Allopatric hybridization and introgression between *Juniperus maritima* R. P. Adams and *J. scopulorum* Sarg.: Evidence from nuclear and cpDNA and leaf terpenoids

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ABSTRACT

Previous studies of leaf terpenoid variation from throughout the range of *Juniperus scopulorum* found the populations in Wallowa, OR and British Columbia (northern Rocky Mountains) were differentiated from the central Rocky Mountain populations. Re-assessment of that data in concert with the leaf essential oil of *J. maritima*, suggest the 'divergent' populations at Wallowa and British Columbia are in fact hybrids and/or introgressants. New data from nrDNA (ITS), maldehy, and petN-psbM (cpDNA) confirm that allopatric hybridization is occurring at Wallowa, eastern WA, and southeastern BC into w Montana. nrDNA was found to be of less use in detecting hybrids than a single copy nuclear gene (SCN), maldehy. This appears to be due to concerted evolution in nrDNA. The uniform presence of either *J. maritima* cpDNA in western BC and WA or *J. scopulorum* cpDNA in eastern BC, WA, OR, and MT suggests allopatric introgression by air-borne pollen. Published on-line **www.phytologia.org** *Phytologia 97(1): 55-66 (Jan 2, 2015).* ISSN 030319430.

KEY WORDS: *Juniperus maritima, J. scopulorum, nrDNA, maldehy, petN-psbM,* leaf terpenoids, hybridization, introgression, Pleistocene refugia, recolonization, Wisconsin glaciation.

In 1983, I published an analysis of geographic variation in leaf oils of *J. scopulorum* (Adams, 1983) and noted that the samples from Puget Sound and Vancouver Island were the most distinct of all populations and that their oils were actually more similar to *J. virginiana* than *J. scopulorum* (Fig. 13, Adams, 1983). Subsequently, Adams (2007, 2014) recognized the Puget Sound and Vancouver Island (including the Strait of Georgia) plants as *J. maritima* based on the combined use of terpenoids, morphology and nrDNA sequence data. In 2011, I re-examined this terpenoid data set (Adams, 2011a), removing the *J. maritima* seaside populations from the analysis, thinking that *J. maritima* only grew in the Puget Sound maritime region. The major trend shows (Fig. 1) the leaf terpenoids of *J. scopulorum* to be relatively uniform throughout the central and southern Rocky Mountains. However, the populations in British Columbia, Wallowa, OR, and Kalispell, MT form a separate group (Fig. 1). There is also some differentiation of the other Montana populations (BM, BR, MM). Adams (1983, 2011a) postulated that the divergent populations (BC, OR, MT) arose from a Wisconsin age refugium group that re-invaded the region after the glacial retreat, bearing a terpenoid profile that differs from the typical *J. scopulorum*, Rocky Mountain terpenoid composition.

The discovery and verification of *J. maritima* growing in the Olympic Mountains as Krumholz plant at 1700m (Adams, Hunter and Fairhall, 2010), marked the first verified report of *J. maritima* growing away from its seaside (maritime) locations. Because the present seaside population sites in Puget Sound and the Strait of Georgia were glaciated during the Wisconsin (Fig. 7, Adams, Hunter and Fairhall, 2010), the authors postulated that *J. maritima* retreated to a refugium in the non-glaciated Olympic Mtns. and/or on newly exposed shore lands on the west coasts of Washington and Oregon (see Buckingham et al., 1995).

Re-analysis of the terpene data, by removing CM (found to be *J. blancoi,* introgressed by *J. scopulorum*, Adams, 2011b) and adding *J. maritima* (MA, Vancouver Island, BC) shows MB (Manning Park) related to *J. maritima* (MA, Fig. 2). There appears to be a cline from MA (*J. maritima*) to Manning Park, to the DB, WO, KM, WB, TB group (Fig. 2).

Recently, Moreno-Letelier, Mastretta-Yanes and Barraclough (2014) used six single copy nuclear (SCN) genes to study geographic variation in *Juniperus blancoi* that had been used by Li et al. (2012). An evaluation of five of these SCN genes was conducted between *J. maritima* (Brentwood Bay, BC) and *J. scopulorum* (Kamas, UT). cc13333 (515bp) gave 0 differences; chs (600 bp) had 1 difference, but was polymorphic in *J. maritima*; LHCA4 (742bp) had 3 differences, but all were polymorphic in one of the taxa; MYB (946bp) had 2 differences, but both were polymorphic in one of the taxa.

However, maldehy (522bp in *J. maritima* and 529 bp in *J. scopulorum*) had a 7 bp indel and 2 differences that separated the taxa. Maldehy is a putative malate dehydrogenase (Dvornyk, et al., 2002; Li et al., 2012).

The purpose of the present paper is to report on analyses of these northwestern populations using nrDNA (ITS), SCN (single copy nuclear) maldehy (Moreno-Letelier et al., 2014) and petN-psbM (cpDNA).

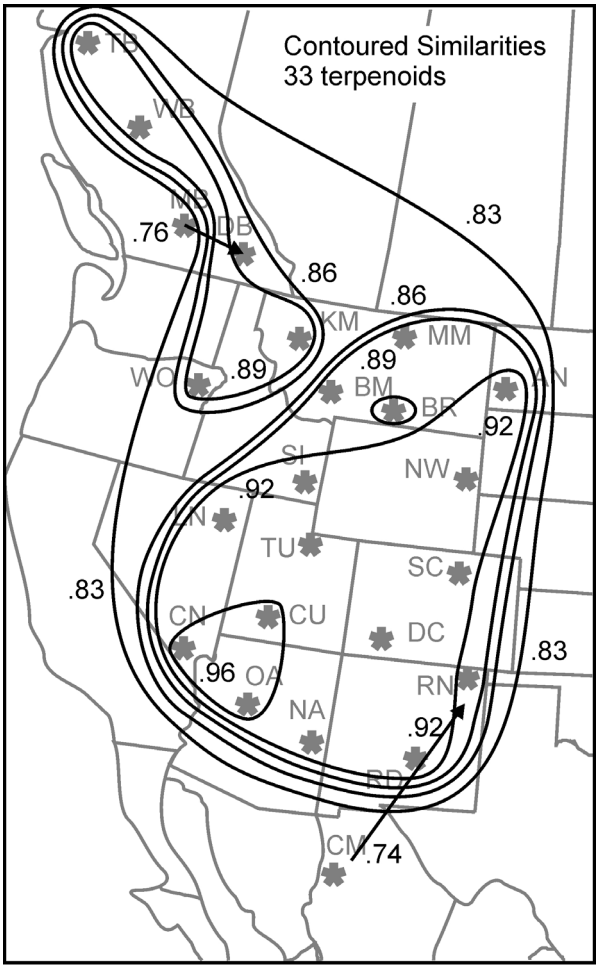

Figure 1. Contoured similarities among populations of *J. scopulorum* based on 33 terpenoids. See text for discussion. From Adams, 2011.

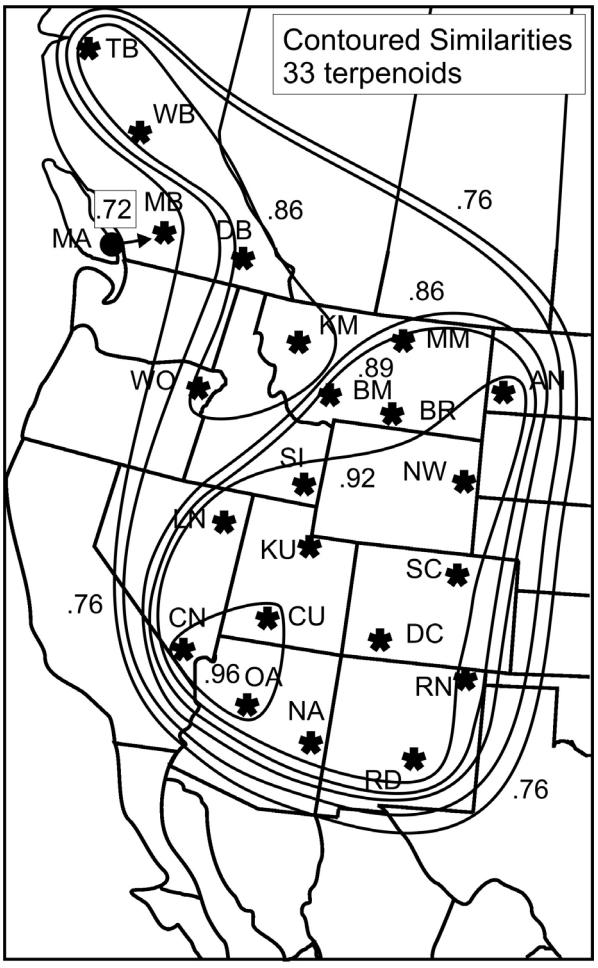

Fig. 2. Modified analysis: CM was removed and MA (*J. maritima*, Vancouver Island) was added. Notice the linkage between MA (*J. maritima*) and MB (Manning Park). Note: KU = TU in Fig. 1.

MATERIALS AND METHODS

Plant material: (species, population acronym, location, vouchers):

J. maritima: BB, Brentwood Bay, Vancouver Island, BC, *Adams 11056-11058;* CB, Cowichan Bay, Vancouver Island, BC, *Adams 11061-11063*; LI, Lesqueti Island, BC, *Adams 11064-11066*; Vancouver Island, BC; PS, San Juan Island, *Adams 11067, 11068*; Whidbey Island, *Adams 11075*; Fidalgo Island State Park, *Adams 11076*; Skagit Island, *Adams 11077-1178* (*11077* is the national big tree for *J. scopulorum*, but should be the *J. maritima*, national big tree); WL, Williams Lake, BC, *Adams 13436-13440*; Cache Creek, BC, *Adams 13431-13435*; MP, Manning Park, BC, *Adams 13426-13430*;

J. scopulorum: Reference, Kamas, UT, *Adams 10895-10899* and Glorieta Pass, NM, *Adams 10933-10935*.

Putative *J. maritima x J. scopulorum*: CR, Creston, BC, *Adams 14026-14030*; FH, Fairmont Hot Springs, BC, *Adams 13421-13425, 14001-14010*; *Adams 14001-14010*; Northport, WA, *Adams 14031-14035*; BV, Beverley, WA, *Adams 14036-14040*; WO, Wallowa Mtns., OR, *Adams 11935-11939*; KM, Kalispell, MT, *Adams 12995-12999*; Voucher specimens are deposited in the Herbarium, Baylor University (BAYLU).

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, 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-psbM), D (maldehy) 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. PCR for maldehy used: maldehyF8 5' GTGATTGGGTGCTTGGTACAC 3'; maldehyR531 5' AGTGGCATCCAGTTTTTCCTT 3', annealing temperature of 60^{o} C, buffer E and 35 cycles.

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.). Minimum spanning networks were using PCO3d and MINSPAN software (Adams et al., 2009; Adams, 1975; Gower, 1966, 1971; Veldman, 1967).

RESULTS AND DISCUSSION

DNA sequencing gave: nrDNA (1270bp), with 5 substitution differences between the reference populations of *J. maritima* (BB) and *J. scopulorum* (KU, GN); petN-psbM (828bp), 8 nucleotide differences plus a 7 bp indel; maldehy (522bp, *maritima*; 529 bp, *scopulorum*), 2 differences plus a 7 bp indel. Each of these sequences displayed fidelity in the reference populations (Table 1). Based on these distinct differences, an effort was made to classify each plant as to species or hybrid for maldehy and nrDNA. Of course, it may be that some positions will be heterozygous by chance or from relictual speciation.

Table 1 shows the classification of 80 individuals for each of these three gene regions. Both *J. maritima* and *J. scopulorum* are uniformly classified in their reference populations. All of the samples from the Puget Sound - Strait of Georgia - Olympic Mtns, plus Manning Park were uniformly classified

as *J. maritima*, except for *11063*, Cowichan Bay, Vancouver Island, for which maldehy was heterogenic for both substitutions, and, thus, classified as a hybrid. nrDNA was much more conserved in detecting hybrids, with only 4 hybrids compared to maldehy that found 16 hybrids (Table 1). In only one case (13421, Fairmont Hot Springs, BC) did nrDNA and maldehy classify the same tree as a hybrid. The conserved nature of the multi-copy nrDNA (up to millions of copies per cell, Liao, 1999) might be due to concerted evolution (Liao, 1999). Liao (1999) argues that because rRNAs are structural molecules, multiple gene copies are necessary to supply the demand for ribosomal subunits in the cell. Since these sub-units function only when assembled into a large complex, homogeneity of rRNAs is critical for regular, functional ribosome assembly and translation to function normally. Liao (1999) concludes that "a possible biological function of concerted evolution is to maintain homogeneous gene copies in a family so that homogeneous transcripts can be produced." However, concerted evolution is thought to be a slow process over numerous generations. Hybrids would seem likely to be heterozygous for both parents nrDNA.

The distribution of cpDNA (petN-psbM) shows a clear trend (Fig. 3) with *J. maritima* petN confined to the western BC, Vancouver Island - Puget Sound, and Olympic Mtns., with the exception of 2 trees in the Wallowa Mtns., OR (WO). Likewise, *J. scopulorum* petN is confined to southeastern BC, eastern WA, Kalispell, MT (KM) and 3 trees in the Wallowa Mtns. (Fig. 3). The pattern is suggestive of *J. scopulorum* pollen flow carrying petN towards the northwest. The four nrDNA hybrids are found in the Williams Lake (WL) and Fairmont Hot Springs (FH) populations (Fig. 4). Interestingly, no typical *J. scopulorum* nrDNA was found in the study area (Fig. 4).

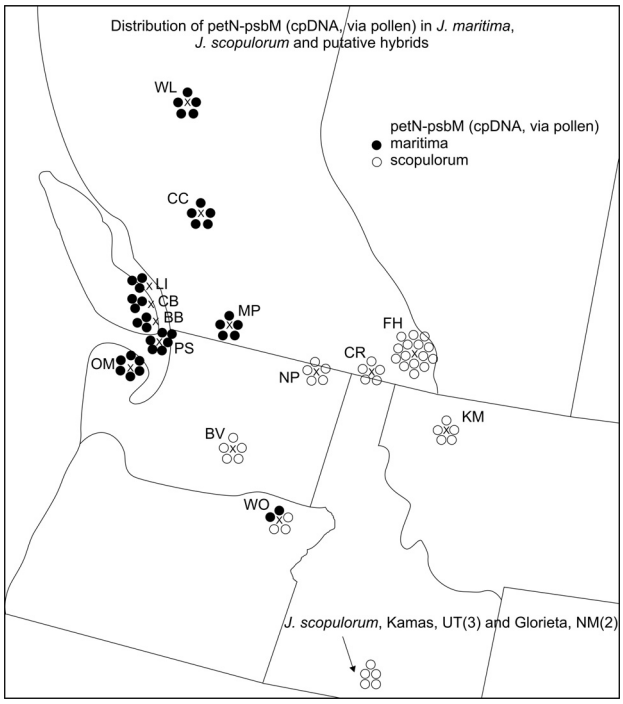

Figure 3. Classification by cpDNA (via pollen). Note the sharp break in western BC in petN.

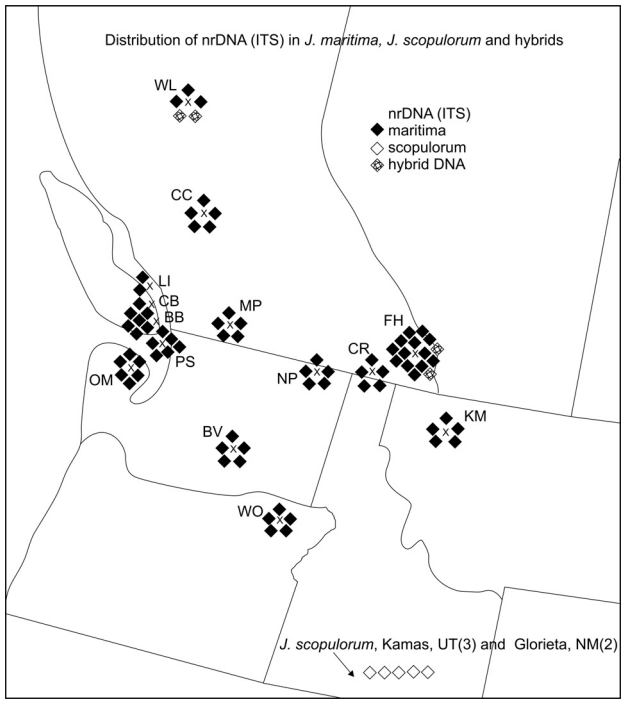

Figure 4. Classification by nrDNA. No typical *J. scopulorum*-nrDNA is present in the study area.

The distribution of maldehy types gives an interesting comparison to nrDNA and petN (Fig. 5). Again, as with nrDNA, no homogenic *J. scopulorum* maldehy trees were found. However, homogenic *J. maritima* maldehy individuals were widespread across the study area (Fig. 5). One hybrid was found in the CC (Cache Creek) population, whereas all the other hybrid maldehy plants were in eastern BC,

Beverly, WA (BV), Wallowa Mtns. (WO) and Kalispell, MT (KM). Kalispell (KM) and Wallowa (WO) are at the northwestern boundary of typical *J. scopulorum* (as judged by the terpene contour map, Figs. 1,2). This distribution is similar to that of petN (via pollen flow) that, again, suggests introgression from *J. scopulorum* via pollen.

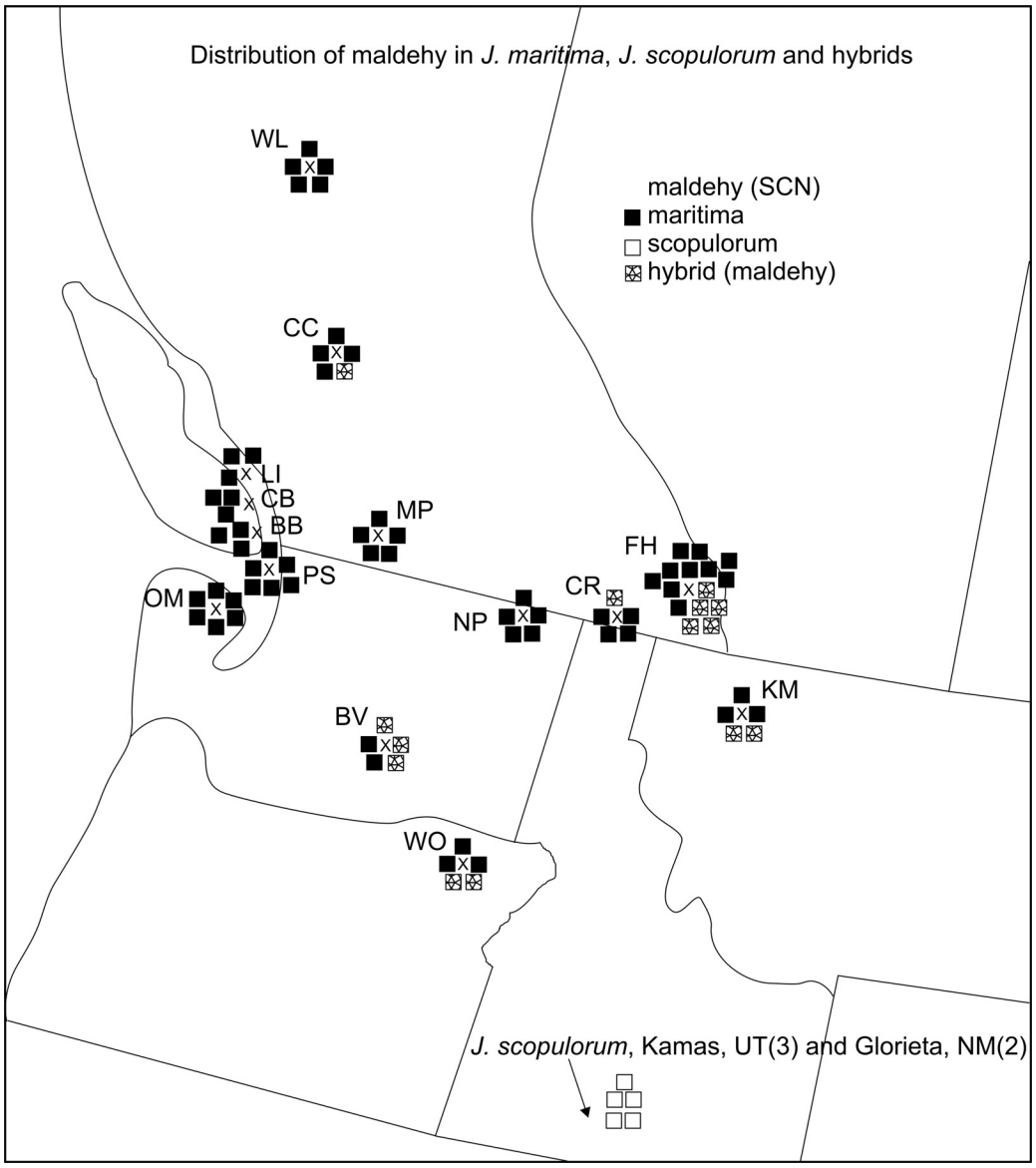

Figure 5. Distribution of *J. maritima, J. scopulorum* and hybrid as per the classification by their maldehy sequences. Notice the lack of any pure, *J. scopulorum*-maldehy individuals.

Figure 6 shows combined mapping using all three gene classifications. The area west of the dashed line appears to separate typical *J. maritima* populations from intermediate individuals (east of the dashed line). Two individuals (in the BB and CC populations) are intermediate in maldehy, along with two individuals at Williams Lake (WL) that are intermediate in nrDNA (Fig. 6). No individuals that are pure in all three genes are present east of the dashed line (central BC and WA). Wallowa (WO) is the only eastern location in which individuals (2) contained *J. maritima* cpDNA (petN). Fairmont Hot

Springs (FH) has the most hybrid individuals as well as the only individual that was classified as an hybrid in both maldehy and nrDNA (Fig. 6).

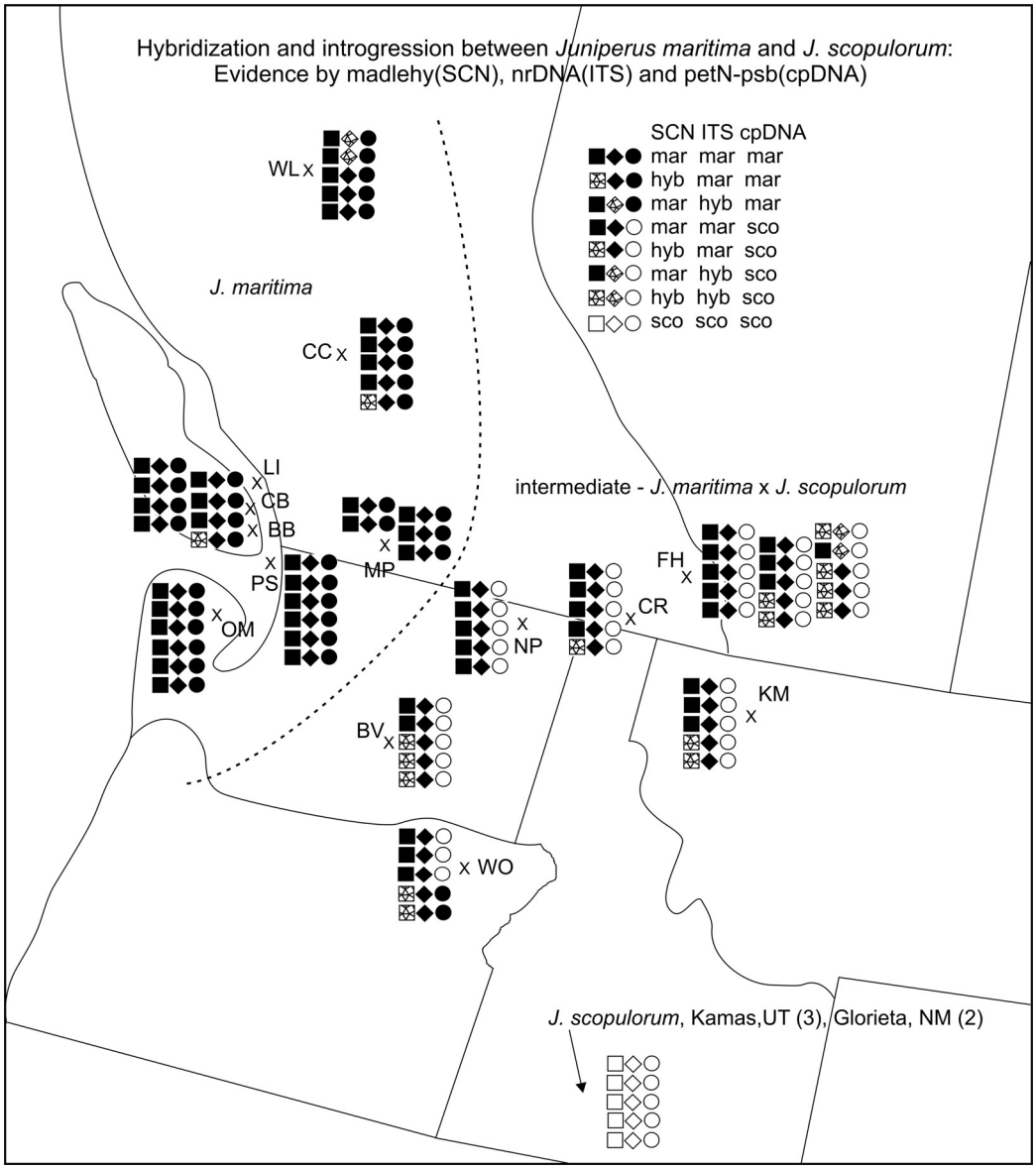

Figure 6. Combined classification based on maldehy, nrDNA, and cpDNA.

Analysis using Principal Coordinates (PCO) of the terpenoid data (Fig. 2) gives a slightly different view from the contoured similarities (Fig. 7). All the central Rocky Mtn. *J. scopulorum* populations are grouped together (shaded oval, Fig. 7). Populations WO, WL, FH, KM, and to a lesser extent, BM, are intermediate, suggesting hybridization and/or introgression. The two divergent northeastern populations (MM, BR) link to RN (Raton, NM), rather than being intermediate to *J. maritima* (Fig. 7), suggesting they are divergent, but not hybrids or introgressants with *J. maritima*. Of course, the divergence of MM and BR could reflect gene flow with *J. virginiana*. The pattern of variation presented by leaf terpenoids (Fig. 7) seems most like that of maldehy (Fig. 5).

It should be noted that although it seems intuitive that hybrids would have intermediate amounts of terpenes, Adams and Tsumura (2012) found that in *Cryptomeria japonica* hybrids, cis-thujopsene,

widdrol and cedrol were inherited in Mendelian fashion with a second (dominant/recessive) gene involved. However, several of the F_1 hybrids had oil very similar to the Haava parent's oil. In a study of the inheritance of the leaf terpenoids of *Pseudotsuga menziesii* var. *menziesii* x var. *glauca*, Adams and Stoehr (2013) found cross *menziesii* 226 x *glauca* 267 produced four hybrids with oils very similar to the glauca parent and 6 F_1 hybrids with intermediate oils. In a second cross, of the 10 major terpenoids, 8 showed dominance with values like one of the parents. Nine of the terpenes were transgressive to both parents. So it may not be unexpected that the PCO of terpenes, in the present study, show the putative hybrids' oils to be more like one of the parents (*J. scopulorum*, Fig. 7).

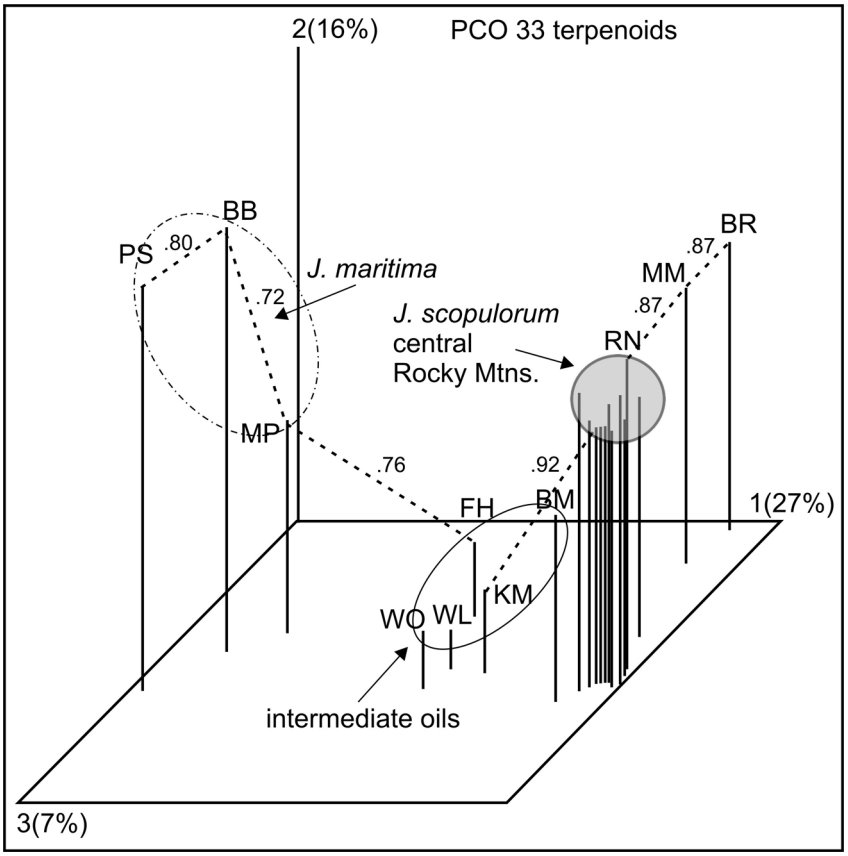

Figure 7. PCO of *J. maritima, J. scopulorum* and putative hybrid populations based on 33 leaf terpenoids. The dashed lines show the minimum spanning network between major groups. Numbers above the lines are similarities.

Pleistocene Patterns

The late Wisconsin maximum ice advance is shown in figure 8 (based on Flint, 1971 and Crandell, 1971). All of the Canadian *J. maritima* and hybrid populations were glaciated. In addition, the Kalispell (KM), Missouri River (MM) and Amidon, ND (AN) populations were probably exterminated. Other populations (BM, BR and NW) were likely displaced by boreal forests and tundra (Flint, 1971; Porter, 1971). *Juniperus scopulorum* is a lower montane species. With the widespread lowering of vegetation zones, it likely moved to lower, drier habitats throughout most of the central Rocky Mountains. Adams (1983) reviewed the literature on packrat middens and pollen profiles. Wells (1970) and Martin and Harwell (1957) suggested that life zones descended 300 to 1100 m throughout the southwest and Great Basin from 13,500 to 10,000 ybp. The current separation of *J. scopulorum* and *J. virginiana* appears to have been bridged with the eastward expansion of *J. scopulorum* and the western expansion of *J. virginiana*. Trees of *J. scopulorum* are currently growing in ravines in northeastern New

Mexico and western Oklahoma panhandle, while *J. virginiana* has now migrated westward into the Canadian River canyons in the Texas panhandle. The population of *J. scopulorum/virginiana* in Palo Duro Canyon resembles both species and is likely a relictual stand of hybrid origin (Adams, 1983).

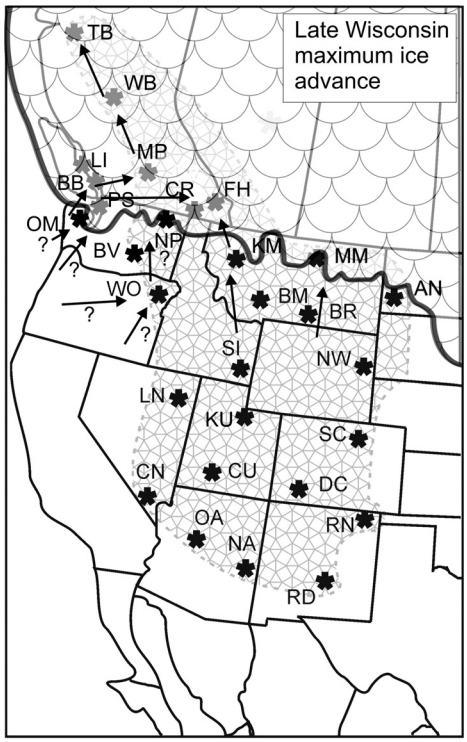

Figure 8. Putative re-colonization routes *J. maritima* and *J. scopulorum* following maximum ice advance during the late Wisconsin (ice boundary based on Flint, 1971;Crandell, 1971).

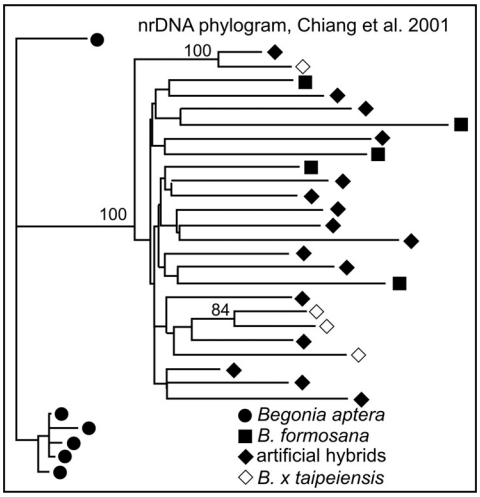

Figure 9. Phyogram based on nrDNA for *Begonia* and hybrids (adapted from Chiang, et al. 2001).

With the retreat of the Wisconsin glacial ice, and the subsequent altithermal period 9000 to 5000 ybp (Wells, 1970), *Juniperus* expanded into the drying, higher elevation habitats that it occupies today. Figure 8 shows the proposed post-Pleistocene re-colonization of the northern portion of the ranges of *J. maritima* and *J. scopulorum*. The *J. maritima* BC populations could have been recolonized by seed from a Wallowa Mtns. refugium (WA, Fig. 8) and thence northward to the present day northern-most population at Telkwa, BC (TB). At Telkwa, *J. scopulorum* is found on dry, southeast facing slopes (ca. 45º - 60º). It seems likely that *J. maritima* that grows along the seashore in western BC and Puget Sound, WA was re-colonized from a refugium south of the Olympic Mtns. or western WA/ Oregon.

Of course, the Wallowa population may have been displaced lower, and perhaps a bit to the south during the Wisconsin. The Amidon, ND (AN) population is similar to populations in the central Rocky Mountains and seems likely to have been derived by seed from the nearest *J. scopulorum* population (perhaps near Newcastle, WY, NW) or any of the scarp-land *J. scopulorum* populations to the south.

It may be that heterozygous trees are the result of previous hybridization(s) or a relict from speciation. Or, the nrDNA may reflect concerted evolution in homogenizing individuals. However, Chaing et al. (2001) found that in the artificial hybrids between *Begonia aptera* (pollen) and *B. formosana* (ovule), nrDNA was predominantly that of the maternal parent, *B. formosana* (Fig. 9). Volkov, et al. (1999) reported that one of the parental nrDNAs was eliminated the allopolyploid genome of cultivated tobacco. Fukuoka et al. (1994) found that the nrDNA in γ-ray irradiated tetraploid rice was homogenized in a short time.

Aguilar et al. (1999) made artificial hybrids between *Armeria villosa* ssp. *longiaristata* and *A. colorata;* then examined the inheritance of nrDNA in F_1 and F_2 generations. They found the expected additive pattern in polymorphisms for five of the six variable sites in F_1 plants. However, in the F_2 generation, there was a bias towards one parent (*A. colorata*). Backcrosses showed homogenization of five of the polymorphic sites to the recurrent parent.

Okuyama et al. (2005) examined introgression in *Mitella* using nrDNA ITS and ETS, and cpDNA and found

that cpDNAs revealed the most introgression, ITS regions showed a moderate amount and the ETS region gave no evidence of introgression. They concluded that non-uniform concerted evolution between the ETS region and ITS regions may explain these different patterns of introgression.

Additional studies are needed to resolve this and to determine the extent of introgression of *J. maritima* genes into the range of *J. scopulorum* in Montana and adjacent areas (research in progress).

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Table 1. Classification of 80 *Juniperus* individuals based on maldehy (SCN), nrDNA and petN-psbM (cpDNA).

Samples (trees)	maldehy	nrDNA	petN/psbM
10895 scopulorum, Kamas, UT	scop	scop	scop
10896 scopulorum, Kamas, UT	scop	scop	scop
10897 scopulorum, Kamas, UT	scop	scop	scop
10933 scopulorum, Glorietta, NM	scop	scop	scop
10934 scopulorum, Glorietta, NM	scop	scop	scop
11056 maritima, Brentwood Bay, VI	marit	marit	marit
11057 maritima, Brentwood Bay, VI	marit	marit	marit
11058 maritima, Brentwood Bay, VI	marit	marit	marit
11061 maritima, Cowichan Bay, VI	marit	marit	marit
11062 maritima, Cowichan Bay, VI	marit	marit	marit
11063 maritima, Cowichan Bay, VI	**hybrid**	marit	marit
11999 maritima, Olympic Mtns., 912m,	marit	marit	marit
12000 maritima, Olympic Mtns., 912m,	marit	marit	marit
12001 maritima, Olympic Mtns., 912m,	marit	marit	marit
12002 maritima, Olympic Mtns., 1671m,	marit	marit	marit
12003 maritima, Olympic Mtns., 1671m,	marit	marit	marit
12004 maritima, Olympic Mtns., 1671m,	marit	marit	marit
11064 maritima, Yellow Point Lodge, VI	marit	marit	marit
11065 maritima, Lesqueti Island, BC	marit	marit	marit
11066 maritima, Lesqueti Island, BC	marit	marit	marit
11067 maritima, Friday Harbor, San Juan	marit	marit	marit
11068 maritima, English Camp, San Juan	marit	marit	marit
11075 maritima, sand dune, Whidbey Isl.	marit	marit	marit
11076 maritima, Fidalgo Isl. St. Pk	marit	marit	marit
11077 maritima, Skagit Isl. ca 360 yr old	marit	marit	marit
11078 maritima, Skagit Isl., WA	marit	marit	marit
13426 maritima, Manning Park, BC	marit	marit	marit
13427 maritima, Manning Park, BC	marit	marit	marit
13428 maritima, Manning Park, BC	marit	marit	marit
13429 maritima, Manning Park, BC	marit	marit	marit
13430 maritima, Manning Park, BC	marit	marit	marit
13431 Cache Ck, BC	marit	marit	marit
13432 Cache Ck, BC	marit	marit	marit
13433 Cache Ck, BC	**hybrid**	marit	marit
13434 Cache Ck, BC	marit	marit	marit
13435 Cache Ck, BC	marit	marit	marit
13436 Williams Lake, BC	marit	**hybrid**	marit
13437 Williams Lake, BC	marit	marit	marit
13438 Williams Lake, BC	marit	**hybrid**	marit
13439 Williams Lake, BC	marit	marit	marit
13440 Williams Lake, BC	marit	marit	marit
13421 Fairmont Hot Sprs, BC	**hybrid**	**hybrid**	**scop**
13422 Fairmont Hot Sprs, BC	marit	marit	scop
13423 Fairmont Hot Sprs, BC	marit	marit	scop
13424 Fairmont Hot Sprs, BC	marit	marit	scop
13425 Fairmont Hot Sprs, BC	marit	marit	scop
14001 Fairmont Hot Sprs, BC	marit	**hybrid**	scop

Samples (trees)	maldehy	nrDNA	petN/psbM
14002 Fairmont Hot Sprs, BC	marit	marit	scop
14003 Fairmont Hot Sprs, BC	marit	marit	scop
14004 Fairmont Hot Sprs, BC	**hybrid**	marit	scop
14005 Fairmont Hot Sprs, BC	marit	marit	scop
14006 Fairmont Hot Sprs, BC	**hybrid**	marit	scop
14007 Fairmont Hot Sprs, BC	**hybrid**	marit	scop
14008 Fairmont Hot Sprs, BC	**hybrid**	marit	scop
14009 Fairmont Hot Sprs, BC	**hybrid**	marit	scop
14010 Fairmont Hot Sprs, BC	marit	marit	scop
14026 Creston, BC	marit	marit	scop
14027 Creston, BC	marit	marit	scop
14028 Creston, BC	**hybrid**	marit	scop
14029 Creston, BC	marit	marit	scop
14030 Creston, BC	marit	marit	scop
14031 Northport, WA	marit	marit	scop
14032 Northport, WA	marit	marit	scop
14033 Northport, WA	marit	marit	scop
14034 Northport, WA	marit	marit	scop
14035 Northport, WA	marit	marit	scop
14036 Beverly, WA	**hybrid**	marit	scop
14037 Beverly, WA	marit	marit	scop
14038 Beverly, WA	marit	marit	scop
14039 Beverly, WA	**hybrid**	marit	scop
14040 Beverly, WA	**hybrid**	marit	scop
12995 Kalispell, MT	marit	marit	scop
12996 Kalispell, MT	marit	marit	scop
12997 Kalispell, MT	**hybrid**	marit	scop
12998 Kalispell, MT	**hybrid**	marit	scop
12999 Kalispell, MT	marit	marit	scop
11935 Wallowa Mtns, OR	**hybrid**	marit	scop
11936 Wallowa Mtns, OR	**hybrid**	marit	scop
11937 Wallowa Mtns, OR	marit	marit	scop
11938 Wallowa Mtns, OR	marit	marit	scop
11939 Wallowa Mtns, OR	marit	marit	scop