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**Long distance gene flow facilitated by bird-dispersed seeds in wind-pollinated species: A story of hybridization and introgression between *Juniperus ashei* and *J. ovata* told by nrDNA and cpDNA**

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**ABSTRACT**

nrDNA and cpDNA were sequenced of *J. ashei* and *J. ovata* from populations throughout their ranges. No *J. ashei* populations were found to be pure in their nrDNA for every tree, however all *J. ashei* trees in every population contained only the pure *J. ashei* chloroplast type. Populations of *J. ovata* in trans-Pecos Texas were almost pure in both nrDNA and cp DNA. Several plants in the *J. ashei* range contained *J. ovata*-type nrDNA (White Cliffs, AR, 3/10); Ranger, TX (1/5); Waco, TX (1/12). Every *J. ashei* population contained at least 1 plant with hybrid (heterozygous) nrDNA and 3 *J. ovata* populations contained putative hybrids (by nrDNA), but one population had only pure *J. ovata* trees. The presence of *ovata* germplasm within *J. ashei* populations seems best explained by long distance bird dispersal of *J. ovata* seeds (thence seedlings and *J. ovata* trees and hybrids) in the disjunct *J. ashei* populations. The reason for the absence of *ovata* paternal cp, which is distributed by pollen in *J. ashei* populations is not known. Judged by distribution of cp data, there is very little movement of cp genomes. In contrast, nrDNA polymorphisms indicate there is considerable gene flow between *J. ashei* and *J. ovata*, but primarily in the direction of *J. ovata* to *J. ashei* which may be explained by a combination of bird migration pattern and recurring and preferential F1-hybrid formation. *Published on-line www.phytologia.org Phytologia 102(2): 55-74 (June 24, 2020). ISSN 030319430.*

**KEY WORDS:** *Juniperus*, *J. ashei*, *J. ovata*, essential oils, distribution, Cupressaceae

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About 50 years ago, one of the authors (RPA) began a series of studies using leaf volatile oils examining variation in *J. ashei* populations (Adams, 1969). Surprisingly, it was discovered that the volatile leaf terpenoids had almost no variation (Adams and Turner 1970; Adams 1969; 1975; 1977) across hundreds of miles (Fig. 1) from the Texas hill country to the Ozark mountains in Arkansas-Missouri (the type locality is Sylamore, AR, Buchholz 1930). In fact, the gas chromatogram traces were so similar that one could lay the chromatograms on top of each other and see no differences. Over the ensuing 50 years of terpenoid analyses on all the 76 *Juniperus* species (Adams 2014), this uniformity has not been encountered in any other *Juniperus* species. However, several divergent populations were subsequently identified in the semi-arid trans-Pecos, Texas region (Ozona, Comstock) and adjacent Mexico (116, Sierra del Carman, Fig. 1). In addition, trees from New Braunfels, TX were found to have leaf terpenoid composition most similar to that found in the trans-Pecos/ Mexico region (Fig. 1). Follow-up research using RAPDs (Random Amplified Polymorphic DNAs) combined with morphological, and terpenoid differences led to the naming of the divergent populations as *J. ashei* var. *ovata* R. P. Adams (Adams and Baker 2007). Later use of DNA sequencing led to the recognition of *J. ashei* var. *ovata* at the specific level, *J. ovata* (R. P. Adams) R. P. Adams (Adams and Schwarzbach 2013). Thus, we will use *J. ovata* in place of the term ‘divergent populations’ of Adams (1977) throughout the remainder of this paper.

Comparing the leaf essential oils of *J. ashei* with *J. ovata*, revealed that they differ mostly in a quantitative fashion (Appdx. 1). —Camphor content is considerably larger in *J. ashei* (69.1%) than in *J. ovata* (53.5%, Appdx. 1). In contrast, bornyl acetate concentration is much larger in *J. ovata* (15.6%) than in *J. ashei* (6.3%)(Appdx. 1). In addition, four (non-trace) compounds differ qualitatively: trans-sabinene hydrate, trans-p-menth-2-en-1-ol, verbenone, and sandaracopimara-8(14),15-diene (Appdx.1) with all four occurring in *J. ashei*, but not *J. ovata*. Several other compounds differ quantitatively:  $\alpha$ -pinene, myrcene, p-cymene, limonene,  $\gamma$ -terpinene, linalool, trans-carveol, carvone and elemol (Appdx. 1).

*Juniperus ovata* is generally easy to identify by the oval (elliptical) glands, especially on the whip (decurent) leaves (Fig. 2). Notice hemispherical glands on *J. ashei* (Fig. 2, left) and the raised, oval to elongated glands on *J. ovata* (Fig. 2, right).

It should be noted that a few nearly hemispherical glands are present on whip leaves of *J. ovata*. This is informative, as these characters can be used to distinguish *J. ovata* from *J. ashei*, yet exclude other nearby junipers species such as *J. monosperma* (Englem.) Sarg. *J. pinchotii* Sudw. and *J. coahuilensis*. (Mart.) Gausson ex R. P. Adams. *Juniperus ovata* also has smaller cones, and more seeds per cone than *J. ashei*. (Table 1).

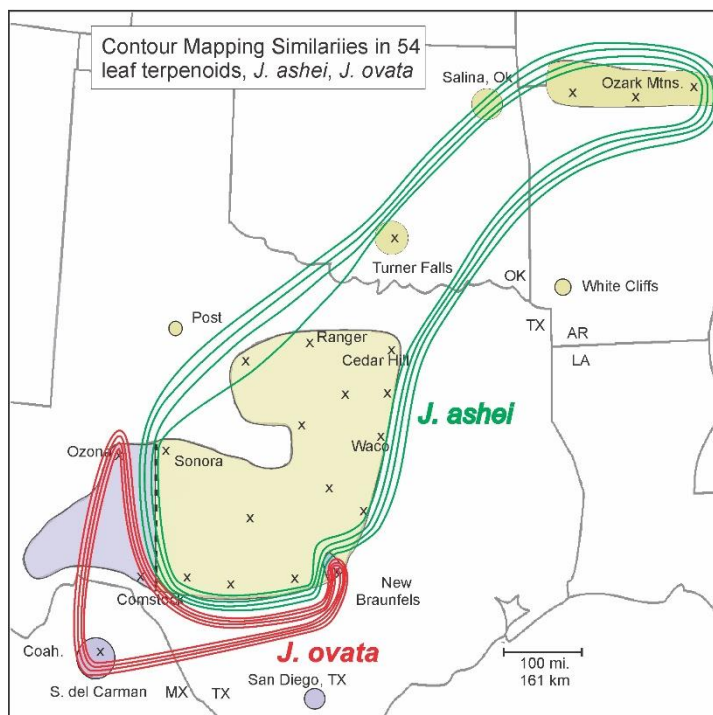


Figure 1. Contoured leaf oil similarities of populations of *J. ashei* and *J. ovata*. Adapted from Adams, 1977.

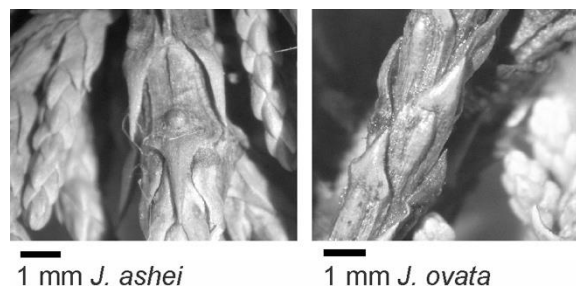


Figure 2. Comparison of whip leaf glands of *J. ashei* and *J. ovata*.

Table 1. Morphological differences between *J. ashei* and *J. ovata*.

| Character                   | <i>J. ashei</i>                 | <i>J. ovata</i>                     |
|-----------------------------|---------------------------------|-------------------------------------|
| female cone diameter        | larger (8)9(10) mm              | smaller (5)6(8) mm                  |
| seeds per cone              | fewer (1.01)                    | more (1.7)                          |
| seed size (L x W)           | larger (16-27 mm <sup>2</sup> ) | smaller (13-16 mm <sup>2</sup> )    |
| whip leaf gland L/ sheath L | smaller ratio (0.20-0.30)       | larger ratio (> 0.40)               |
| whip leaf gland shape       | hemispherical (1.0 - 1.5)       | raised, oval to ellipse (2.0 - 2.5) |
| branching angle             | narrow (45 - 40°)               | wider (45 - 55°)                    |

In the original study (Adams, 1977), the New Braunfels population of *Juniperus ovata* was represented by samples from 15 individuals from a single population 8 km west of New Braunfels. The nearest populations sampled (Adams, 1977) were at Bandera and Hyde (80 - 100 km w and nw of New Braunfels) and these had typical *J. ashei* leaf terpenoids. To determine if *J. ovata* extended further west, Adams (2008) obtained new samples from New Braunfels to the junction of US 281 and TX Hwy 46. Because tricyclene is fairly constant in *J. ashei*, by merely examining if the height of the  $\alpha$ -pinene peak (that runs just after tricyclene on DB-5) is greater than tricyclene, one can determine that the oil is from *J. ovata*, whereas if  $\alpha$ -pinene is less than tricyclene, the oil is from *J. ashei*.

Figure 3 shows that the samples taken along TX 46 from US 281 to near loop 337 are all low in  $\alpha$ -pinene. This is typical for *J. ashei*. The samples from loop 337 (L) are high in  $\alpha$ -pinene that is typical of *J. ovata*. The samples of *J. ovata* from the National Big Tree site (N) and nearby are uniformly high in  $\alpha$ -pinene. Two of the samples along FM 482 are typical var. *ovata*, but the third sample is more like *J. ashei*.

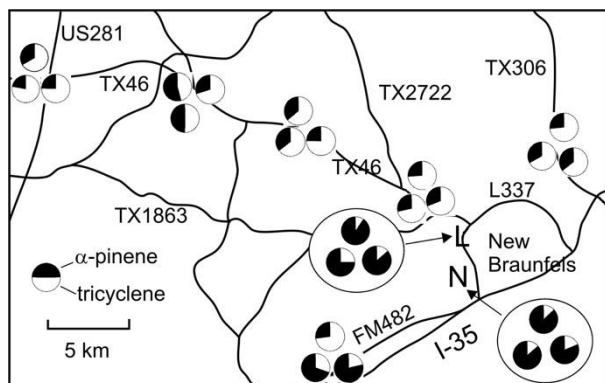


Figure 3. Distribution of *J. ashei* and *J. ovata* in the New Braunfels area based on the concentration of tricyclene and  $\alpha$ -pinene. From Adams (2008).

Geographic variation in camphor and bornyl acetate show the same pattern (Fig. 4). However, at least one individual in both the FM 482 and the TX 306 populations appear to be intermediate between *J. ashei* and *J. ovata*, suggesting some hybridization between the taxa.

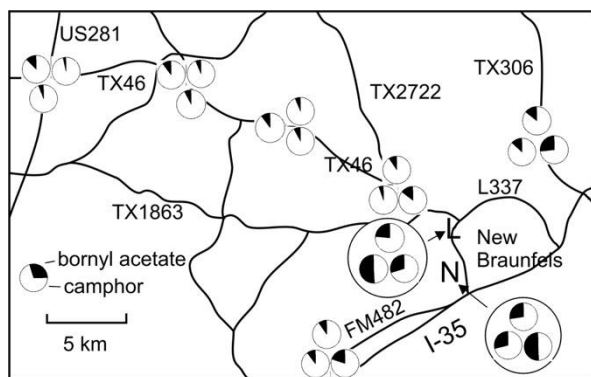


Figure 4. Distribution of *J. ashei* and *J. ovata* based on the concentration of bornyl acetate and camphor. From Adams (2008).

A preliminary study of nrDNA and cpDNA from the samples of Adams (2008) near New Braunfels, revealed a more complex pattern of hybridization and potential introgression than originally seen in the terpene analyses. The purpose of this paper is to present the results of a more exhaustive study of variation in nrDNA (ITS) and cpDNA throughout the ranges of *J. ashei* and *J. ovata*.

## MATERIALS AND METHODS

Figure 5 shows the distribution of *J. ashei* and *J. ovata* with the populations sampled for this study. Additional samples were collected in the Comal Co. - New Braunfels areas (Fig. 6).

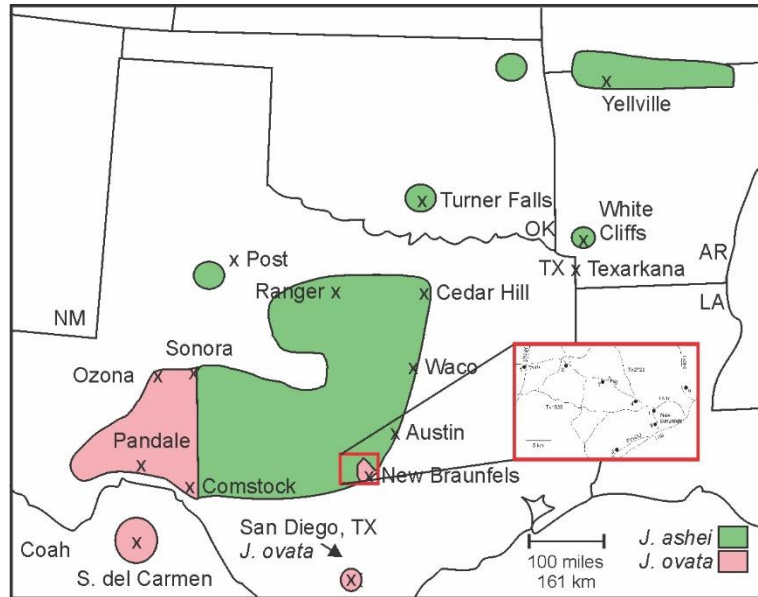


Figure 5. Distribution of *J. ashei* and *J. ovata*. Adapted from Adams (2014). The detailed Comal Co. study area is indicated by the fly-out box. X's mark the populations studied.

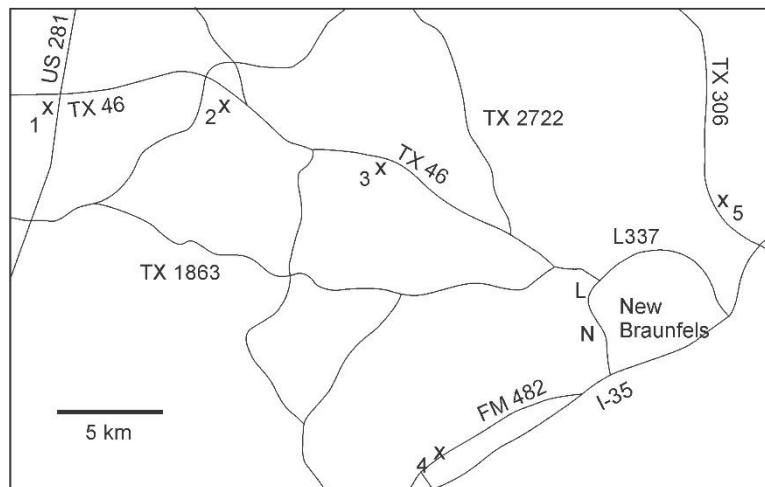


Figure 6. Populations sampled in Comal Co. and New Braunfels area in the fly-out box.

### Specimens used in this study:

*Juniperus ashei*: Comal Co., TX: Popn. 1, jct TX46 & US 281, Adams 11295, 11296, 11297; Popn. 2, on TX 46, 8 km e of jct TX 46 and US 281, Adams 11298, 11299, 11300; Popn. 3, on TX 46, 16 km e of jct TX 46 and US 281, Adams 11301, Popn. 5, on TX 306, 1 km nw of Hunter Rd, Adams 11322, 11323, 11324.

Yellville AR: Adams 10215-10219; White Cliffs, AR: Adams 14071-14080; Turner Falls, OK, Adams 14094-14100; Cedar Hill, TX: Adams 12007-12011; Benbrook Lake, TX Adams 14091-14092; Ranger, TX: Adams 12012-12015; Cameron Park, Waco, TX: Adams 14081-14090; Bosque Blvd and Hwy 6, Waco,

TX: Adams 6746, 6752; Texas A & M Extension Station (TAES), Sonora, TX: Adams 12250-12269, as part of a study on deer browsing (Adams et al. 2013);

*J. ovata*: Comal Co., TX: Popn. L, Loop 337, 1 km s of jct TX 46 and Loop 337, Adams 11314, 11315, 11316; Popn. N, 40 m w of jct Cedar Elm St. and Madeline St. on Madeline St. (site of the National Big Tree for *J. ashei*), New Braunfels, Adams 11309, 11317, 11318; Popn. 4, 100 m n of jct Hubertus Rd. and FM 482 on FM 482, Adams 11319, 11320, 11321.

Ozona, TX: Adams 7470, 7473, 12280-12284; Comstock, TX 12270-12274; Pandale, TX: Adams 12275-12279; San Diego, TX, escaped seed from tree(s) planted in San Diego Cemetery: Adams 12532-12533. Voucher specimens are deposited at 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° 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 (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<sub>2</sub> according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS primers utilized. Two indels (at sites 194 & 802) prevented single pass sequencing of the 1270 bp ITS area, so two internal primers were designed (ITS426for and rev (site 410 in sample 12271(*J. ovata*), ITS426for = CCC GTT GAG ATT CCA TG). The primers for 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 were determined by Chromas 2.31 (Technelysium Pty Ltd.).

## RESULTS

Four informative cp gene regions were sequenced to compare *J. ashei* with *J. ovata*. These four cp regions had the following informative SNPs and indels: petN-psbM (798 bp), one SNP and 2 indels; trnD-trnT (684bp), 2 SNPs, one indel; trnL-trnF (701bp), 2 SNPs, no indel; and trnS-trnG (823bp), 2 SNPs and 2 indels. All these 4 cp regions distinguished *J. ashei* from *J. ovata*. However, trnS-trnG (trnSG) contained a large, 133bp indel (present in *J. ashei* and absent in *J. ovata*) that could be easily scored on a 1.5% agarose gel. Thus, this marker could be used for an easy detection of the 'ashei' or 'ovata' cp type. Table 2 contains the summary of the DNA analyses as well as the cp classification for individuals, ordered by location. Because cp are inherited from the male (pollen) in the Cupressaceae section that includes *Juniperus* (Adams, 2019; Adams, Miller and Low 2016), the paternal (pollen) parent can be determined for any hybrids found. Every plant collected as *J. ashei* (green highlight in Table 2), except 11320, *ovata* cp, Table 2) had the *ashei* cp DNA. And, every plant collected as *J. ovata* (red highlight, Table 2) had the *ovata* cp DNA.

Sequencing nrDNA (ITS), yielded 1270 bp, with 4 SNPs (sites 258, 302, 303, 758) and 2 indels (sites 194, 802) that distinguish *J. ashei* and *J. ovata*. About half of the plants collected as *J. ashei* were homozygous at the 4 SNP and 2 indel sites and about half were found to be heterozygous at the 4 SNP and 2 indel sites, implying they are hybrids or backcrossed to *J. ovata* (Table 2). About two-thirds of the trees collected in the trans-Pecos Texas area were homozygous at the 4 SNP and 2 indel sites, but four were hybrids or backcrosses and one was homozygous for *J. ashei* in their nrDNA (ITS)<sub>r</sub>, but had a *J. ovata* cp type (Table 2).

There is a noticeable trend from New Braunfels (with mostly red, *ovata*) to Austin and Waco, with considerable hybrids and introgressants (IG symbol, Fig. 7), then Cedar Hills and Turner Falls (nearly all green, *ashei*). White Cliffs, AR is nearly half *J. ashei* and half *J. ovata* with 3 'pure' *J. ovata* (red) ITS

plants and 3 introgressants (IG), whereas Yellville, AR has only pure *J. ashei* or hybrid ITS, no introgressants (Fig. 7). No plants in the *J. ashei* range had *J. ovata* chloroplasts and no plants in the *J. ovata* range had *J. ashei* chloroplasts. The *J. ashei* chloroplast plants end abruptly west of Sonora; thence westward, all plants have *J. ovata* chloroplasts, although hybrid ITS, and introgressants are found in Comstock and Ozona, respectively. The Pandale plants are the purest *J. ovata* found in this study.

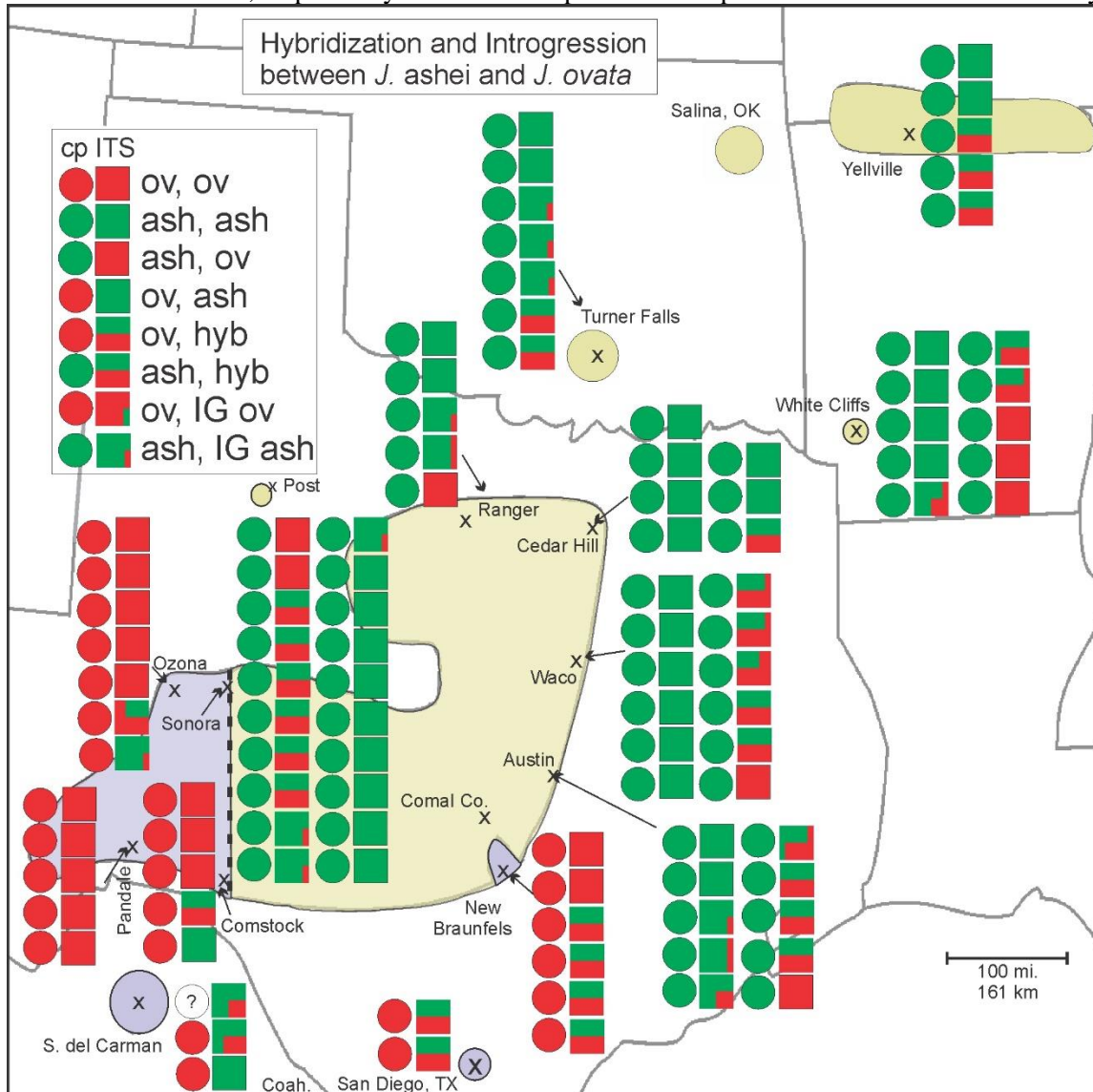


Figure 7. Hybridization and introgression between *J. ashei* and *J. ovata*, in cp DNA and nrDNA (ITS).

The Sonora population also has considerable polymorphic ITS DNA (hybrids and introgressants), but no *ovata* cp DNA. Is there wind in the correct direction to move pollen? Wind data shows that the most wind in January comes from the south and west and north (northerner winds) from Midland to Waco and northward to Oklahoma City and Tulsa (Fig. 8). However, San Antonio has low frequency of wind from the south and Ft. Smith winds are deflected by the Ozark Mtns. east and westward. Because *J. ashei* and *J. ovata* shed pollen in Dec.-Jan.-early Feb., it is useful to examine winds from near Sonora (Midland, Fig. 8). This shows the major January winds are from south and west. Clearly copious amounts of *ovata* pollen from the trans-Pecos are near to Sonora. But, none of the ITS hybrids in the Sonora population had *ovata* cp DNA.

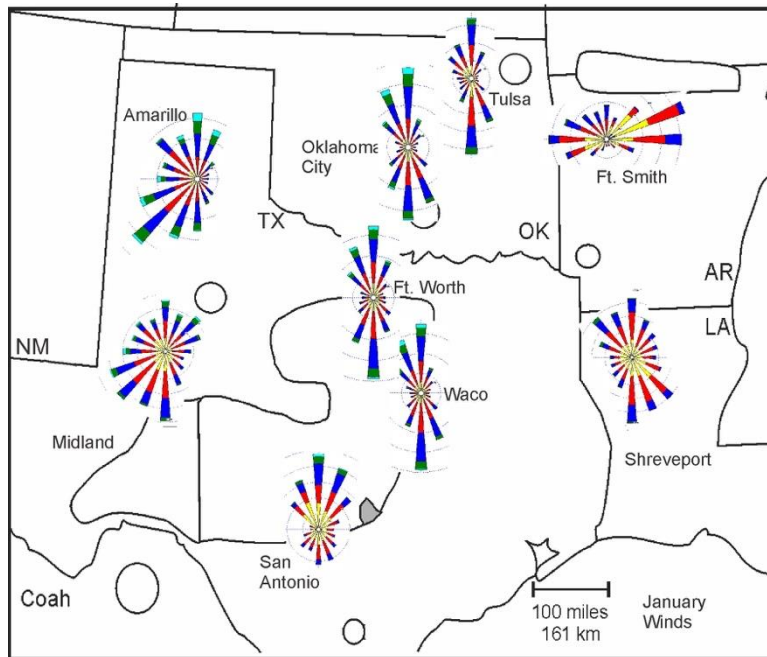


Figure 8. Wind direction (from the arrow outer tips) and velocity for sites in the study area. Velocity normalized, not to scale. Adapted from Wind Rose Resources. [wcc.nrcs.duda.gov/climate/windrose.html](http://wcc.nrcs.duda.gov/climate/windrose.html).

Several other studies in conifers have reported long distance transport (LDT) of pollen from a few km to several hundred km (Szczepanek et al. 2017; Neale and Wheeler 2019; Stewart et al. 2012; Sarvas 1962; Koski 1970; Nichols et al. 1978; Campbell et al. 1999). To effect pollination in distant populations, one needs long distance pollen transport, but also viable pollen when it arrives at a distant population. In fact, several studies have reported that LDT pollen has maintained its viability (Lindgren et al. 1995; Varis et al. 2009; Williams 2010). Pollen from *Juniperus communis*, in the western Alps, was stored at ambient conditions and found to be 40-90% viable for fresh pollen, 20-40% viable after two weeks and 0-10% viable after two months storage (Carmeliello et al. 1990). Finally, it should be mentioned that in a preliminary study on LDT pollen viability, Levetin (Dr. Estelle Levetin, U. Tulsa, per. comm.) found viable *Juniperus* (*J. ashei*) LDT airborne pollen in Tulsa, OK, after having traveled at least 200 mi. because Tulsa has a prevailing south wind in Dec. - Feb. (Fig. 8), the nearest *J. ashei* populations to the south are White Cliffs, AR, Turner Falls, OK or Cedar Hill, TX.

Unfortunately, as attractive as long-distance transport (LDT) of pollen and subsequent fertilization is, it cannot explain the pattern of an absolute lack of *J. ovata* cp in any population of *J. ashei*, nor that none of the F1s have *J. ovata* cp (i.e., obtained by *J. ovata* pollen fertilizing *J. ashei* receptive female cones). Nor are pollen crossing barriers an explanation, because of the existence of F1 hybrids, arising from crosses of pure *J. ovata* with pure *J. ashei*. The lack of *J. ovata* chloroplasts found in the range of *J. ashei*, supports the idea that *J. ovata* genes are introgressed by the movement of F1 hybrid seeds and thence seedlings and eventually, mature F1 hybrid trees.

Birds are well known to eat juniper seed cones ('berries') and widely disperse the seeds (Adams and Thornburg (2010); Phillips 2010; Adams 2014; Holthuijzen, Sharik and Fraser 1987). In fact, cases of junipers endemic to islands are attributed to long distance transport (LDT) by birds. These include *J.*



*bermudiana*, Bermuda; *J. brevifolia*, Azores, and *J. cedrorum*, Canary Islands. Cedar waxwing (*Ampelis cedrorum*) is a major consumer of *J. ashei*, *J. ovata* and *J. virginiana* berries in the winter in central Texas (Phillips, 1910). In fact, Phillips (1910) lists 17 bird species that feed on *J. virginiana* berries. Brugger et al. (1994) researched the winter ranges of cedar waxwings, banded in their summer range in June - August, by recapture in their winter ranges (Dec. - Feb.). It is useful to examine their results concerning banded cedar waxwings in their winter ranges in Texas and Louisiana (Fig. 9). The group included two from WI (Wisconsin), 2 from PA (Pennsylvania), and one each from North (ND) and South Dakota (SD). Their shortest return routes to their summer ranges take the Dakota birds over the Ranger population. The Wisconsin cedar waxwings would fly over Turner Falls, Salina, OK, White Cliffs, AR and the Ozarks - Yellville populations. The Pennsylvania (PA) birds would fly over the White Cliffs, AR and the Ozarks - Yellville populations. It is clear from even this limited study, that there is certainly ample opportunity for cedar waxwings to disperse juniper seeds into all the disjunct population areas, and one should remember that this event has happened every year for thousands of years! So, it is not surprising that *J. ovata* and/or hybrids seeds from New Braunfels, and trans-Pecos Texas have been sown by birds in every conceivable habitat both within Texas and north-northwest of Texas. The same is true for *J. ashei* seeds. It is possible that repeatably some *J. ovata* and/or hybrid seeds fall on suitable sites in the disjunct populations, germinate and grow into reproductive trees, thus injecting germplasm into these '*J. ashei*' populations.

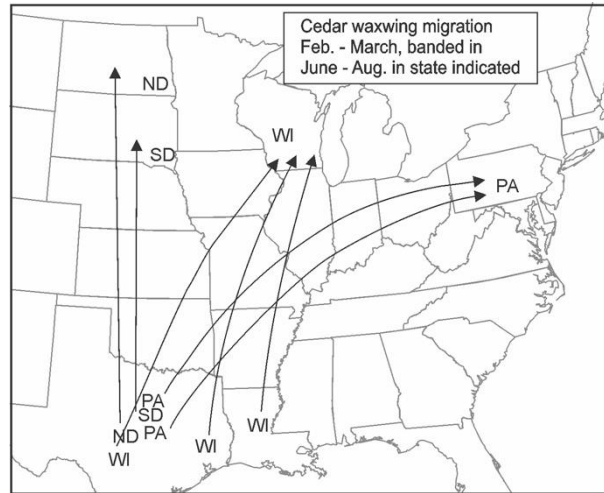


Fig. 9. Cedar waxwing migration in March from their winter home to their summer home states. Adapted from data in Brugger et al. 1994.

It should be noted that the New Braunfels, trans-Pecos, and Sierra del Carman *J. ovata* populations do not appear to be affected by the southern - southwestern migration of birds from ND, SD, WI, PA etc. in the fall, because their summer ranges are outside the distribution of *J. ashei* and *J. ovata*. If birds brought juniper seeds southward, it would most likely be *J. virginiana* and/ or *J. horizontalis*.

The geographic pattern shows no *J. ashei* population examined had only pure *J. ashei* trees (Fig. 7). This is surprising in view of the uniformity found in the terpenoids (Fig. 1). However, terpenoids are well known to be involved as chemical defenses in plants. Seminal papers in the 1970s (Rhoades and Cates, 1976; Cates and Rhoades, 1977; Feeny, 1976) enlightened biologists that plants produce defensive compounds against herbivores. Terpenes and tannins are two types of compounds produced by juniper that are known to deter herbivores (Bernays et al. 1989; Gershenson and Dudareva 2007). Terpenes can act as feeding deterrents (Gershenson and Dudareva 2007) and have numerous toxic actions such as central nervous system depression, contact dermatitis, lung function impairment, liver and kidney cysts and even death (Sperling et al., 1967; Savolainen, 1978; Falk et al., 1990) as well as alter microbial fermentation (Schwartz et al. 1980; Nagy et al. 1964). More recently, deer and goats have been shown to selectively browse on *J. ashei* trees that are lower in leaf essential oil concentration (Adams et al. 2014). Woodrats (*Neotoma*) have also been found to sense the leaf terpenoids to select *J. osteosperma* trees to feed on (Skopec, Adams and Muir, 2019). So, it is very likely that the *J. ashei* (and *J. ovata*) leaf terpenoids are under considerable selection pressure to maintain their chemical defenses. Thus, one might expect widespread herbivores, bacteria and fungi to lead to wide-spread terpenoids patterns, as found in *J. ashei* and *J. ovata* (Adams 1977). In contrast, neutral sites such as ITS can show more variation within an area where hybridization occurs. A hybrid plant that has incorporated unusual terpenoid synthase genes from a parent

from a different habitat, into its genome, may be less likely to survive than a tree that has the nuclear genome parts that carry the terpenoid genes from a local parent that has the local array of defensive terpenoids.

Examination of another area of possible hybridization (New Braunfels - Comal Co.) shows that the purest *J. ashei* trees are in the west area near the US 281 and TX 46 junction, whereas the purest *J. ovata* is in or near New Braunfels (Fig. 10). Of particular interest is that only 2 pure *J. ovata* were found in the New Braunfels populations. Population 4 is interesting because both *J. ashei* and *J. ovata* cp parents are present (Fig. 10) in the population. This is the only population found that has both *J. ashei* and *J. ovata* chloroplasts. This population (4) is 'fence row' population: a recent population with plants growing under the barbed wire fence where bird sit to digest juniper female cones and 'plant' the seeds under the fence wire.

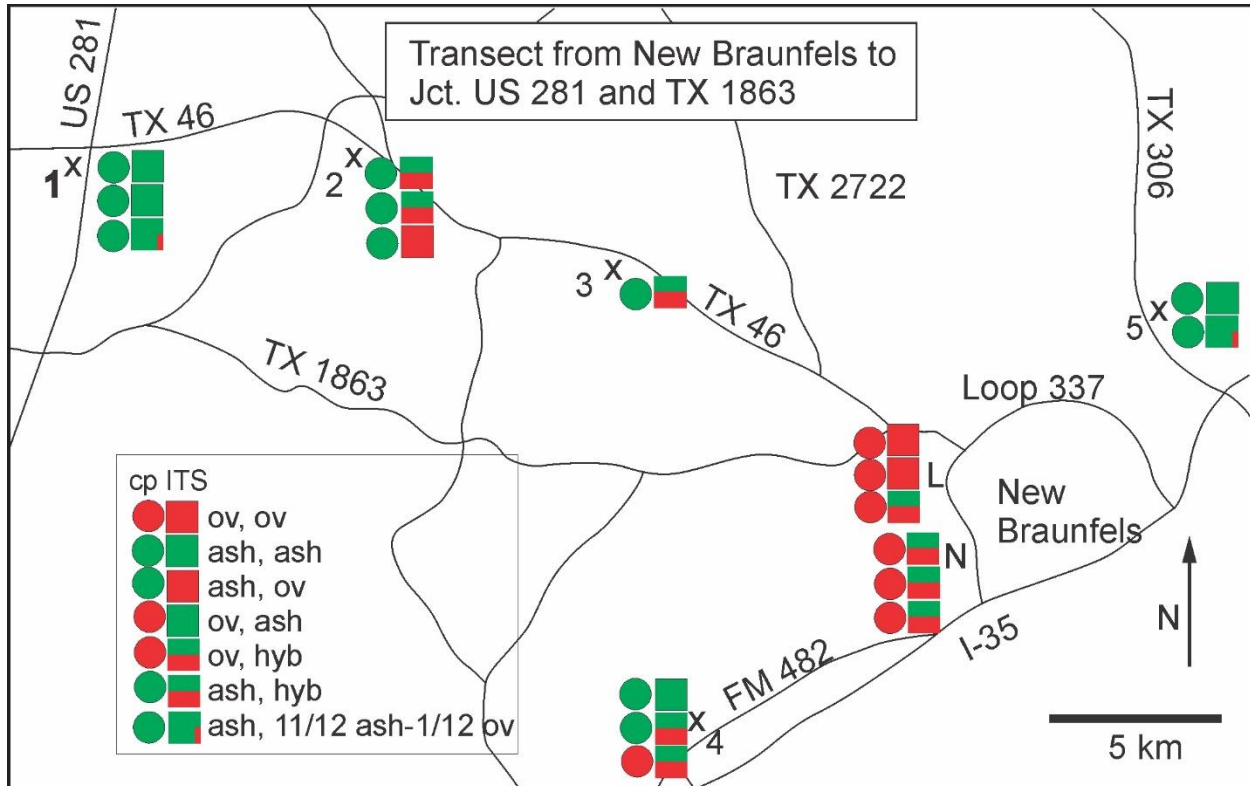


Figure 10. Variation in the New Braunfels - Comal Co. area. Populations 1, 2, 3, differ by 8 km, L = Loop 337/ TX 46 population, N = New Braunfels (city) population.

The two young *J. ovata* trees from near San Diego, TX were growing on a fence-row a few km north of several *J. ovata* trees planted in the San Diego cemetery. The cultivated trees were very likely purchased or dug up from the New Braunfels population due north, rather than the trans-Pecos/ Mexico populations. Both trees were small (2 - 4m) and young. These seem most likely to have been established by birds (cedar waxwings?), that digested the *J. ovata* seed cone fleshy portion and expelled the seeds while sitting on the barbed wire fence. This is a very common dispersal mechanism in junipers and accounts for miles of 'fence-row' junipers in, otherwise, grassland habitats devoid of junipers (Adams 2014). Both plants had *J. ovata* cp, but heterozygous (hybrid) nrDNA (Fig. 7.)

Comparing (using data from the same trees) the terpene classifications (Fig. 4, 5) with the DNA data, both data sets show the purest *J. ovata* in L and N (New Braunfels populations) and purest *J. ashei* in

the western population 1 (Figs. 4, 5, 9). Overall, there is good agreement in the terpenoid pattern and the cp, nrDNA pattern in this small area.

## DISCUSSION

The idea that the current patterns are a result of relictual mixing (i.e., hybridization during the Pleistocene) that formed the pattern observed, seems implausible because of the presence of many current F1 hybrid plants rather than later generation introgressants. This pattern can best be explained by recurring F1 hybrid seeds being brought into the northern disjunct populations by birds in recent times.

It is instructive to compare the recent reports of hybridization and introgression in *Juniperus* in the western United States with the present results. Figure 11 shows an interesting case where *J. maritima* comprises very uniform populations in the Pacific Northwest, then bordered by a broad zone of unusual plants with *J. scopulorum* cp and pure *J. maritima* ITS DNA, then an area of *J. scopulorum* with evidence of introgression from *J. maritima*, and finally an area of relatively pure *J. scopulorum*. Notice that one population (WO, Willowa Mtns., OR, Fig. 11) has a mixture of both chloroplast types. There are only 2 plants with [*maritima* cp/hybrid ITS] (WL, Williams Lake), but there are plants with [*scopulorum* cp/hyb or IG] in populations FH, BU, SS, MO and KU in the *J. scopulorum* range. This is very similar to [*ashei* cp/hyb or IG] plants in this study (Figs. 7, 10). It may be that bird-transported seeds are important in the *J. maritima*-*J. scopulorum* case also.

A second example of hybridization between *J. arizonica* and *J. coahuilensis* (Adams 2017) shows (Fig. 12) a zone of hybridization between the species (Hueco Tanks, Quitman Mtns.) with some gene flow in both directions, with a very few F1 hybrids inside the home ranges of *J. arizonica* and *J. coahuilensis*. These hybrid areas appear to be in the overlap areas between the taxa; however, it is notable that only one hybrid ITS plant has *J. coahuilensis* cp DNA (sMF, Fig. 12), a similar pattern as we see in Figure 11 and as well as this present study (Fig. 7).

The third study is of hybridization and introgression between *J. blancoi* and *J. scopulorum* (Adams, et al. 2020). This pattern is somewhat like that of *J. maritima* and *J. scopulorum* (Fig. 11) in having a zone of hybridization and introgression with only *J. scopulorum* cp present. A high frequency of wind from the north in March and April was postulated to have been important in the asymmetric occurrence of *J. scopulorum* cp in plants in north Mexico (Adams, et al. 2020). However, again we see (Fig. 13) plants in the hybrid zone with [*scopulorum* cp/pure *blancoi* ITS]. It should be noted that the taxon in the hybrid zone is *J. blancoi* var. *mucronata* (RP Adams) RP Adams. The taxon is thought to have experienced a *scopulorum* chloroplast capture event (Adams et al. 2020), thus explaining the [*scopulorum* cp/*blancoi* v. *mucronata* ITS] genome.

Two of these examples (Figs. 11, 13) have plants in a hybrid zone with a genotype of (sp. A cp, pure sp. B ITS), which is what we found in this study [*ashei* cp, pure *ovata* ITS: Sonora, Ranger, White Cliffs, Waco, Austin, Comal Co, #2] and [*ovata* cp, pure *ashei* ITS: Comstock, S. del Carman].

In addition, all three of these examples share an interesting aspect: the hybrid areas are located in areas with lower juniper densities. In the *J. maritima* - *J. scopulorum* study, Eastern Washington and Oregon, and Northern Idaho, and Southeastern British Columbia juniper populations are localized and often very small, separated from adjacent populations by 10s or 100s of miles (Fig. 11). In the *J. arizonica* and *J. coahuilensis* study (Fig. 12), junipers are quite rare in the zone of hybridization (Hueco Tanks, Quitman Mtns.). And, the *J. blancoi* and *J. scopulorum* study, *J. blancoi* var. *mucronata* in northern Mexico has only small, isolated populations (Fig. 13).

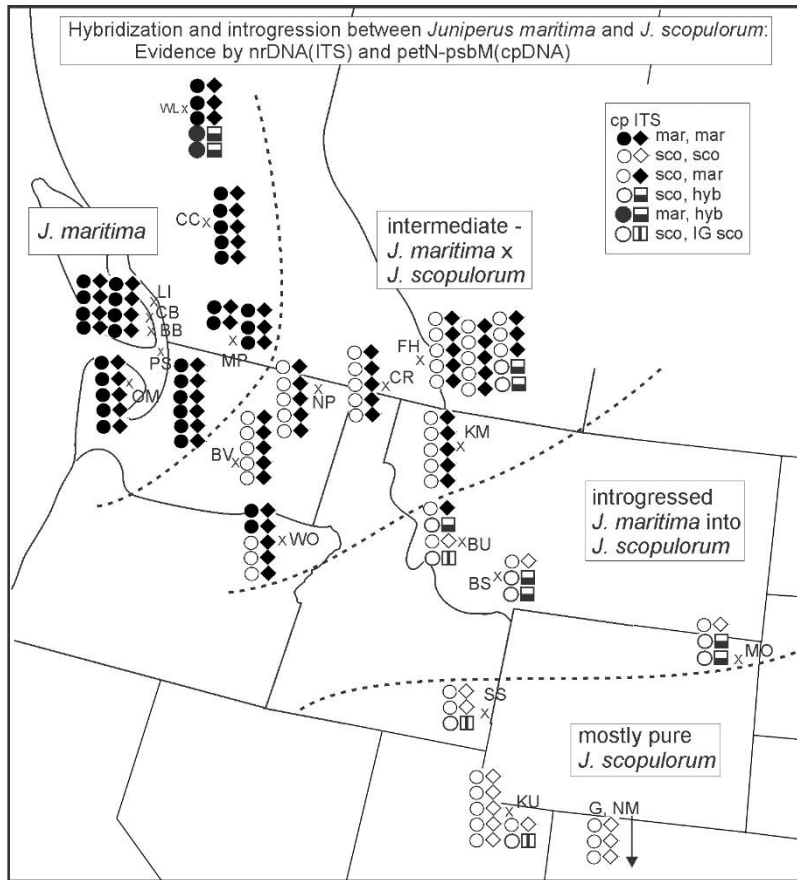


Fig. 11. Hybridization and introgression between *J. maritima* and *J. scopulorum*. Modified from Adams 2015. Note the broad areas of hybridization and introgression. These are areas of low density in these junipers, the hybrids and introgressants.

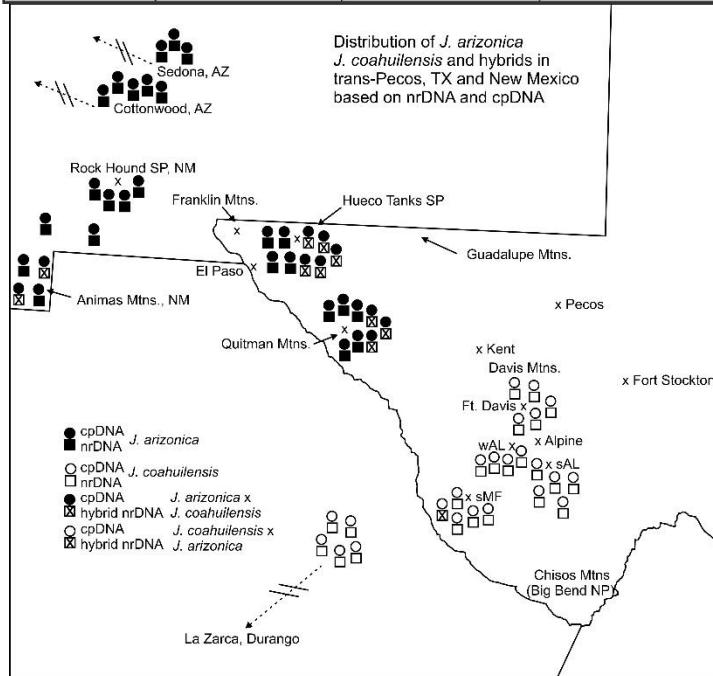


Fig. 12. Hybridization / introgression between *J. arizonica* and *J. coahuilensis* (from Adams 2017). The hybrid zone has a low density of juniper trees.

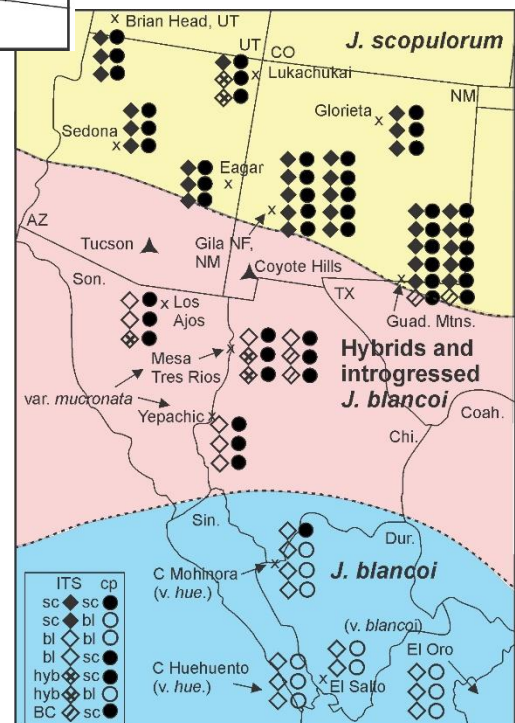


Fig. 13. Hybridization between *J. blancoi* and *J. scopulorum* (from Adams et al. 2020). Red zone has a low density of juniper trees.

In contrast, *J. ashei* is so aggressive that it often forms closed canopy stands of pure *J. ashei* trees in the central Texas limestone hill country and the Arbuckle Mtns, OK. However, the White Cliffs, AR population is very small and mixed in a mesic forest setting. At the Yellville, AR site, *J. ashei* can form cedar glades on limestone outcrops, but these outcrops are often small areas with large gaps between populations.

Friedman and Barrett (2009) reviewed pollination in wind-pollinated plants and consider the leptokurtic distribution of pollen. In *Festuca pratensis* most of its pollen is spread only about 75 m from the source (Rognli et al. 2000). Trees in populations that have been fragmented by disturbances may have pollen dispersal distances of only 65 m (Knapp et al. 2001; Sork et al. 2002). However, although there is good evidence of long-distance transport (LDT) of viable pollen (see above), the presence of copious amounts of local pollen versus limited amounts of LDT pollen would greatly favor pollination by local pollen. Thus, the lack of *J. ovata* cp in hybrids in the Texas Hill country and northern disjunct populations may be just a matter of the overwhelming abundance of local, nearby pollen (of *J. ashei*) compared to the LDT *J. ovata* pollen. It might be noted that all juniper trees in a population normally produce pollen at nearly the same time, so great clouds of pollen are common.

It is interesting to note that Austin, Waco, Sonora, Ranger, and White Cliffs populations each have 1 to 3 trees with perfect *J. ovata* nrDNA (ITS) (Fig. 7). Obviously seeds of this odd combination [*ashei* cp/homozygous *J. ovata* nrDNA] are being produced in the New Braunfels area as one sees one of these plants in population 2 (Fig. 10). A cross of [*ashei* cp/*ovata* ITS] x [*ashei* cp/*ashei* ITS] would yield an ITS hybrid [*ashei* cp/heterozygous ITS]. Birds can easily carry these seeds to the Austin, Waco, Sonora, Ranger, and White Cliffs populations, as well as [*ashei* cp/hybrid ITS] and [*ashei* cp/hybrid ITS] seeds to these and other populations. Holthuijzen and Sharik (1985) reported that juniper seeds that had passed thru the digestive tract of warblers and waxwings germinated at a rate of 55.0% and 27.6% compared to the control rate of 16.1%. It is thought that passage through the digestive tract scarifies the seeds making seeds easier to absorb water and germinate. Johnson (1962) studied the cumulative % germination of *J. monosperma* seeds and found seeds passed through birds germinated sooner (ex. 20%, wk 3, vs. 6% control), but after 10 weeks, both bird and control seeds reached the same level of germination (44%). However, it is very important for a juniper to germinate quickly after rainfall, as the opportunity to establish deep and/ or widespread roots must be done before the moisture is exhausted. This being said, it might be that bird transported seeds, having a long residence time in the digestive tract, may germinate more readily than local seeds and thus have an advantage in the establishment of alien seedlings over indigenous seedlings.

### **Pleistocene ranges, refugia and re-colonization and the formation of present-day ranges**

Although there is considerable evidence of a continuous band of sclerophyllous vegetation from central Texas into northern Mexico during the Tertiary (Axelrod, 1975), it is more productive to focus on events in the Pleistocene, particularly the last Wisconsin and subsequent eras. According to King (1973), the western Ozarks were covered with boreal spruce forest from about 25,000 to at least 13,000 B.P., with pine parkland preceding the boreal spruce forest. The pine parkland and boreal spruce forest both appeared to have been pushed southward from the north (Dillon, 1956).

#### **pre-Wisconsin era:**

Based on the uniformity of the current *J. ashei* and *J. ovata* populations (excluding *J. ovata* near New Braunfels), there must have been uniform populations of *J. ashei* on the exposed limestone Edwards Plateau in the pre-Wisconsin era, and likely on the exposed limestone outcrops of White Cliffs, Arbuckle Mtns, and Ozark Mtns. Whether *J. ovata* was wide-spread in the trans-Pecos is not known, but for this discussion we assume it occupied the lower, dryer trans-Pecos and perhaps the northern Chihuahuan desert in Texas, Chihuahua and Coahuila. It seems likely that the current, isolated *J. ovata* population at New Braunfels was not present much more recently.

### Wisconsin era vegetation:

Figure 14 shows the hypothetical vegetation during the pluvial period (modified from Adams, 1977). The area south of the Ozarks may have been pine woodland or parkland (see Bryant, 1969). A pine-spruce woodland seems likely on the Llano Estacado of northwest Texas according to Hafsten (1961). Bryant (1969) suggested that, based on pollen profiles, the present Chihuahuan desert area around Del Rio, TX (430 m) was pinyon woodland. Wells (1966), using data obtained from rat middens from the Big Bend region of Texas, concluded that life zones descended about 800 m for pinyon-juniper (*J. pinchotii* in that case), allowing the advance of pinyon-juniper into most of the present desert region between the Big Bend and Del Rio. Typical *J. pinchotii*, and *J. ovata*, have been found growing just south of the Sierra de Carmen

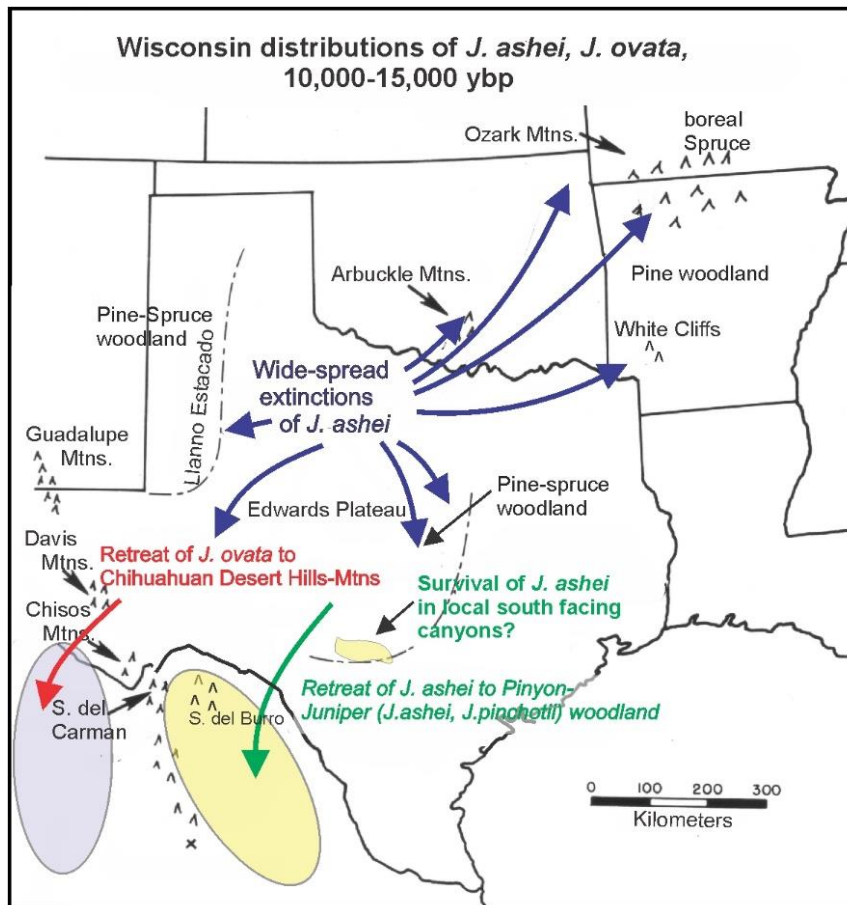


Figure 14. Possible *J. ashei* distribution during the Wisconsin era (from Adams, 1977, 2004).

mountains of the Big Bend region (Adams, 2004). It appears that the Serranias del Burro, Mexico, may have been an important refugium or "island point" in the pinyon-juniper woodland. A mixed deciduous woodland with conifers is postulated in central Texas (Bryant, 1969) based on pollen profiles.

At the end of the Wisconsin glacial advance (10,000 - 13,000 yr bp), the central Texas, Oklahoma and Arkansas populations of *J. ashei* were likely extinct, because this area was a much wetter and cooler spruce woodland (Fig. 14). However, it is possible that some local population(s) of *J. ashei* may have survived on dry, sunny, south facing limestone slopes, especially on the steep south and southeast sides of the Balcones Escarpment. During the cool-wet Wisconsin period, *J. ashei* may have expanded south and west into the Chihuahuan desert (Wells, 1966), but not as far south as Cuatro Ciénegas, Coahuila, Mexico (Meyer, 1973). Migration of populations to regions west of the Sierra del Carmen was also possible because *J. ovata* grows at the top of La Cuesta pass just south of the Sierra del Carmen (Adams, 1977). So, it is possible that sympatry of *J. ashei* and *J. ovata* occurred in the Pleistocene. However, if *J. ashei* and *J. ovata* were sympatric in Mexico during the glacial era, and there were hybridization and introgression

between the taxa, a variety of genotypes would have been available including a mixture of *J. ashei*, hybrids, introgressants, and even some 'pure' *J. ovata* for recolonization of central Texas, Arbuckle Mtns., White Cliffs and the Ozarks. However, the presence of only *J. ashei* pollen throughout the range of *J. ashei* supports the idea that pure *J. ashei* first recolonized central Texas, Oklahoma and the Ozarks. Sometime later *J. ovata* invaded the trans-Pecos area.

### Post-glacial (Holocene) re-colonization:

As mentioned above, both *J. ashei* and *J. ovata*, today, have uniform cp within their respective ranges, that supports the idea that the *J. ashei* re-colonization immigrants had uniform *J. ashei* cp. If populations of *J. ashei* were forced to extinction in central Texas, Oklahoma, Arkansas, and Missouri, and subsequent recolonization in the Holocene took place as depicted in figure 15, over a very short time, from a refugium in Mexico (or a relictual population in central Texas) the species may have gone through a selection 'bottleneck' perhaps coupled with genetic drift. The rapid colonization of limestone outcrops (Fig. 15) could then lead to a uniform taxon from central Texas to the Ozarks. Rapid colonization is supported because *J. ashei* has evolved into a very invasive species that, today, is invading disturbed grasslands in the region (Adams et al. 1998; Smith and Rechenthin 1964).

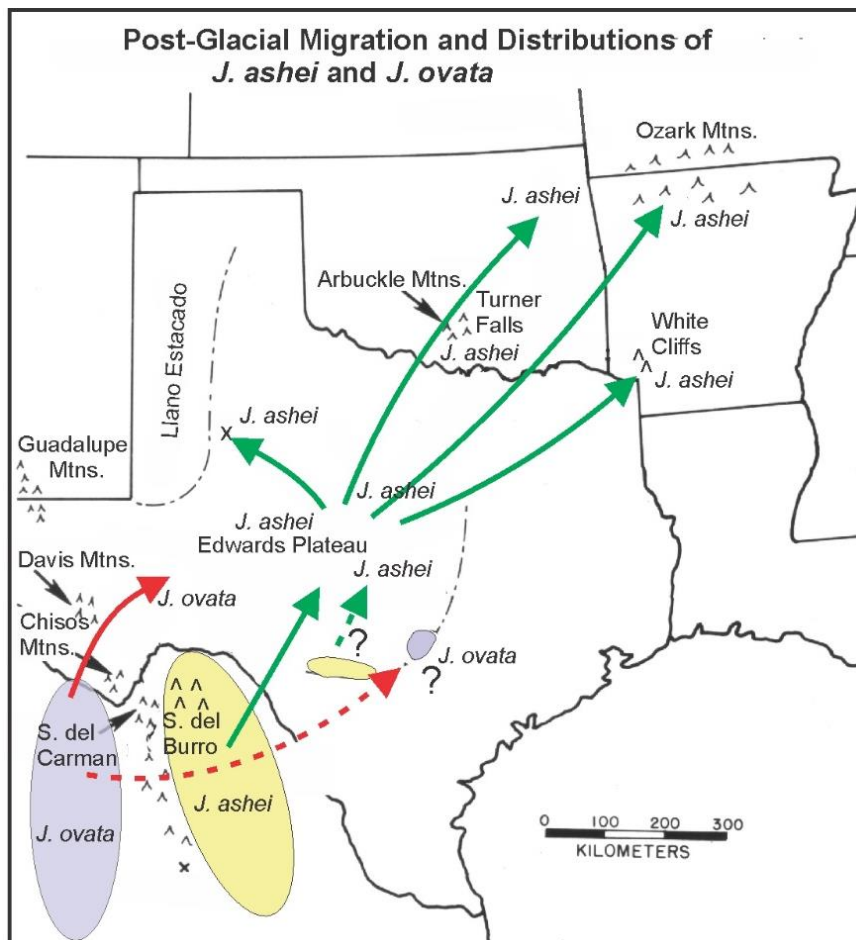


Figure 15. Postulated post-glacial recolonization of *J. ashei* onto limestone producing very uniform populations (Adams, 1977, 2004).

The same argument can be made for uniform ancestral *J. ovata*, that quickly invaded open habitat in the Holocene in the trans-Pecos region. The disjunct, New Braunfels population seems most likely to be a long-distance transport event by birds from the trans-Pecos or northern Mexico *J. ovata* populations. Additional research should resolve some of the un-answered questions in this study.

## ACKNOWLEDGEMENTS

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Table 2. Classification of *J. ashei* and *J. ovata* samples based on trnSG and nr ITS DNA. **Orange highlights** are putative hybrid sites. Note that ITS sites 194 and 802 are indel sites scored (when aligned) as: T or - (no T), and A or - (no A), respectively.

| coll # | location, field id                | trnSG | ITS            | 194 | 258 | 302 | 303 | 758 | 802 |
|--------|-----------------------------------|-------|----------------|-----|-----|-----|-----|-----|-----|
| 10215  | Yellville, AR ashei               | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 10218  | Yellville, AR ashei               | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 10216  | Yellville, AR ashei               | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 10217  | Yellville, AR ashei               | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 10219  | Yellville, AR ashei               | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 14071  | White Cliffs, AR ashei            | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14076  | White Cliffs, AR ashei            | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14079  | White Cliffs, AR ashei            | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14080  | White Cliffs, AR ashei            | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14072  | White Cliffs, AR ashei            | ashei | ovata          | T   | T   | A   | C   | G   | -   |
| 14073  | White Cliffs, AR ashei            | ashei | ovata          | T   | T   | A   | C   | G   | -   |
| 14074  | White Cliffs, AR ashei            | ashei | ovata          | T   | T   | A   | C   | G   | -   |
| 14075  | White Cliffs, AR ashei            | ashei | F2 hyb?        | T/- | C/T | A   | T   | A   | A   |
| 14077  | White Cliffs, AR ashei            | ashei | hyb BC ovata   | T/- | C/T | A   | C/T | A/G | A/- |
| 14078  | White Cliffs, AR ashei            | ashei | hyb BC ashei   | T/- | C/T | A/G | C/T | A/G | A   |
| 14096  | Turner Falls, OK ashei            | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14100  | Turner Falls, OK ashei            | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14094  | Turner Falls, OK ashei            | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 14095  | Turner Falls, OK ashei            | ashei | ashei BC ovata | -   | C   | A/G | T   | A   | A   |
| 14097  | Turner Falls, OK ashei            | ashei | ashei BC ovata | -   | C   | A/G | T   | A   | A   |
| 14098  | Turner Falls, OK ashei            | ashei | ashei BC ovata | -   | C   | A/G | T   | A   | A   |
| 14099  | Turner Falls, OK ashei            | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12007  | Cedar Hill, TX ashei              | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12008  | Cedar Hill, TX ashei              | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12010  | Cedar Hill, TX ashei              | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12011  | Cedar Hill, TX ashei              | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12009  | Cedar Hill, TX ashei              | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 14091  | Benbrook TX ashei                 | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14092  | Benbrook TX ashei                 | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12012  | Ranger, TX ashei                  | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12016  | Ranger, TX ashei                  | ashei | ashei          | -   | C   | G   | T   | A   | na  |
| 12013  | Ranger, TX ashei                  | ashei | ovata          | T   | T   | A   | C   | G   | -   |
| 12014  | Ranger, TX ashei                  | ashei | ashei BC ovata | -   | C   | A   | T   | A   | na  |
| 12015  | Ranger, TX ashei                  | ashei | ashei BC ovata | -   | C   | A/G | T   | A   | na  |
| 14082  | Cameron Pk, Waco, TX ashei        | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14084  | Cameron Pk, Waco, TX ashei        | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14086  | Cameron Pk, Waco, TX ashei        | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14089  | Cameron Pk, Waco, TX ashei        | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14090  | Cameron Pk, Waco, TX ashei        | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 14081  | Cameron Pk, Waco, TX ashei        | ashei | hyb BC ovata   | T/- | C/T | A/G | C/T | G   | -   |
| 14083  | Cameron Pk, Waco, TX ashei        | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 14085  | Cameron Pk, Waco, TX ashei        | ashei | hyb BC ovata   | T/- | C/T | A   | C/T | A/G | A/- |
| 14087  | Cameron Pk, Waco, TX ashei        | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 14088  | Cameron Pk, Waco, TX ashei        | ashei | hyb BC ovata   | T/- | C/T | A   | C/T | A/G | A/- |
| 6746   | Bosque Blvd., Waco, TX ashei      | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 6752   | Bosque Blvd., Waco, TX ashei      | ashei | ovata          | T   | T   | A   | C   | G   | -   |
| 12030  | West Lake Hills, Austin, TX ashei | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12031  | West Lake Hills, Austin, TX ashei | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12032  | West Lake Hills, Austin, TX ashei | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12033  | West Lake Hills, Austin, TX ashei | ashei | ashei BC ovata | -   | C/T | A   | C/T | A/G | A/- |
| 12034  | West Lake Hills, Austin, TX ashei | ashei | ashei BC ovata | -   | C/T | A   | C/T | A/G | A/- |
| 12035  | West Lake Hills, Austin, TX ashei | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12036  | West Lake Hills, Austin, TX ashei | ashei | ashei BC ovata | -   | C   | A/G | T   | ?   | na  |
| 12037  | West Lake Hills, Austin, TX ashei | ashei | ashei BC ovata | -   | C   | A   | T   | A   | A   |
| 12038  | West Lake Hills, Austin, TX ashei | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12039  | West Lake Hills, Austin, TX ashei | ashei | ovata          | T   | T   | A   | C   | G   | -   |
| 11296  | Comal Co, TX ashei T46&281        | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 11297  | Comal Co, TX ashei T46&281        | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 11295  | Comal Co, TX ashei T46&281        | ashei | ashei BC ovata | -   | C   | A/G | T   | A   | A   |
| 11298  | Comal Co, TX ashei T46,5mi E.     | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 11299  | Comal Co, TX ashei T46,5mi E.     | ashei | ovata          | T   | T   | A   | C   | G   | -   |
| 11300  | Comal Co, TX ashei T46,5mi E.     | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |

| coll # | location, field id                | trnSG | ITS            | 194 | 258 | 302 | 303 | 758 | 802 |
|--------|-----------------------------------|-------|----------------|-----|-----|-----|-----|-----|-----|
| 11301  | Comal Co, TX ashei T46 10mi E     | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 11319  | Comal Co, TX ashei Huber. Rd.     | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 11320  | Comal Co, TX ashei Huber. Rd.     | ovata | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 11321  | Comal Co, TX ashei Huber. Rd.     | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 11323  | Comal Co, TX ashei FM306          | ashei | ashei          | -   | C   | G   | T   | na  | na  |
| 11322  | Comal Co, TX ashei FM306          | ashei | ashei BC ovata | -   | C   | A/G | T   | A   | A   |
| 11309  | New Braunfels ovata Cedar Elm St. | ovata | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 11317  | New Braunfels ovata Cedar Elm St. | ovata | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 11318  | New Braunfels ovata Cedar Elm St. | ovata | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 11314  | New Braunfels ovata Loop 337      | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 11316  | New Braunfels ovata Loop 337      | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 11315  | New Braunfels ovata Loop 337      | ovata | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12251  | Sonora, TX TAES ashei             | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12256  | Sonora, TX TAES ashei             | ashei | ashei          | -   | C   | A   | T   | A   | na  |
| 12257  | Sonora, TX TAES ashei             | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12258  | Sonora, TX TAES ashei             | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12259  | Sonora, TX TAES ashei             | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12263  | Sonora, TX TAES ashei             | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12266  | Sonora, TX TAES ashei             | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12268  | Sonora, TX TAES ashei             | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12269  | Sonora, TX TAES ashei             | ashei | ashei          | -   | C   | G   | T   | A   | A   |
| 12250  | Sonora, TX TAES ashei             | ashei | ovata          | T   | T   | A   | C   | G   | na  |
| 12252  | Sonora, TX TAES ashei             | ashei | ovata          | T   | T   | A   | C   | G   | -   |
| 12253  | Sonora, TX TAES ashei             | ashei | ashei BC ovata | T/- | C/T | A/G | T   | A   | na  |
| 12254  | Sonora, TX TAES ashei             | ashei | ashei BC ovata | -   | C   | A/G | T   | A   | na  |
| 12255  | Sonora, TX TAES ashei             | ashei | ashei BC ovata | -   | C   | A/G | T   | A   | A   |
| 12260  | Sonora, TX TAES ashei             | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12261  | Sonora, TX TAES ashei             | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12262  | Sonora, TX TAES ashei             | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12264  | Sonora, TX TAES ashei             | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12265  | Sonora, TX TAES ashei             | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12267  | Sonora, TX TAES ashei             | ashei | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 7470   | Ozona, TX ovata holotype tree     | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12281  | Ozona, TX ovata                   | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12282  | Ozona, TX ovata                   | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12283  | Ozona, TX ovata                   | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12284  | Ozona, TX ovata                   | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 7473   | Ozona, TX ovata                   | ovata | ashei BC ovata | -   | C   | A/G | T   | A   | A   |
| 12280  | Ozona, TX ovata                   | ovata | ovata BC ashei | T/- | C/T | A   | C/T | G   | A/- |
| 12270  | Comstock, TX ovata                | ovata | ashei          | -   | C   | G   | T   | A   | A   |
| 12271  | Comstock, TX ovata                | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12272  | Comstock, TX ovata                | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12273  | Comstock, TX ovata                | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12274  | Comstock, TX ovata                | ovata | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12275  | Pandale, TX ovata                 | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12276  | Pandale, TX ovata                 | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12277  | Pandale, TX ovata                 | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12278  | Pandale, TX ovata                 | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12279  | Pandale, TX ovata                 | ovata | ovata          | T   | T   | A   | C   | G   | -   |
| 12532  | San Diego, TX ovata               | ovata | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 12533  | San Diego, TX ovata               | ovata | ashei X ovata  | T/- | C/T | A/G | C/T | A/G | A/- |
| 0098   | Sierra de. Carman, MX ovata       | ovata | ashei BC ovata | -   | C/T | A/G | T   | A/G | A   |
| 0099   | Sierra de. Carman, MX ovata       | ovata | ashei BC ovata | T/- | C/T | A/G | T   | A/G | A   |
| 1092   | Sierra de. Carman, MX ovata       | ovata | ashei          | -   | C   | G   | T   | A   | A   |

194 indel CCTTT= T insert=ovata; CCTT=del = ashei. 258: xGAATGCC; 302: GAAGAGx; 303: xTCGGAC; 758: xAAGTGCAT; 802:xAAAAAACAT 8As in ashei, -7As in ovata.

Appendix 1. Comparisons of the per cent total oil for the leaf essential oils of *J. ashei* and *J. ovata*. Large differences in concentrations are highlighted in boldface.

| KI          | Compound                                    | <i>J. ashei</i> | <i>J. ovata</i> |
|-------------|---|-----------------|-----------------|
| 921         | tricyclene                                  | 1.3             | 1.1             |
| <b>933</b>  | <b><math>\alpha</math>-pinene</b>           | <b>0.4</b>      | <b>3.8</b>      |
| 946         | camphene                                    | 1.6             | 1.6             |
| 969         | sabinene                                    | t               | 0.3             |
| 974         | $\beta$ -pinene                             | t               | -               |
| <b>988</b>  | <b>myrcene</b>                              | <b>0.5</b>      | <b>2.6</b>      |
| 1001        | $\delta$ -2-carene                          | t               | -               |
| 1002        | $\alpha$ -phellandrene                      | t               | t               |
| 1008        | $\delta$ -3-carene                          | t               | 0.1             |
| 1014        | $\alpha$ -terpinene                         | t               | t               |
| <b>1020</b> | <b>p-cymene</b>                             | <b>2.0</b>      | <b>0.7</b>      |
| <b>1024</b> | <b>limonene</b>                             | <b>3.5</b>      | <b>7.7</b>      |
| 1025        | $\beta$ -phellandrene                       | t               | t               |
| <b>1054</b> | <b><math>\gamma</math>-terpinene</b>        | <b>0.2</b>      | <b>0.8</b>      |
| 1067        | cis-linalool oxide<br>(furanoid)            | t               | -               |
| 1078        | camphenilone                                | t               | -               |
| 1084        | trans-linalool oxide<br>(furanoid)          | 0.3             | 0.4             |
| 1086        | terpinolene                                 | t               | t               |
| <b>1095</b> | <b>linalool</b>                             | <b>1.4</b>      | <b>0.4</b>      |
| <b>1098</b> | <b>trans-sabinene hydrate</b>               | <b>0.2</b>      | -               |
| 1100        | isopentyl 2-methyl<br>butanoate             | t               | -               |
| 1112        | 3-methyl butanoate, 3-<br>methyl-3-butenyl- | t               | t               |
| 1118        | cis-p-menth-2-en-1-ol                       | t               | t               |
| 1122        | $\alpha$ -campholenal                       | t               | -               |
| <b>1136</b> | <b>trans-p-menth-2-en-1-ol</b>              | <b>0.2</b>      | -               |

|             |  |             |             |
|-------------|--|-------------|-------------|
| <b>1141</b> | <b>camphor</b>                             | <b>69.1</b> | <b>53.3</b> |
| 1145        | camphene hydrate                           | 0.3         | 0.3         |
| 1165        | borneol                                    | 2.2         | 2.8         |
| 1174        | terpinen-4-ol                              | 0.3         | 0.5         |
| 1179        | p-cymen-8-ol                               | 0.3         | 0.1         |
| 1186        | $\alpha$ -terpineol                        | 0.1         | t           |
| <b>1204</b> | <b>verbenone</b>                           | <b>0.1</b>  | -           |
| 1207        | trans-piperitol                            | 0.2         | t           |
| <b>1215</b> | <b>trans-carveol</b>                       | <b>0.7</b>  | <b>t</b>    |
| 1218        | endo-fenchyl acetate                       | t           | -           |
| 1226        | cis-carveol                                | t           | t           |
| <b>1239</b> | <b>carvone</b>                             | <b>0.8</b>  | <b>t</b>    |
| 1249        | piperitone                                 | t           | -           |
| 1273        | trans-carvone oxide                        | t           | -           |
| <b>1287</b> | <b>bornyl acetate</b>                      | <b>6.3</b>  | <b>15.6</b> |
| 1289        | p-cymen-7-ol                               | t           | -           |
| 1298        | carvacrol                                  | t           | -           |
| 1339        | trans-carvyl acetate                       | t           | t           |
| 1340        | piperitenone                               | t           | -           |
| <b>1548</b> | <b>elemol</b>                              | <b>0.2</b>  | <b>0.9</b>  |
| 1649        | $\beta$ -eudesmol                          | t           | 0.4         |
| 1652        | $\alpha$ -eudesmol                         | t           | 0.5         |
| <b>1968</b> | <b>sandaracopimara-<br/>8(14),15-diene</b> | <b>0.2</b>  | -           |
| 1987        | manoyl oxide                               | 3.6         | 3.2         |
| 2055        | abietatriene                               | 0.2         | 0.2         |
| 2087        | abietadiene                                | 0.2         | 0.3         |
| 2282        | sempervirol                                | 1.1         | 0.5         |
| 2314        | trans-totarol                              | 0.7         | 0.3         |
| 2331        | trans-ferruginol                           | 0.2         | 0.1         |

values of 0.05% or less are denoted as traces (t). Unidentified components less than 0.5% are not reported. KI is the arithmetic retention index in DB-5.