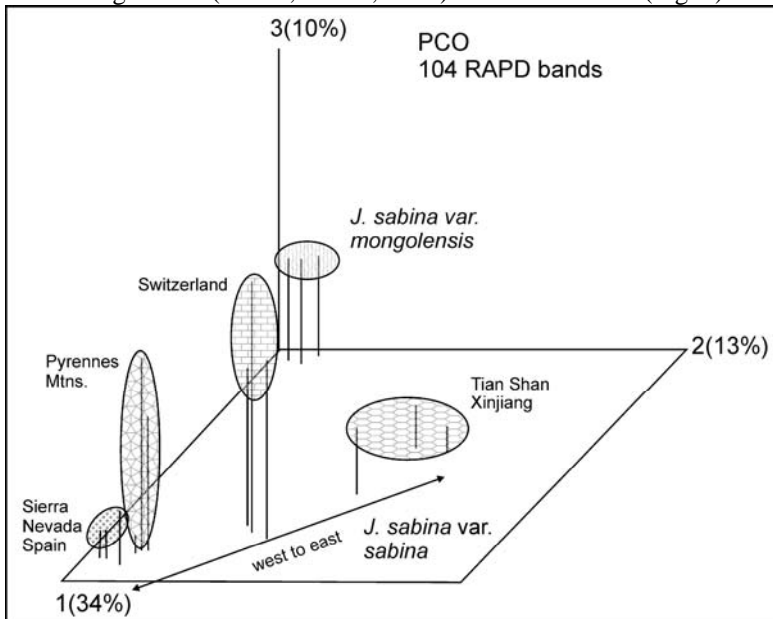


Fig. 7. PCO showing west to east clinal variation within *J. s.* var. *sabina*.

shows a west to east trend from Spain to Tian Shan, Xinjiang.

Many of the populations of *J. sabina* examined in this study were glaciated areas during the last ice age (14,000 - 70,000 ybp, Flint, 1971). The plants now growing in the Iberian peninsula likely survived at lower elevations or perhaps by retreating into the Atlas mountains of northern Africa. The Switzerland population was likely recolonized from seed from populations in Italy. The central Asian populations may have been recolonized from southern refugia or forced to lower elevation (warmer) habitats. Yet, in spite of the large geographic displacements of populations during recent glacial events and the distances separating European and central Asia populations (Fig. 1), there persists strong genetic affinities between these populations.

CONCLUSIONS

An important aspect of this study is discovery of the distinct nature of the *J. sabina* var. *mongolensis* plants that grow on the sand dunes sw of Ulan Batar in both their sequence data (Fig. 2) and RAPD data (Fig. 5, 6). These data support the recent recognition (Adams, 2006) of this morphologically cryptic variety. Comparing *J. sabina* var. *mongolensis* versus *J. sabina* var. *arenaria*, the seeds are 2-4 per cone vs. (1) 2 (3-4), and are flattened globose with an obtuse tip vs. an elongated ellipsoid with an acute tip that resemble a duck bill, and with ultimate branchlets that grow from the top (upper) side of long lateral branches vs. radially distributed branching. In both taxa, the female cones are borne on long, curved peduncles.

More detailed population level studies are clearly needed in order to reconstruct postglacial migration routes and to better explain present distribution patterns.

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