

**DISCOVERY AND SNPS ANALYSES OF POPULATIONS OF
JUNIPERUS MARITIMA IN THE OLYMPIC PENINSULA,
A PLEISTOCENE REFUGIUM?**

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ABSTRACT

Trees from two populations of *Juniperus* commonly identified as *J. scopulorum* growing on the northeast side of the Olympic mountains were analyzed by SNPs from nrDNA and petN-psbM (cpDNA) and compared with *J. scopulorum* (UT), *J. virginiana* (TN) and *J. maritima* from Puget Sound. The trees of the Olympic Mountains were found to be *J. maritima*. The habitat of these newly investigated populations of *J. maritima* are atypical in that they occupy high elevation sites (600 - 1700 m), whereas most previous *J. maritima* sites are seaside. The Deer Park population (1700 m) plants are Krumholz due to the high winds and snowpack conditions. The Cordilleran ice sheet is mapped and the Olympic peninsula is proposed as the site of a Pleistocene refugium for *J. maritima*. *Phytologia* 92(1): 68-81 (April, 2010).

KEY WORDS: *Juniperus maritima*, *J. scopulorum*, Olympic Peninsula, Olympic Mountains, nrDNA, petN-psbM, SNPs.

Juniperus maritima R. P. Adams, the seaside juniper, is a rare species found on the seashores in the Puget Sound - Strait of Georgia area (Adams, 2007, 2008). Examination of herbarium specimens revealed that *Juniperus* trees identified as *J. scopulorum* Sarg. have been reported from the dry side (northeast) of the Olympic Mountains on the Olympic Peninsula of Washington. The female cones on these specimens had insect damage and the exerted seeds typical of *J. maritima*. Specimens were analyzed from 2-4 m trees at 3 o'clock ridge (912 - 960 m) and Krumholz shrubs (25-30 cm tall) at Deer Park (1670 m) on the northeast side of the Olympics.

The purpose of this paper is to report on the analyses of SNPs from nrDNA and petN-psbM (cpDNA) for these putative *J. maritima* plants in comparison with *J. maritima* from seaside locations and with typical *J. scopulorum* from Utah.

MATERIALS AND METHODS

Specimens used in this study: *J. maritima*, Adams 11056-58, Brentwood Bay, Vancouver Isl., BC, Adams 11061-63, Cowichan Bay, Vancouver Isl., BC, Adams 11064, Yellow Point, Vancouver Isl., BC, Adams 11065-66, Lesqueti Isl., BC, Adams 11067-68, Friday Harbor, San Juan Isl., WA, Adams 11075, Whidbey Isl., Cranberry L., WA, Adams 11076, Washington Park, Fidalgo Island, Anacortes, WA, Adams 11077-78, Skagit Isl., WA; Adams 11999-12001, 912-960m, 3 o'clock ridge, Olympic National Forest, WA, Adams 12002-12004, 1670 m, Dear Park, Olympic National Park, WA, *J. virginiana*, Adams 10230-10232, Knoxville, TN; *J. scopulorum*, Adams 10895- 10897, Kamas, UT. 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 using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia CA).

PCR amplification ITS (nrDNA), petN-psbM 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) 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 PCR reaction was subjected to purification by agarose gel electrophoresis (1.5% agarose, 70 v, 55 min.). In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit. The gel purified DNA band with the appropriate 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.). Alignments and NJ trees were made using MAFFT (<http://align.bmr.kyushu-u.ac.jp/mafft/>). Minimum spanning networks were constructed from SNPs data using PCODNA software (Adams et al., 2009).

Associational measures were computed using absolute compound value differences (Manhattan metric), divided by the maximum observed value for that compound over all taxa (= Gower metric, Gower, 1971; Adams, 1975). Minimum spanning networks were constructed (Adams et al. 2009). Principal coordinate analysis was performed by factoring the associational matrix based on the formulation of Gower (1966) and Veldman (1967).

RESULTS AND DISCUSSION

Analysis of the nrDNA (ITS) sequences revealed 36 mutational events of which 19 mutations were found in only one individual and removed from the data. This resulted in 17 SNPs among *J. maritima*, *J. scopulorum*, *J. virginiana*, and the junipers from the Olympics for analysis. A minimum spanning network based on these 17 SNPs from nrDNA is shown in figure 1. The individuals are arranged in three groups: *J. virginiana*, Knoxville, TN, no variation; *J. scopulorum*, Kamas, UT with some variation; and the central group consisting of *J. maritima* from Puget Sound - Strait of Georgia and the

plants from the Olympic mountains with a few SNPs separating individuals, but no pattern among them. Individuals from the Olympic mountains are intermixed with *J. maritima* from seaside locations.

Analysis of petN-psbM (cpDNA) sequences yielded 52 mutational events. Fourteen mutations were found in only one individual and removed from the data leaving 38 SNPs among *J. maritima*, *J. scopulorum*, *J. virginiana*, and the junipers from the Olympics for analysis. A minimum spanning network based on these 38 SNPs from petN-psbM sequences is shown in figure 2.

Two plants (11075, Whidbey Isl., Cranberry L., WA, Adams 11076, Washington Park, Anacortes, WA) share a haplotype that differs by 17 SNPs from other *J. maritima* plants. In contrast with the nrDNA (Fig. 1), only two groups are present: *J. scopulorum* (bottom) and a diverse group in the center consisting of *J. virginiana*, *J. maritima* from seaside locations and the Olympic mountains plants. Adams (2007) reported that cpDNA did not fully separate *J. maritima* and *J. virginiana* plants. It is interesting that the Olympics plants do form distinct groups (Fig. 3). However, the 3 o'clock ridge (960m) plants are intermixed with the Deer Park (1670 m) Krumholz plants (Fig. 3).

Removing the 2 divergent haplotypes of *J. maritima* and combining the sequences (nrDNA plus petN-psbM) resulted in 34 SNPs. A minimum spanning network based on these 34 SNPs (Fig. 3) shows three groups: *J. virginiana*, *J. scopulorum* and *J. maritima* from seaside locations and the Olympic Mountains plants. It is interesting that the Olympic Mountains plants are in the center of the network, between *J. scopulorum*, *J. virginiana* and *J. maritima* seaside plants. There is considerable variation among the *J. maritima* plants, but it is clear that the Olympic Mountains plants are part of *J. maritima*, and not *J. scopulorum* nor *J. virginiana*.

The presently understood distribution of *J. maritima* is shown in figure 4. It seems likely that additional populations will be found on islands and the seashore of the Strait of Georgia. It appears that *J.*

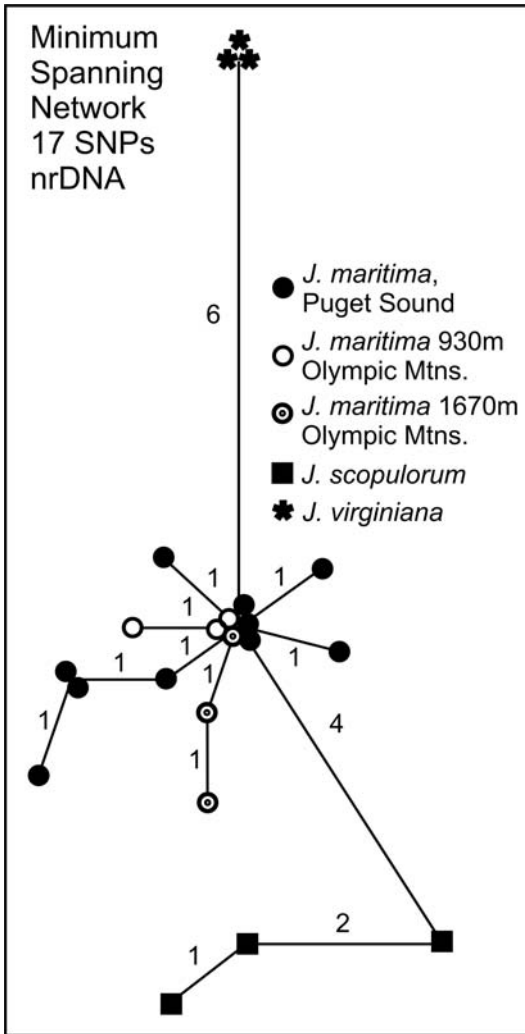


Figure 1. Minimum spanning network based on 17 SNPs from nrDNA. Notice the central group composed of seaside *J. maritima* from Puget Sound and the putative *J. maritima* from the Olympic mountains. Numbers next to the lines are the number of SNPs in that link.

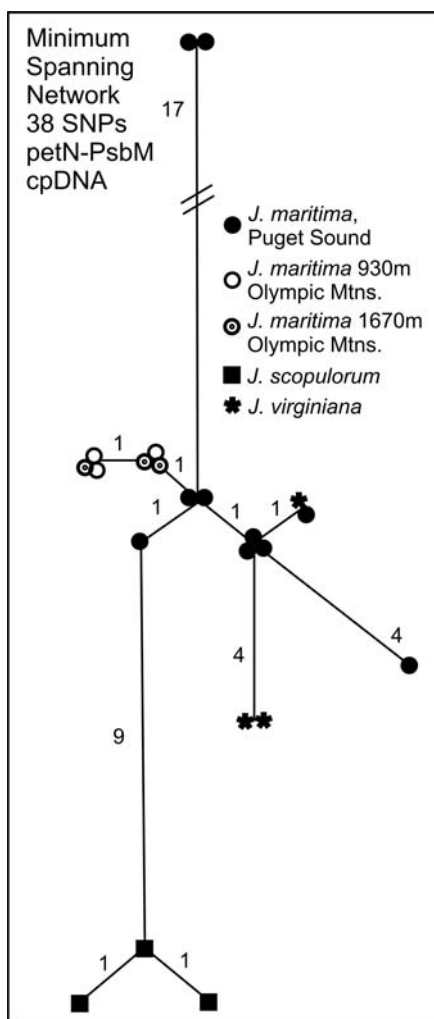


Figure 2. Minimum spanning network based on 38 SNPs from petN-psbM sequences. Note that two *J. maritima* plants (Fidalgo and Whidbey Islands) share a haplotype that differs by 17 SNPs from other *J. maritima* plants.

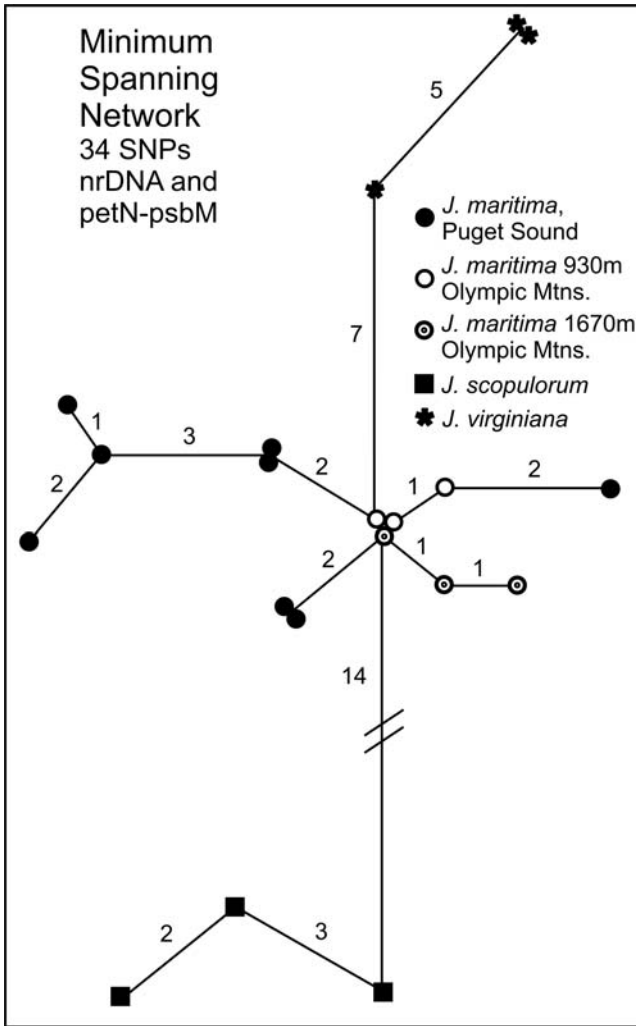


Figure 3. Minimum spanning network based on 34 SNPs from both nrDNA and petN-psbM.

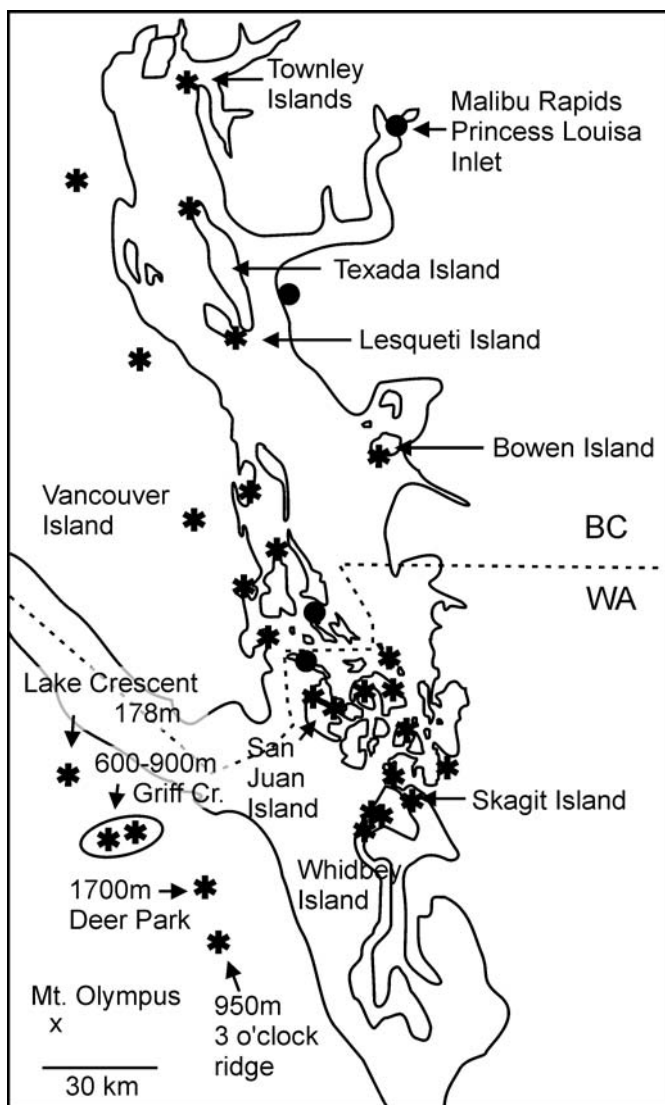


Figure 4. Distribution of *Juniperus maritima* based on Adams field collections, herbarium specimens from V, WS, and WTU (asterisks), and sight observations by Tom Fairhall (closed circles).

maritima's northernmost populations are on the Townley Islands and at Malibu Rapids at the Princess Louisa Inlet (Fig. 4). *Juniperus maritima* trees in the Olympics Mountains below 960 m are shorter than the seaside trees in Puget Sound - Strait of Georgia (Fig. 5). The population of *J. maritima* at Deer Park is Krumholz (Fig. 6) and only about 25-30 cm tall.



Fig. 5. *Juniperus maritima* as a tree on 3 o'clock ridge, 960m, Olympic National Forest, mixed with *Pseudotsuga menziesii*.

Adams (1983), using terpenoid data, considered the Puget Sound junipers to be divergent populations of *J. scopulorum*. He examined Pleistocene refugia and recolonization and hypothesized that the Puget Sound populations prior to extinction moved south of the Olympic peninsula, then recolonized Puget Sound when the Cordilleran ice sheet retreated (Figs. 9, 10; Adams, 1983). All of the present seaside populations of *J. maritima* in Puget Sound and the Strait of Georgia were under the Cordilleran ice sheet (Fig. 7). The present-day high elevation populations in the Olympic Mts. were likely at the very edge or under alpine glaciers (Fig. 4, Buckingham et al. 1995).

However, most of the Olympic Mountains were thought to be ice free (Fig. 7), except for glaciers at higher elevations.



Fig. 6. *Juniperus maritima* as a Krumholz shrub at 1670 m at Deer Park, Olympic National Park.

Buckingham et al. (1995) suggest a strip of land along the west coast of the Olympic peninsula as wide as 50 km was exposed during the Wisconsin to provide additional plant refugia. They considered it unlikely that *J. scopulorum* (*J. maritima*) retreated to northwestern Oregon, but rather, found a refugium in the Olympic Mountains itself. Lacking any fossil evidence, one can only conjecture that *J. maritima* was in fact a 'seaside' juniper in the Strait of Georgia and Puget Sound before the Pleistocene.

Although it has been widely held view that species retreated from glacial ice into warmer refugia, Opgenoorth et al. (2010) present DNA evidence that populations of *Juniperus* on the Tibetan plateau may have persisted in cryptic or micro-refugia within a few hundred

meters of glacial ice during the Pleistocene. The micro-refugia theory is controversial and additional cases need to be examined to validate it. Nevertheless, it does lead to an interesting hypothesis that *J. maritima* might have survived near the front edge of the Puget Lobe of the Cordilleran ice field. With the discovery of *J. maritima* at high elevation sites (this paper), it does seem possible that *J. maritima* survived the Pleistocene in the Olympic peninsula. It may be that before the Pleistocene, *J. maritima* was a montane species and that it merely spread to open habitat on rocky seashores in the Strait of Georgia and Puget Sound during the Hypsithermal (10,000 - 6,000 ybp, see Buckingham et al. 1995). The DNA data presented in this study did not show much differentiation between the seaside populations and the montane populations of the Olympic mountains. Additional analyses of more sensitive markers may reveal differentiation, but at present it appears that the seaside populations of *J. maritima* are of very recent origin with little differences from the montane (ancestral) populations.

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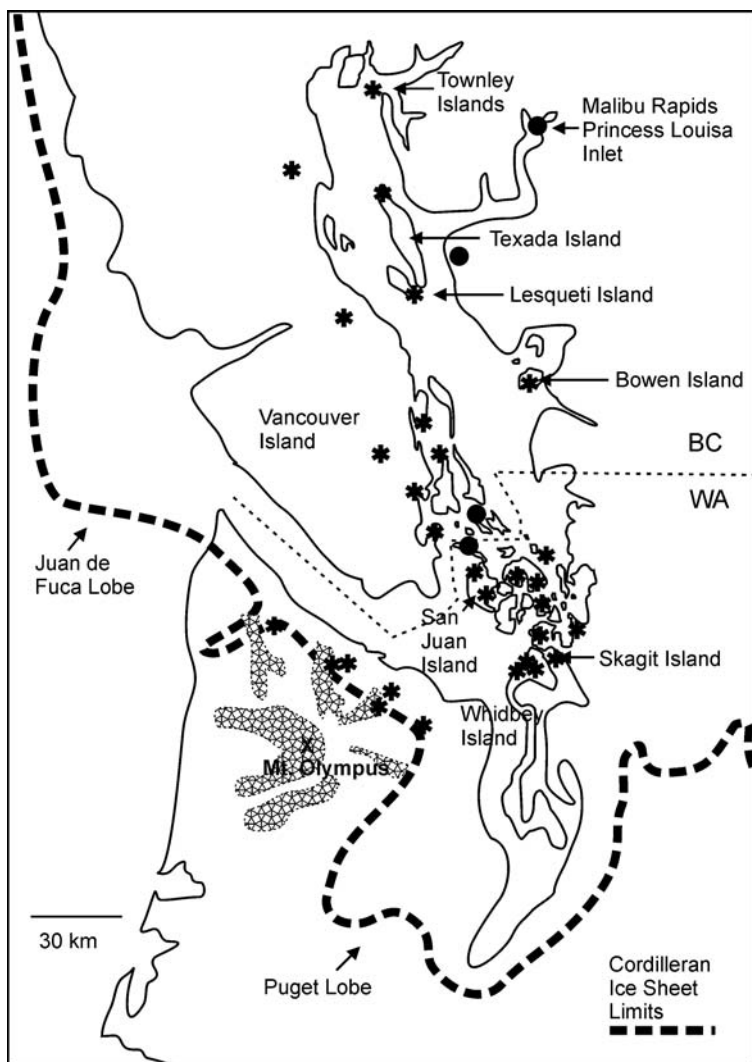


Figure 7. Cordilleran ice sheet limits. Cross-hatched areas around Mt. Olympus are hypothetical glaciers. Based on Booth et al. (2004) and Buckingham, et al. (1995).

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