Phylogeny of *Juniperus* using nrDNA and four cpDNA regions.

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ABSTRACT

The Phylogeny of *Juniperus* is presented based on nrDNA (ITS), petN-psbM, trnS-trnG, trnD-trnT, trnL-trnF sequencing (4411 bp) utilizing all currently recognized species. The major clades of the phylogenetic tree were congruent with the previously published phylogenetic tree of Mao et al. (2010) that had a subset of taxa of the current study. The lone species with serrate leaves in the eastern hemisphere, *J. phoenicea*, was found to be in a clade quite separated from the serrate junipers of North America. *Juniperus phoenicea* is referred to as 'pseudoserrate' to distinguish it from the semi-arid, serrate leaf junipers of the western hemisphere. Section *Sabina* is the most derived group and has radiated into niches in both the eastern and western hemispheres with approx. 60 species.
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The genus *Juniperus* is comprised of approx. 75 species in 3 sections:
sect. *Caryocedrus*, 1 species with large, blue, woody, 3-seeded cones, showing the fusing of 3 cone scales, with an Old World Mediterranean distribution (Adams, 2011, Adams and Schwarzbach, 2012a),

sect. *Juniperus*, 14 species, 12 only in the eastern hemisphere, one endemic to North America and one species, *J. communis*, being circumboreal, seed cones blue or red, often with 3 seeds (Adams and Schwarzbach, 2012a) and

sect. *Sabina* (approx. 60 species) with species about equally divided between the eastern and western hemispheres, seed cones with 1 to 13 seeds, blue, red-copper, rose, or brown (Adams, 2011).

Section *Sabina* is divided into three major clades (Mao et al., 2010, Adams 2011):
1. Serrate-leaf junipers of North America (21 species, Adams and Schwarzbach, 2011, 2013d),
2. Turbinate-seed cones, single-seeded, entire-leaf junipers, eastern hemisphere (16 species, Adams and Schwarzbach, 2012b, 2013a, Zanoni and Adams, 1976, 1979) and
3. Multi-seeded, entire-leaf junipers, both eastern and western hemispheres (23 species, Adams and Schwarzbach, 2012c, 2013b).
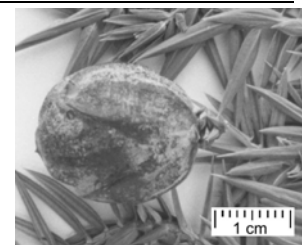
J. drupacea
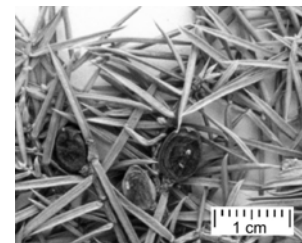
J. communis var. *communis*
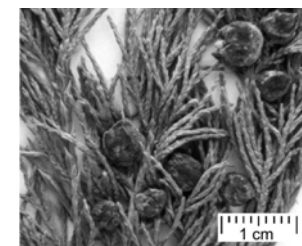
J. sabina

The phylogenetic position of *Juniperus* (and Cupressaceae) in the plant kingdom (Fig. 1) shows *Juniperus* as a terminal clade and as one of the most advanced conifer genera (Rai et al., 2008). Mao et al. (2010) demonstrated that the closest relatives of *Juniperus* are *Cupressus* (of eastern hemisphere) and the *Hesperocyparis - Callitropsis - Xanthocyparis* clade (Fig. 2).

Although Mao et al. (2010) published a robust phylogeny of *Juniperus*, their principal purpose was to investigate the origins and evolutionary radiations of the major clades of *Juniperus*. As such, they utilized representative species from all clades, but not complete coverage of all known taxa. During the past few years, we have utilized DNA sequences to investigate the taxonomy of sections *Caryocedrus* and *Juniperus* (Adams and Schwarzbach, 2012a), section *Sabina*: serrate *Juniperus* of North America (Adams and Schwarzbach, 2006, 2012b, 2013a, 2013c, 2013d; Adams and Nguyen, 2005); the turbinate seed cones, eastern hemisphere (Adams and Schwarzbach, 2012b, 2013a) and the multi-seeded, entire-leaf junipers, both eastern and western hemispheres (Adams and Schwarzbach, 2012c, 2013b).
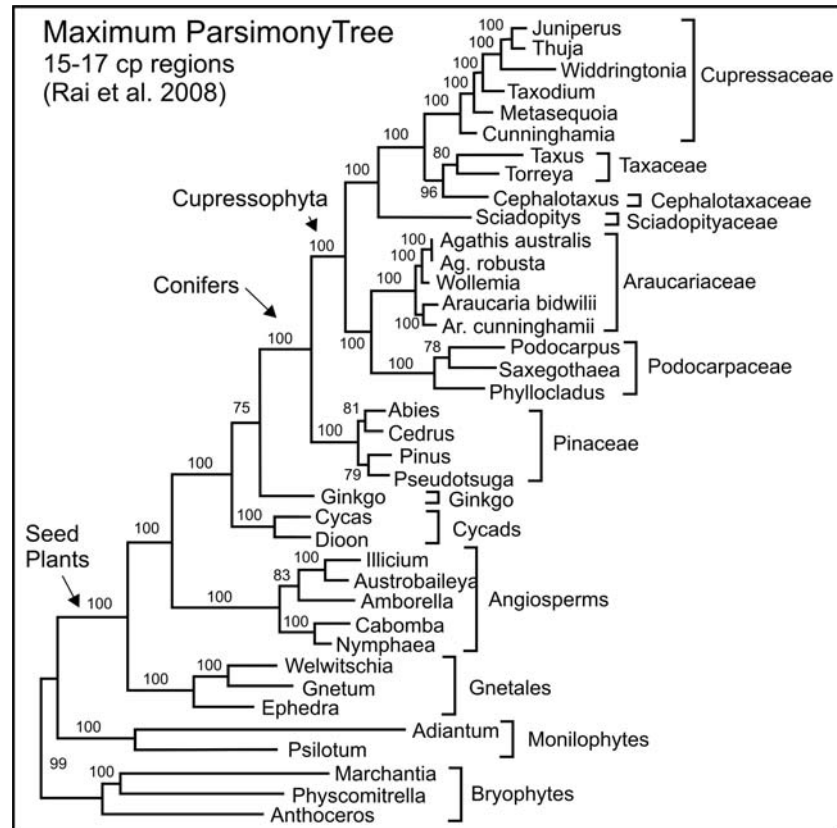

Figure 1. Maximum parsimony tree showing the position of *Juniperus*. Adapted from Rai et al. (2008).
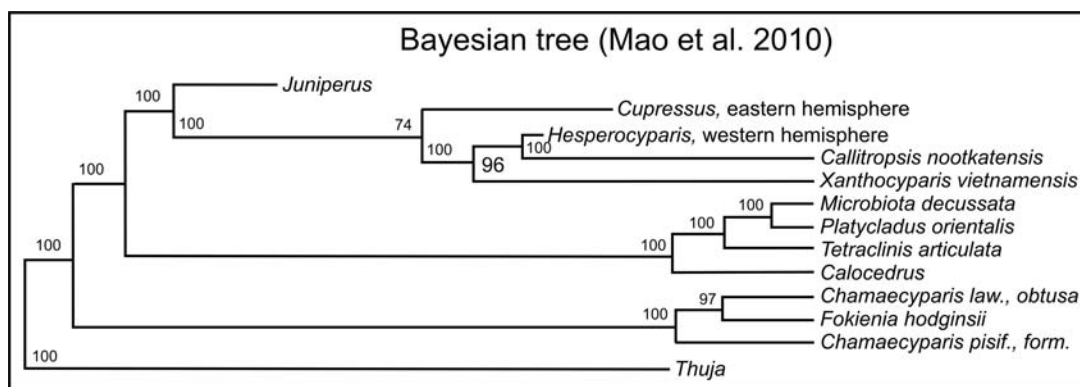

Figure 2. Simplified Bayesian tree with genera collapsed showing *Juniperus* relationship to *Cupressus* and *Hesperocyparis*. Adapted from Mao et al., 2010.

With the taxonomy and nomenclature having been addressed, the present report is to integrate these data into a robust phylogeny of *Juniperus* based on sequencing of nrDNA (ITS), petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF including all known *Juniperus* species.

MATERIALS AND METHODS

Specimens used in this study: see Adams and Schwarzbach (2012a, 2012b, 2012c, 2013a, 2013b, 2013c). Voucher specimens are deposited at BAYLU herbarium Baylor University.

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 $MgCl_2$ 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. R6-1 (Biomatters. Available from http://www.geneious.com/), the MAFFT alignment program. 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.

RESULTS AND DISCUSSION

The phylogeny of *Juniperus* utilized the most informative gene regions (nrDNA, petN-psbM, trnSG, trnDT and trnLF). The tree is similar (Fig. 3) to Mao et al. (2010), except the positions of *J. californica*, *J. durangensis*, and for their clade IV which is now resolved into *excelsa* and *chinensis* groups (Fig. 3). The use of duplicate samples for most taxa appears to have stabilized the Bayesian tree in many places increasing branch support. In addition, all known taxa are included (approx. 100), compared with 51 taxa by Mao et al. (2010) and this has strengthened the tree.

Several 'problem' taxa present difficulties: *J. phoenicea* and *J. p.* var. *turbinata* stand loosely affiliated with sect. Sabina (Fig. 3). These taxa have small serrations on the leaf margins, but are denoted as 'pseudoserrate' (Fig. 3). It seems unlikely that serrate leaf margins in the eastern and western hemispheres is a homologous character, but has arisen independently as *J. phoenicea* is not in the clade with the serrate, semi-arid junipers of the western hemisphere. Also, *J. erectopatens* and *J. microsperma* form an unusual clade that does not nest into the *J. chinensis* clade (Fig. 3*). Juniperus ashei* and *J. a.* var. *ovata,* now *J. ovata* (R. P. Adams) R. P. Adams, are in separate clades (see Adams, 2008; Adams and Schwarzbach, 2013d for discussion).

Mao et al. (2010) used three *Juniperus* fossil dates: *J. pauli* (ca. $\geq$ 33.0 mya, cf. extant *J. sabina* and allies), *J. creedensis* (ca. $\geq$ 23.0 mya, cf. *J. californica* / *J. osteosperma*), and *J. desatoyana* (ca. $\geq$ 16.0 mya, cf. *J. occidentalis* / *J. osteosperma*). They postulated the serrate, semi-arid junipers migrated from the eastern to the western hemisphere via the North American Land Bridge (NALB) ca. 47 - 30.3 mya (Fig. 4).
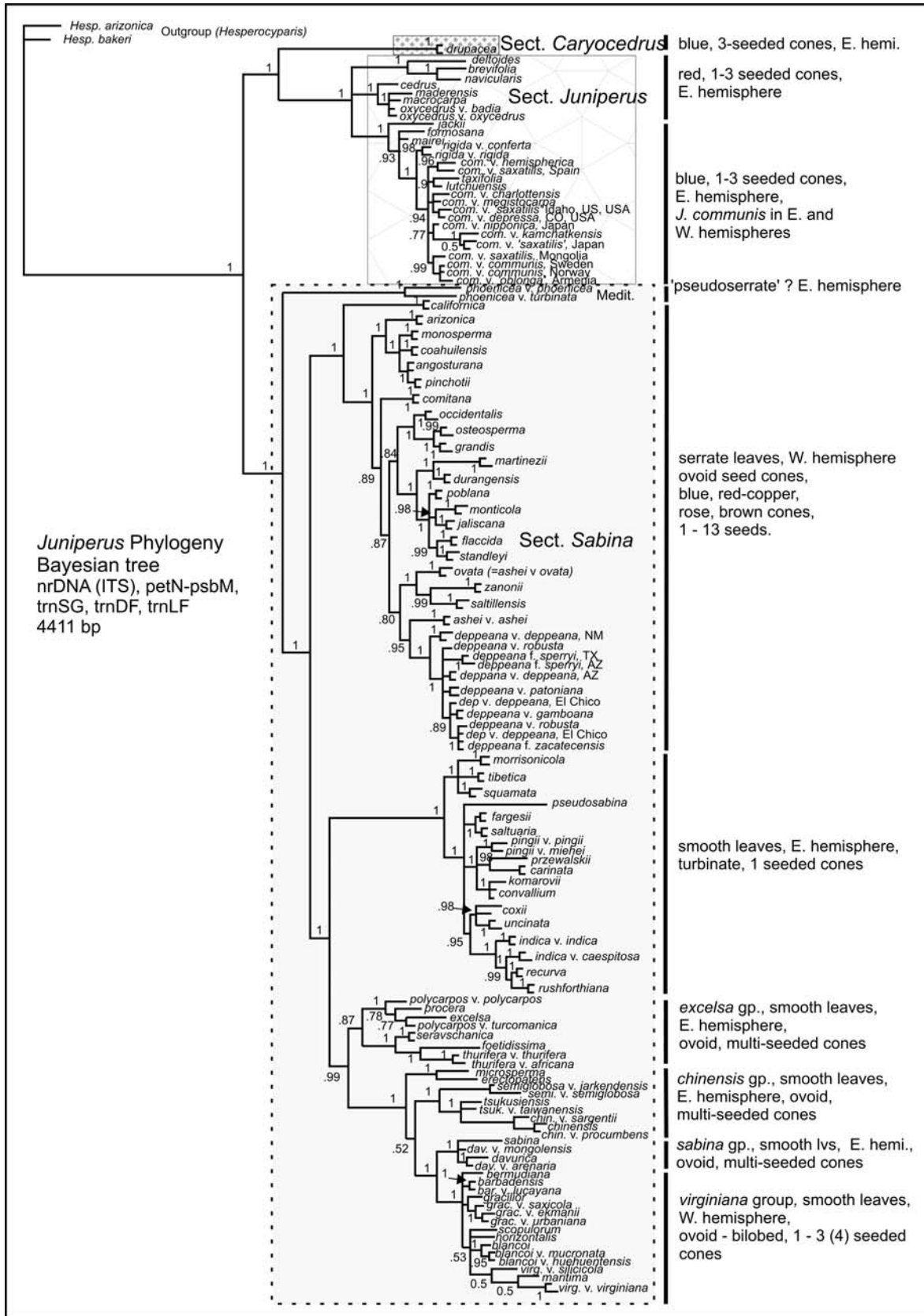
Figure 3. Bayesian tree of the Phylogeny of *Juniperus.* Numbers on lines are posterior probabilities.
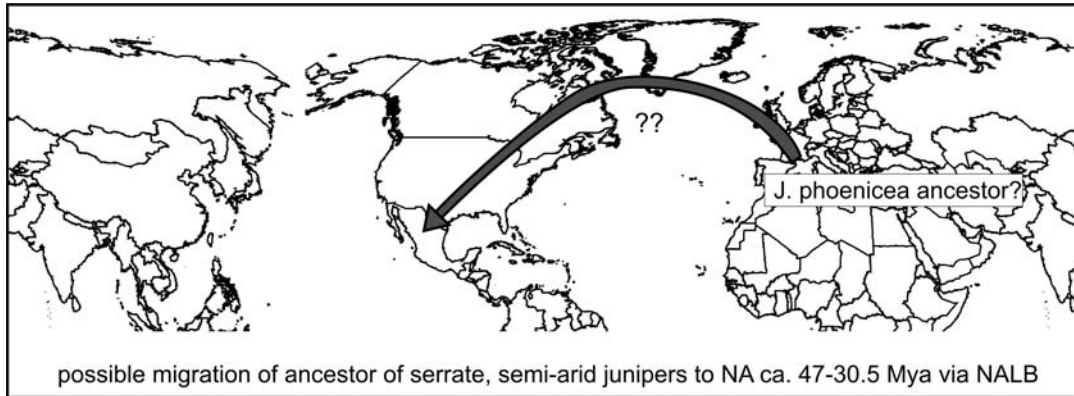
Figure 4. Possible migration of ancestor of the serrate, semi-arid junipers from the Mediterranean to the western hemisphere via the NALB.

The fossil *J. creedensis* of the Creede geoflora (ca. $\geq$ 23.0 mya) bears a striking resemblance to present-day *J. californica* (Fig. 5). Because the present-day *J. californica* appears little changed from the fossil, *J. creedensis*, it may be that the serrate junipers in North America are much older than thought. It might be noted that Axelrod (1987) described a second juniper from the Creede geoflora as *J. gracillensis* that he thought was similar to extant *J. flaccida*, but Wolfe and Schorn (1990) have identified the specimen as *Eleopoldia lipmanii* (Rosaceae).
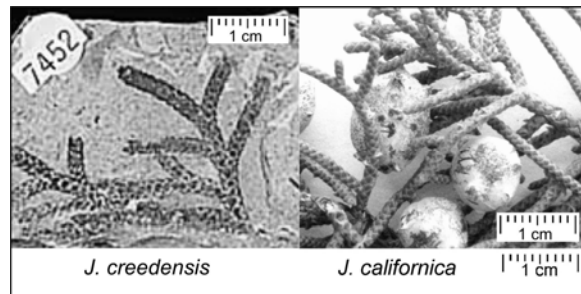

Fig. 5. *Juniperus creedensis* Axelrod paratype and present-day *J. californica*.

The Madrean-Tethyan vegetation belts in Eurasia and North America may have been continuous during the Eocene and Oligocene (Axelrod, 1975, Wen and Ickert-Bond, 2009), such that *Juniperus* section *Sabina* might have had a wider distribution (Fig. 6). So it is possible that the serrate-leaf junipers may have existed in the Madrean-Tethyan vegetation belts in both Eurasia and North America during the same period (Fig. 6), and there may have been exchanges via the North Atlantic Land Bridge (NALB). However, one should note the Madrean-Tethyan vegetation, depicted in Figure 6, predates the ages of any known juniper fossils.
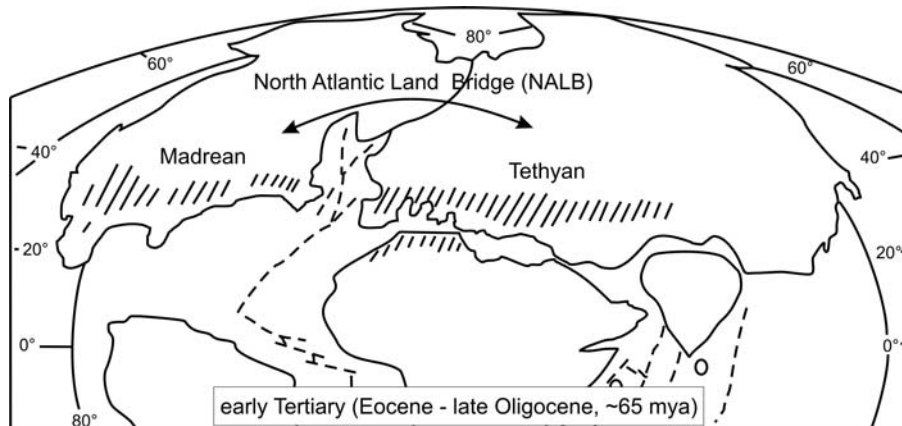

Figure 6. Madrean - Tethyan vegetation zones of Axelrod (adapted from Axelrod (1975) and Wen and Ickert-Bond (2009).

It is unfortunate that the only serrate-leaf species (*J. phoenicea*) that is extant in the eastern hemisphere, has DNA so different that it is poorly grouped with any clade (Fig. 3). It is quite removed from the serrate junipers clade (Fig. 3). At present, it seems appropriate to consider *J. phoenicea* as 'pseudoserrate' and of a different lineage than the serrate junipers of North America. If the serrate leaves of *J. phoenicea* are not homologous to the serrate leaves of junipers in North America, then we are left with no extant (or known fossils) of truly serrate junipers in the eastern hemisphere.

The migration of the smooth leaf members of sect. *Sabina* to the western hemisphere is thought to be more recent (17.6-5.5 mya, Mao et al., 2010) and those dates are younger than the fossil *J. creedensis* of the Creede geoflora (Axelrod, 1987). Since *J. phoenicea* does not appear to be a true member of the serrate junipers and no serrate juniper fossils have been found in the eastern hemisphere, the serrate junipers may be endemic to the western hemisphere. Undoubtedly, additional fossils will be found some day to help resolve the question.

Mao et al. (2009) argues that the movement of sect. *Sabina* to the western (17.6-5.5 mya) is too young for migration across the North Atlantic Land Bridge (NALB), but possible via the Bering Land Bridge (BLB). Because sect. *Sabina* species such as *J. sabina* and *J. davurica* are quite cold adapted, they could have migrated to produce the ancestors that gave rise to the current, cold climate, western hemisphere species such as *J. horizontalis* and *J. scopulorum*. *Juniperus davurica* is the northeastern-most species in northeast Asia (in sect. *Sabina*) and could have provided ancestral stock to migrate across the Bering Land Bridge (Fig. 7). Notice that *J. davurica - J. sabina* are in a sister clade to the smooth-leaf juniper of North America (Fig. 3), supporting the concept of migration from northeastern Asia via the BLB (Fig. 7.)
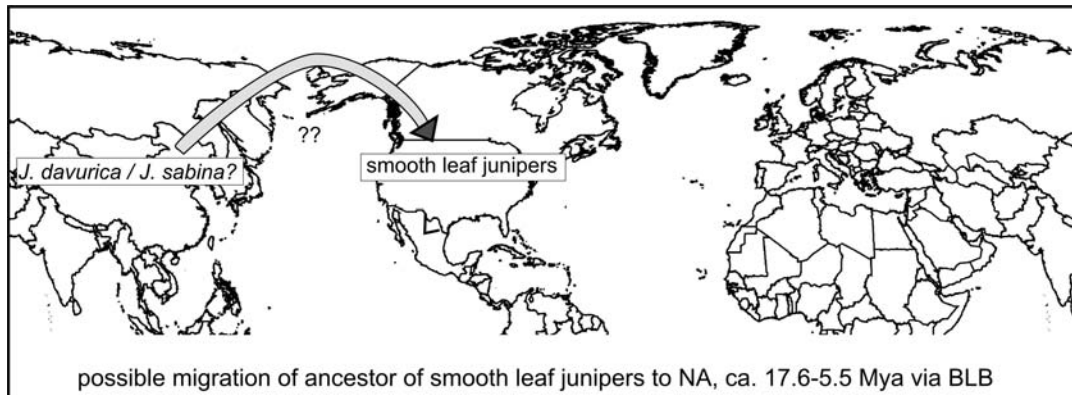

Figure 7. Possible migration pathway of the smooth leaf junipers of North America.

Juniperus communis is an interesting taxon in that it is the most weedy (invasive) species in sect. *Juniperus*. Its seed cones are especially juicy and attractive to birds. It is found in disturbed habitats as an invasive weed in Hungary and central Europe as well as in North America (as *J. c.* var. *depressa*). *Juniperus communis* and *J. c.* var. *depressa* form a boreal distribution in the higher latitudes around the northern hemispheres. Adams and Schwarzbach (2012a) examined the taxonomy of *J. communis* and found it to be very complex. The species is comprised of several morphological varieties that are closely linked by only a few mutations (Fig. 8). Notice the Kamchatka group (Fig. 8) is closely linked (6 mutational events, MEs) to *J. communis* var. *nipponica*, Japan, thence to *J. c.* var. *megistocarpa* (NA, 5 MEs).

The North America *communis* group is equally linked between the Japan and Europe-Central Asia groups. Thus, the linkage map gives equal support to the Bering Land Bridge and North Atlantic island hopping model for the origin of *J. communis* in North America. The situation was previously more

unclear when *J. jackii* was included in *J. communis* (*J. c.* var. *jackii*). However, *J. jackii* is clearly quite differentiated (20 MEs from *J. mairei*, Gansu, China; 21 MEs from *J. c.* var. *megistocarpa*, NA and Fig. 3), but the data is equivocal as to whether its origin is from the BLB or North Atlantic island hopping model. It grows on serpentine and volcanic basalt of quite recent origin in the Cascade Range of western Oregon/ northern California.
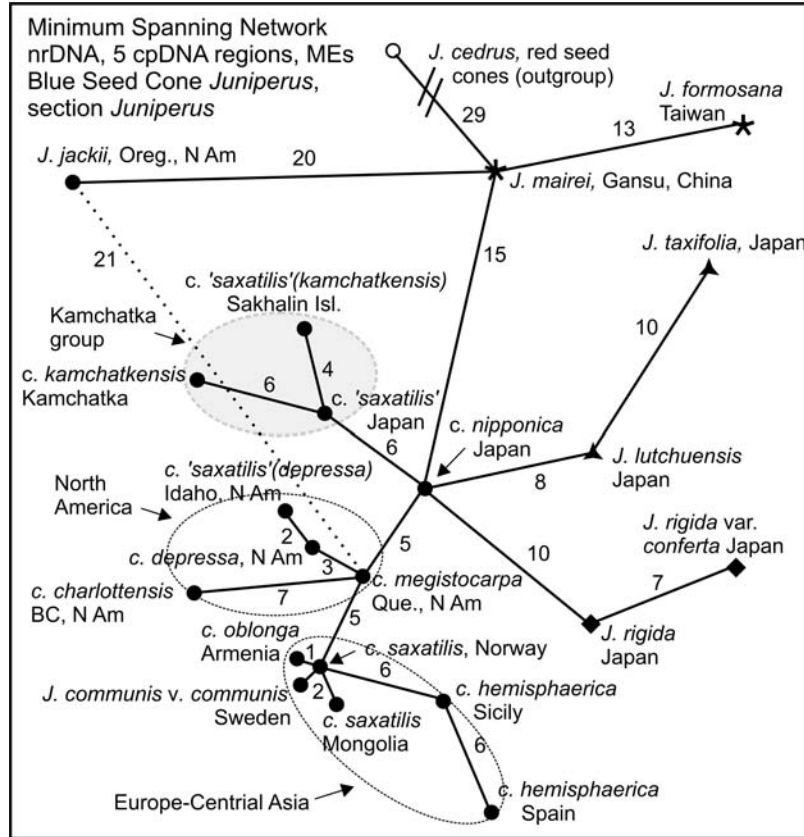

Figure 8. Minimum spanning network of blue seed cone junipers. Numbers on the links are MEs (mutational events). Adapted from Adams and Schwarzbach (2012a).

A diagrammatic representation of the possible migrations of *J. communis* (and *J. jackii*) is shown in Figure 9. The migration dates proposed by Mao et al. (2010) seem consistent with the recent habitat availability for *J. jackii* and support the observed lack of differentiation among morphological varieties of *J. communis* (Adams and Schwarzbach, 2012a).
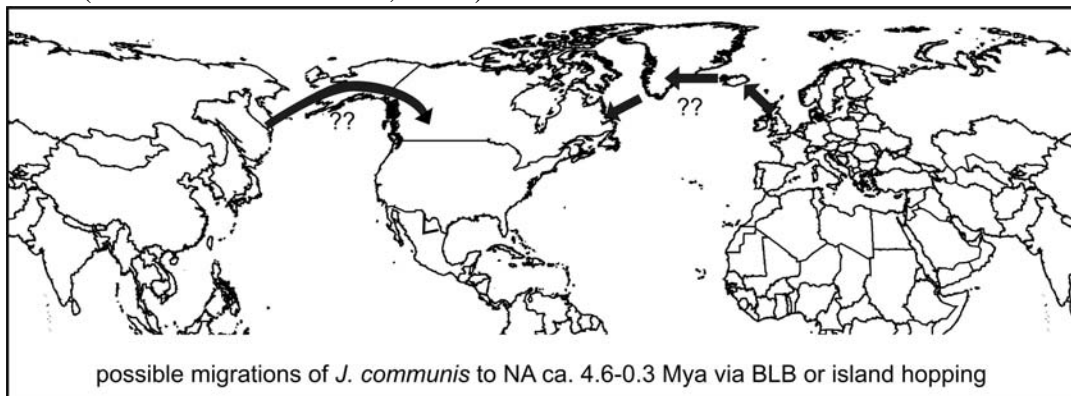

Figure 9. Possible migrations of *J. communis* to North America. Based on data in Mao et al. (2010).

Wen and Ickert-Bond (2009) summarized data from 17 studies concerning Madrean-Tethyan disjunctions. Their summaries are useful in the present discussion. They concluded (Fig. 10) that: 53% of the inter-continental migrations was by the North Atlantic Land Bridge; 40% was by long distance dispersal and 7% by the Bering Land Bridge (BLB). Their summary of the directional data indicated the origins as: 86% from eastern to western hemisphere; 7% from western to eastern hemisphere and for 7% the direction was uncertain (Fig. 10). This trend broadly supports the conclusions of Mao et al. (2010) and the present study.
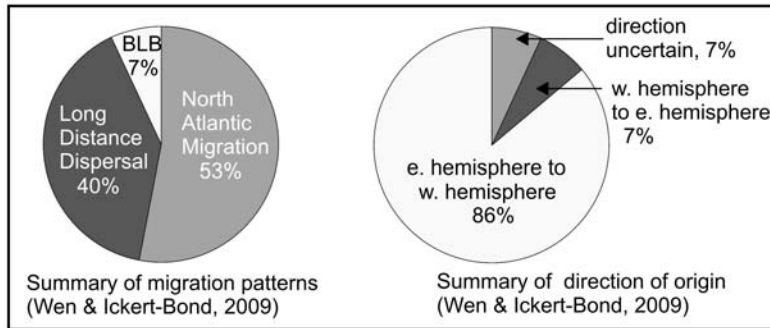

Figure 10. Summary of trends based on 17 studies of Madrean-Tethyan disjunctions. Adapted from Wen and Ickert-Bond (2009).

CONCLUSIONS

The present phylogenetic results are compatible with the results of Mao et al. (2010). In the present report, the clades are better defined and clade IV (Mao et al. 2010) is now resolved into three clades of the *J. excelsa* group and the *J. chinensis* group, with *J. erectopatens* and *J. microsperma* forming a somewhat intermediate clade. The lone species with serrate leaves in the eastern hemisphere, *J. phoenicea*, was found to be in a clade quite separated from the serrate junipers of North America. It appears that the evolution of serrate leaves occurred independently in the eastern hemisphere. *Juniperus phoenicea* is referred to as 'pseudoserrate' to distinguish it from the semi-arid, serrate leaf junipers of the western hemisphere. Section *Sabina* is the most advanced group and has radiated into niches in both the eastern and western hemispheres with approx. 60 species. Additional fossils are needed from older formations to clarify the evolution of the genus.

ACKNOWLEDGEMENTS

LITERATURE CITED

Adams, R. P. 2008. Distribution of *Juniperus ashei* var. *ashei* and var. *ovata* around New Braunfels, Texas. Phytologia 90: 97-102.

Adams, R. P. 2011. The junipers of the world: The genus *Juniperus*. 3rd ed. Trafford Publ., Victoria, BC.

Adams, R. P. and S. Nguyen. 2005. Infra-specific variation in *Juniperus deppeana* and f. *sperryi* in the Davis Mountains, Texas: Variation in leaf essential oils and Random Amplified Polymorphic DNAs (RAPDs). Phytologia 87: 96-108.

Adams, R. P. and A. E. Schwarzbach. 2006. Infraspecific adjustments in *Juniperus deppeana* (Cupressaceae) Phytologia 88: 227-232.

Adams, R. P. and A. E. Schwarzbach. 2011. DNA barcoding a juniper: the case of the south Texas Duval county juniper and serrate junipers of North America. Phytologia 93: 146-154.

Adams, R. P. and A. E. Schwarzbach. 2012a. Taxonomy of *Juniperus* section *Juniperus*: Sequence analysis of nrDNA and five cpDNA regions. Phytologia 94: 280-297.

Adams, R. P. and A. E. Schwarzbach. 2012b. Taxonomy of the turbinate seed cone taxa of *Juniperus* section *Sabina*: Sequence analysis of nrDNA and four cpDNA regions. Phytologia 94: 388-403.

Adams, R. P. and A. E. Schwarzbach. 2012c. Taxonomy of the multi-seeded, entire leaf taxa of *Juniperus* section *Sabina*: Sequence analysis of nrDNA and four cpDNA regions. Phytologia 94: 350-368.

Adams, R. P. and A. E. Schwarzbach. 2013a. Taxonomy of the turbinate shaped seed cone taxa of *Juniperus*, section *Sabina*: Revisited. Phytologia 95: 122-124.

Adams, R. P. and A. E. Schwarzbach. 2013b. The multi-seeded, entire leaf taxa of Juniperus section Sabina: inclusion of *Juniperus microsperma*. Phytologia 95: 118-121.

Adams, R. P. and A. E. Schwarzbach. 2013c. Taxonomy *of Juniperus deppeana* varieties and formas based on nrDNA (ITS), petN-psbM, trnS-trnG, trnD-trnT, trnL-trnF sequences. Phytologia 95: 161-166.

Adams, R. P. and A. E. Schwarzbach. 2013d. Taxonomy of the serrate leaf *Juniperus* of North America: Phylogenetic analyses using nrDNA and four cpDNA regions. p. 172-178

Axelrod, D. I. 1975. Evolution and biography of Madrean-Tethyan sclerophyll vegetation. Ann. Missouri Bot. Gard. 62: 280-334.

Axelrod, D. I. 1987. The late Oligocene Creede flora, Colorado. University of California, Publications in Geological Sciences 130: 1-235.

Mao, K, G. Hao, J-Q Liu, R. P. Adams and R. I. Milne. 2010. Diversification and biogeography of *Juniperus* (Cupressaceae): variable diversification rates and multiple intercontinental dispersals. New Phytologist 188: 254-272.

Posada, D. and K. A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817-818.

Rai, H. S., P. A. Reeves, R. Peakall, R. G. Olmstead and S. W. Graham. 2008. Inference of higher-order conifer relationships from a multi-locus plastid data set. Botany 86: 658-669.

Ronquist, F. and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

Wen, J. and S. M. Ickert-Bond. 2009. Evolution of the Madrean-Tethyan disjunctions and the North and South American amphitropical disjunctions in plants. J. Syst. Evol. 47: 331-348.

Wolfe, J. A. and H. E. Shorn. 1990. Taxonomic revision of the spermatopsida of the Oligocene Creede Flora, southern Colorado. U. S. Geological Survey Bull. 1923, USGS, Denver, CO.

Zanoni, T. A. and R. P. Adams. 1976. The genus *Juniperus* (Cupressaceae) in Mexico and Guatemala: Numerical and chemosystematic analysis. Biochem. Syst. Ecol. 4: 147-158.

Zanoni, T. A. and R. P. Adams. 1979. The genus *Juniperus* (Cupressaceae) in Mexico and Guatemala: Synonymy, Key, and distributions of the taxa. Bol. Soc. Bot. Mexico 39: 83-121.