Geographic variation in pentane extractable hydrocarbons in natural populations of *Helianthus annuus* (Asteraceae, Sunflowers)

Robert P. Adams and **Amy K. TeBeest**
Baylor-Gruver Lab, Baylor University, 112 Main Ave., Gruver, TX 79040
robert_Adams@baylor.edu

Walter Holmes
Biology Department, Baylor University, Box 97388, Waco, TX 76798

Jim A. Bartel
San Diego Botanic Garden, P. O. Box 230005, Encinitas, CA 92023

Mark Corbet
7376 SW McVey Ave., Redmond OR 97756

Chauncey Parker
11643 Norse Ave., Truckee, CA 96161

and

David Thornburg
2200 W. Winchester Lane, Cottonwood AZ 86326

ABSTRACT

Populations of *Helianthus annuus*, ranging from eastern Oklahoma to coastal southern California, were sampled and the yields of total hydrocarbons (HC) from leaves determined. The highest yielding populations were in the Texas Panhandle (6.0 - 7.99%) and the lowest yields were in Camp Verde, AZ, NM mountains, Redland, OR, and San Diego, CA. Medium-high yields were found in northern UT and southern ID. Four populations near Waco, TX had large yield differences ranging from 3.6 to 6.2%. Some native populations were contaminated by germplasm from cultivated sunflowers and these populations had very low yields (2.6 - 3.6%). Population variability in HC yields varied geographically and also between nearby populations, suggesting the micro-habitat environments are important as well as limited genetic population size. The frequency distribution (329 individuals) ranged from 1.0 to 12.63% yield and showed a skewed, normal distribution, with a tail towards highest yielding plants. The mean was 5.33%, with the top 5% being larger than 8.7% yield. A very low correlation (r=0.18) was found between leaf size biomass and % yield implying an opportunity to select for high yields and high biomass concurrently. Published on-line www.phytologia.org *Phytologia 99(1): 1-10 (Jan 19, 2017).* ISSN 030319430.

KEY WORDS: *Helianthus annuus,* Sunflower, geographic variation in leaf hydrocarbon yields.

Adams and Seiler (1984) surveyed 39 taxa of sunflowers for their cyclohexane (hydrocarbon) and methanol (resins) concentrations. The highest cyclohexane (bio-crude) yielding taxa were *H. agrestis*, an annual, Bradenton, FL (7.38%) and *H. annuus*, Winton, OK (7.09%). Adams et al. (1986) screened 614 taxa from the western US for their hydrocarbon (hexane soluble) and resin (methanol soluble) yields. They reported 2 plants of *H. annuus* from Idaho with 8.71% and 9.39% hydrocarbon yields.

Seiler, Carr and Bagby (1991) reported on 28 *Helianthus* taxa for their yields of oil, polyphenols, protein and rubber. The rubber was found to be of lower molecular weight than *Hevea* rubber, but still appeared to be useful as a plasticizing additive and for coatings inside pipes and containers. Yields of natural rubber has recently been reported for *H. annuus* (Pearson et al. (2010a) that ranged from 0.9% to 1.7% rubber in cultivated sunflower cultivars (Fig. 4, Pearson et al. 2010b).

There does not appear to be any information on geographic variation in the yields of hydrocarbons for *H. annuus*. The purpose of this report is to present new information on geographic variation of the yields of pentane extractable hydrocarbons in native, annual sunflower. This is continuation of our research on sunflowers (Adams and TeBeest, 2016; Adams, et al. 2016).

MATERIALS AND METHODS

Population locations - see Appendix I.

The lowest growing, non-yellowed, 8 mature leaves were collected at stage R 5.1-5.3 (Figure 1) when the first flower head opened with mature rays. The leaves were air dried in paper bags at 49° C in a plant dryer for 24 hr or until 7% moisture was attained.

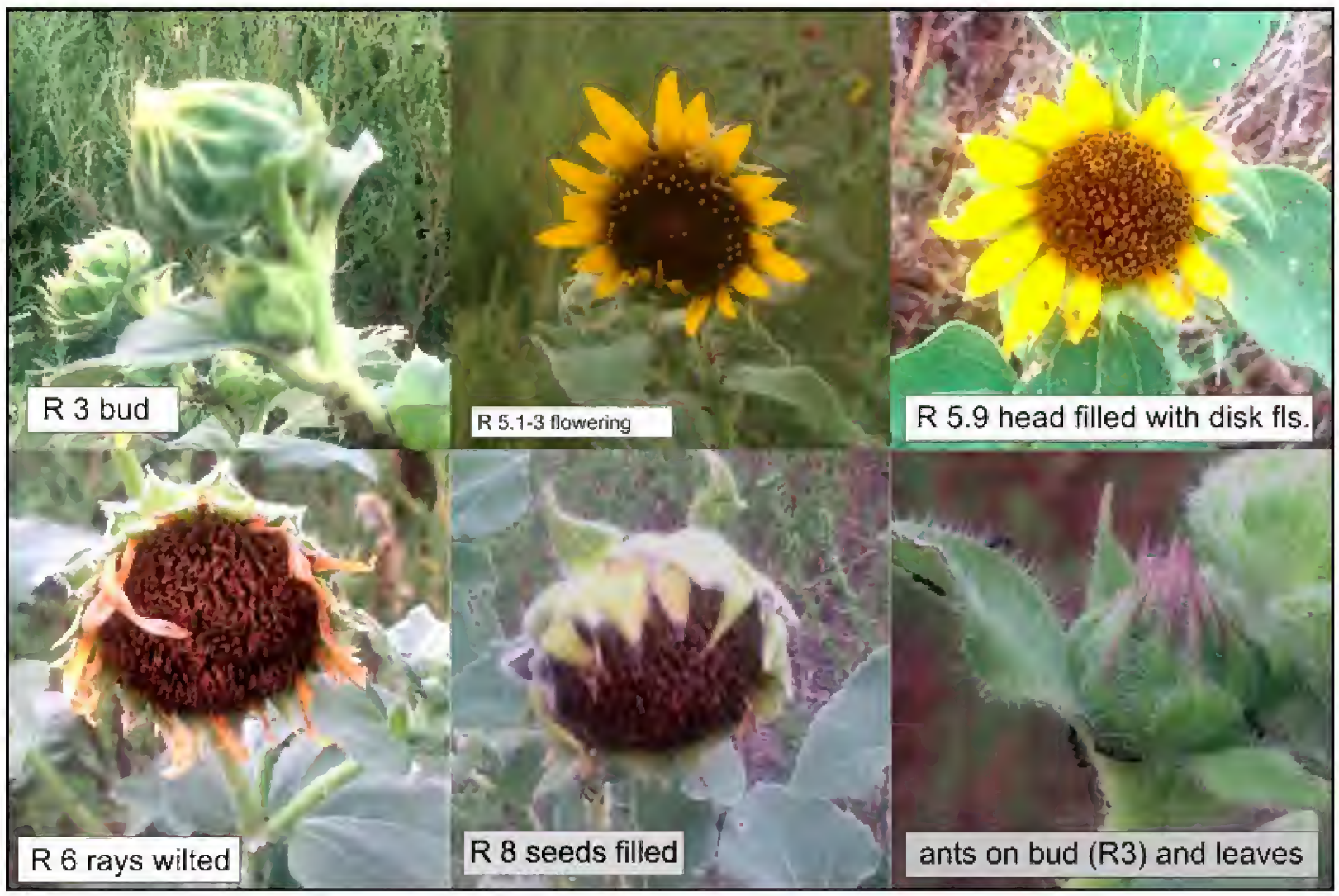

Figure 1. Growth stages of wild (*H. annuus*) sunflowers, Gruver, TX. Note black ants on the bud and leaves in lower right photo (from Adams et al. 2016). Sunflower growth stages termination is from Schnetter and Miller(1981).

Leaves were ground in a coffee mill (1mm). 3 g of air dried material (7% moisture) were placed in a 125 ml, screw cap jar with 20 ml pentane, the jar sealed, then placed on an orbital shaker for 18 hr. The pentane soluble extract was decanted through a Whatman paper filter into a pre-weighed aluminum pan and the pentane evaporated on a hot plate (50°C) in a hood. The pan with hydrocarbon extract was weighed and tared.

RESULTS

The yields of hydrocarbons (HC) by population are given in Table 1. The highest yield (8.60%) was from Gruver, TX followed by Lake Tanglewood, TX (8.47%) in the Texas Panhandle. The lowest yield was from Woodward, OK (2.62%) and Eagle Nest, NM (2.62%) followed by cultivated sunflowers (Oslo, TX)(3.20%). The Woodward population had smooth leaves as found in cultivated sunflowers. The plants and leaves were very large, although the heads were small. It appears that the Woodward population was a product of crosses between native and cultivated sunflowers and this resulted in the very low oil yield.

To visualize the variation in HC yields, the means were contour mapped (Fig. 2). Notice that the highest yields are in the Texas Panhandle. The lowest yields are in the west (EN, AZ, RO) and just off the caprock, east of the Texas Panhandle (PT, QN). The low yield at the WO (Woodward, OK) is in a population that is likely of hybrid origin between native and cultivated sunflowers. The southern Idaho - northern Utah area had medium-high yields. Of interest are the four populations near Waco, TX (MC, FC, LC, HC) that have 6.2, 5.3, 3.6, and 4.9% yields in a very small area. At this time, it is not known if

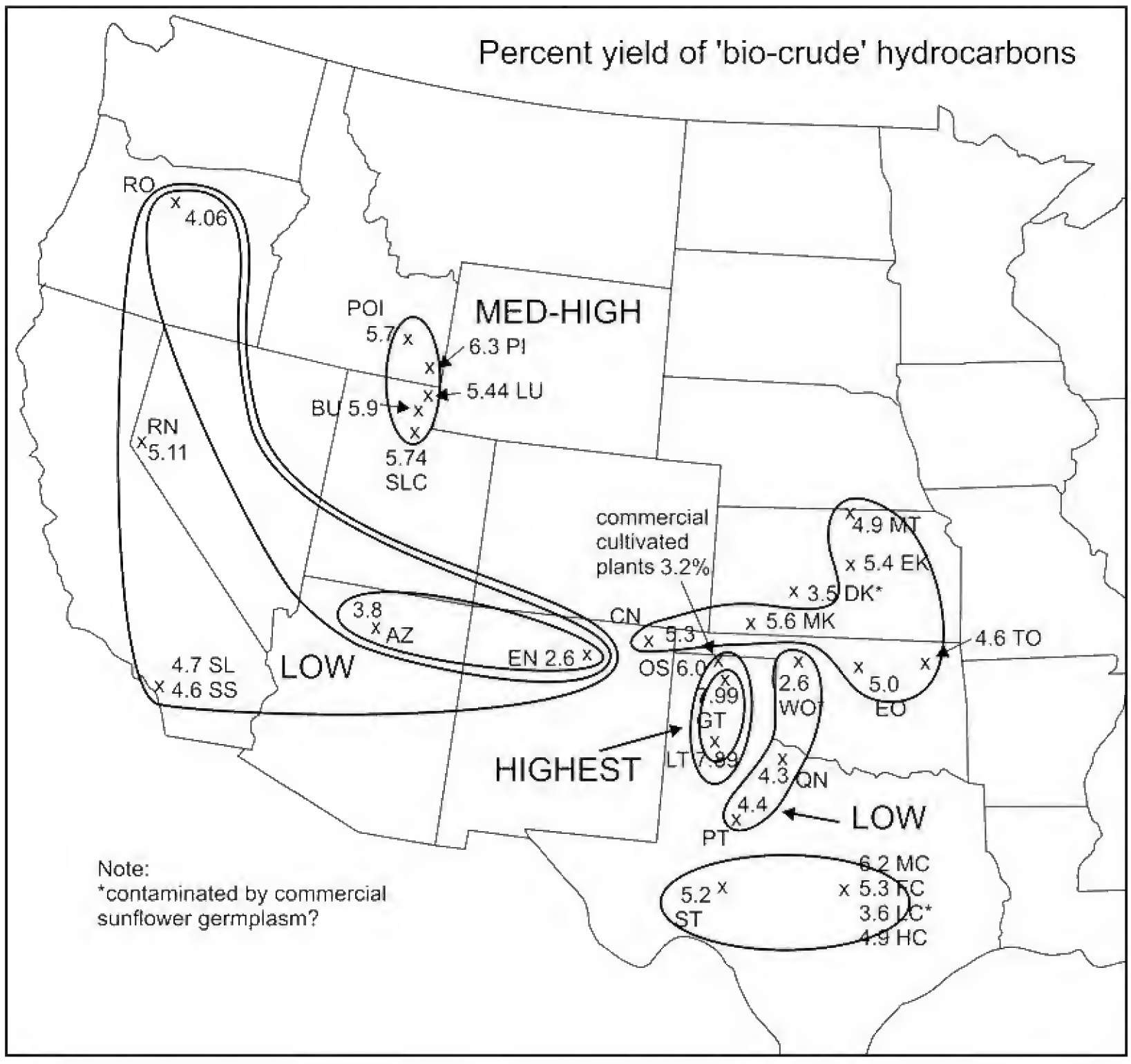

Figure 2. Geographic variation in % yields of HC by population. The asterisk (*) at the WO population indicates that the population is likely of hybrid origin between native and cultivated sunflowers. Note the low yield from a commercial sunflower field near Oslo, TX (lower left). See text for discussion.

the variation in yields is due to genetics or the environment. It is interesting that the correlation between % yield and leaf weight as only r= 0.18 (highly significantly different from zero, df = 327). But the correlation accounts for only 3.24% (r^2) of the variance. Thus, breeding for both increased % yields of HC and biomass seems feasible.

The variability of yields by population is mapped in Figure 3. Population variability in HC yields varied geographically and also between adjacent populations, suggesting the micro-habitat environments are important as well as limited genetic population size. One of the least variable populations was Pocatello, ID (POI, Fig. 3). This was a population of perhaps 50 plants, growing next to the sidewalk at an on-ramp to I15. It seems likely that POI is very inbred. The Brigham City, UT (BU) population, in a disturbed vacant lot where a new mall was recently built, was much more variable (Fig. 3). BU contained perhaps 100 plants, but a more extensive group of sunflowers grew nearby.

Clearly the most unusual situation was the Waco, TX area where 4 nearby populations (MC, FC, LC, HC, Fig. 3) showed very small to large amounts of variation in their HC yields. MT (Montrose, KS) was from only 3 cultivated plants raised from seed, so its small variability may be just chance.

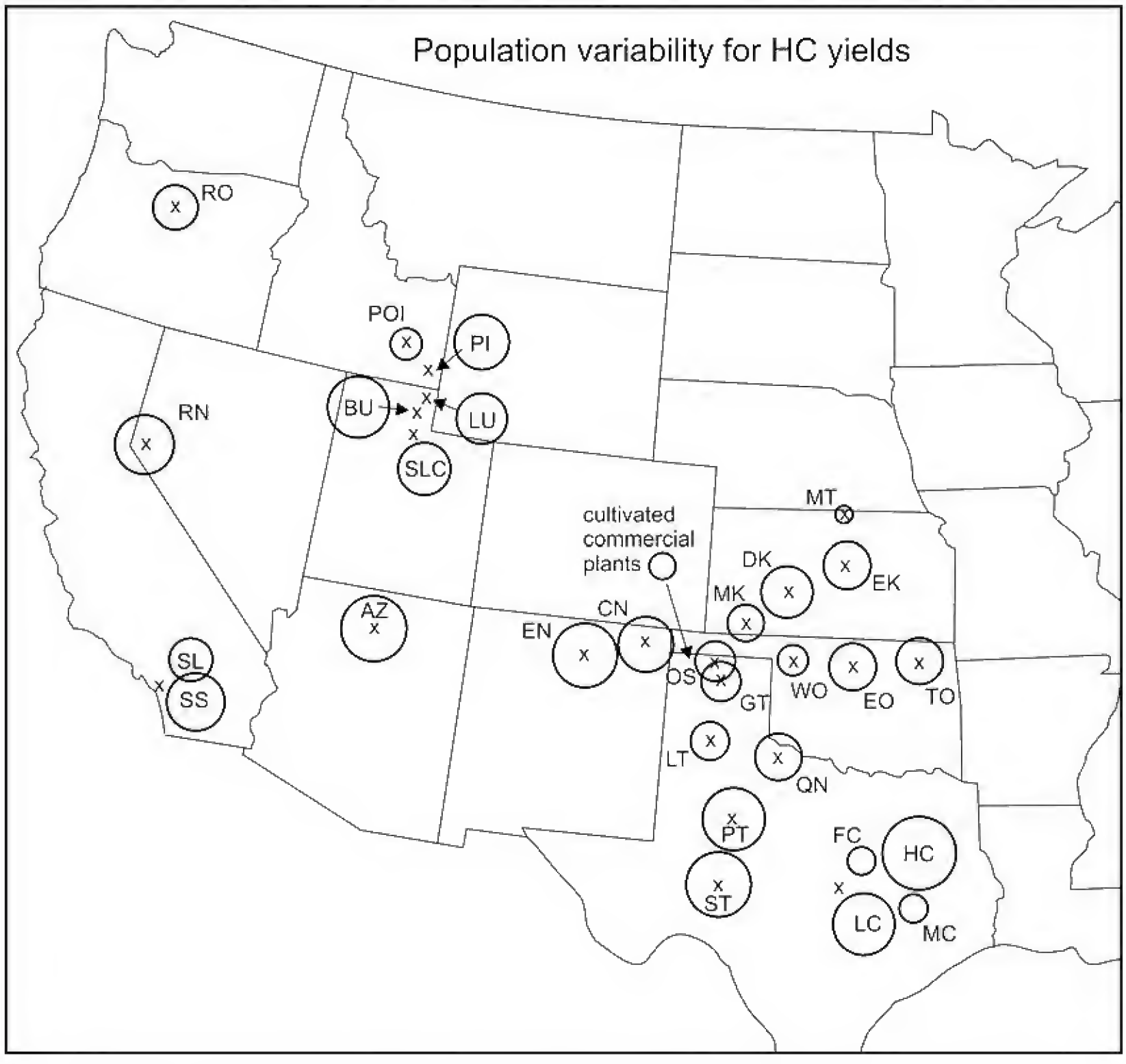

Figure 3. Population variability (coefficient of variation in HC yields) for the 29 populations sampled. The size (diameter) of the circles is proportional to their coefficient of variation.

The total yields of HC per the weight of 8 mature leaves is a measure of the grams of HC per plant (likely larger than if the entire plant were extracted). In Table 1, the yields range from 0.114 g/ 8 mature leaves (Eagle Nest, NM) to 1.428 g (Gruver, TX). Variation in yields shows (Fig. 4) the highest yields were in the Texas Panhandle (1.20 g - 1.23 g) and Ellsworth, KS (1.0 g) and Enid OK (0.88 g). The lowest yields were in the southwestern United States. Note the difference between the San Diego, large leaves (SL, .43 g) and small leaves (SS, .27 g). These are plants collected from the same population. Recall that the % yields were quite similar (Table 1, SS, 4.59%; SL, 4.68%).

The four populations near Waco, TX are quite variable and yields ranged from 0.26 g to 0.74 g. Whether this is due to micro-habitat environments or genetically isolated populations is not known at this time.

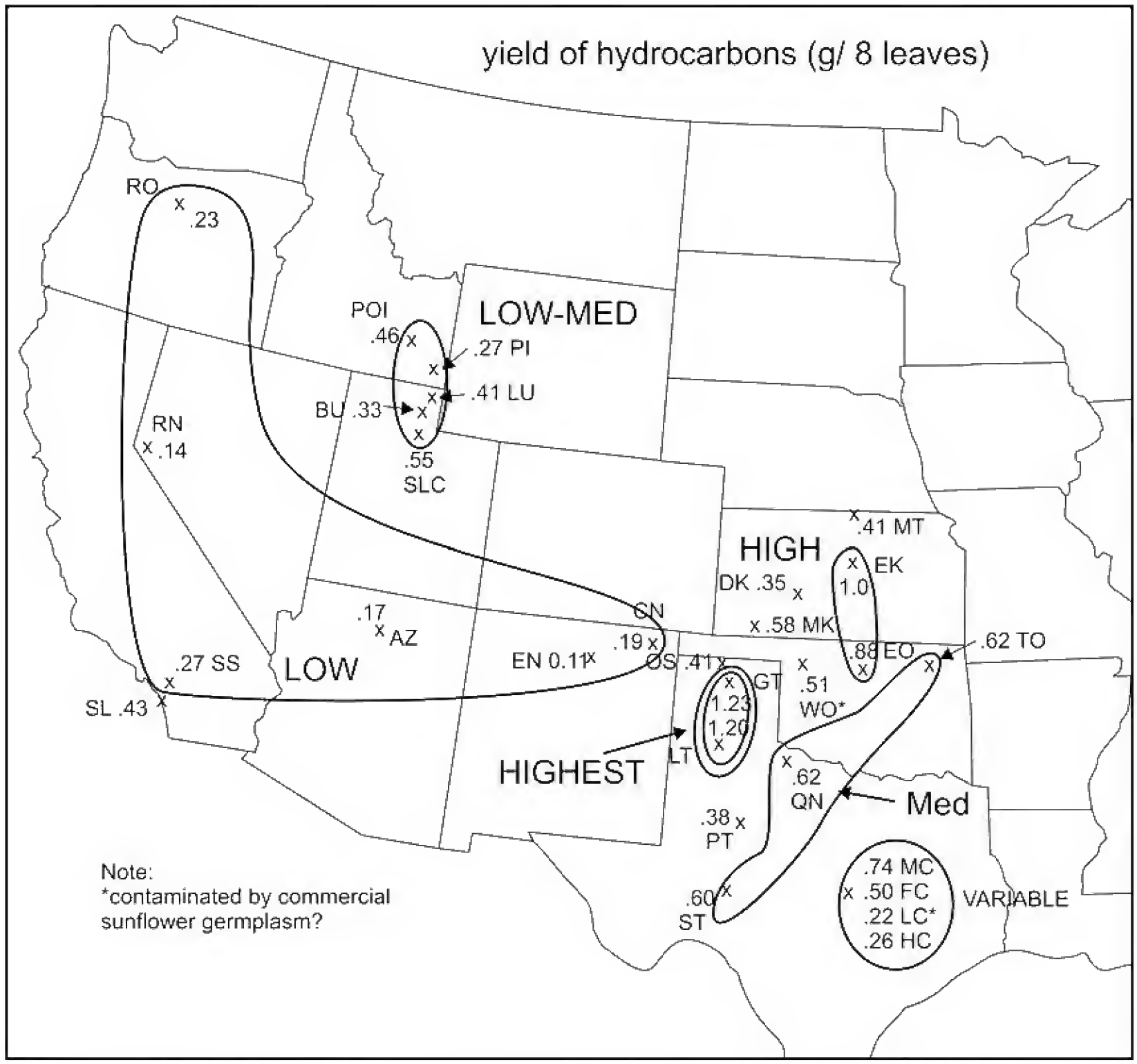

Figure 4. Geographic variation in the HC yields (g/ weight of 8 mature, dried leaves, basis).

The frequency distribution (329 individuals, Fig. 5) shows yields ranged from 1.0 to 12.63% with a skewed, normal distribution, and tailing towards the highest yielding plants (Fig. 5). The mean was 5.33%, with the top 5% being larger than a 8.7% yield. Seed from high yielding plants have been collected in preparation to examine genetic and environmental factors.

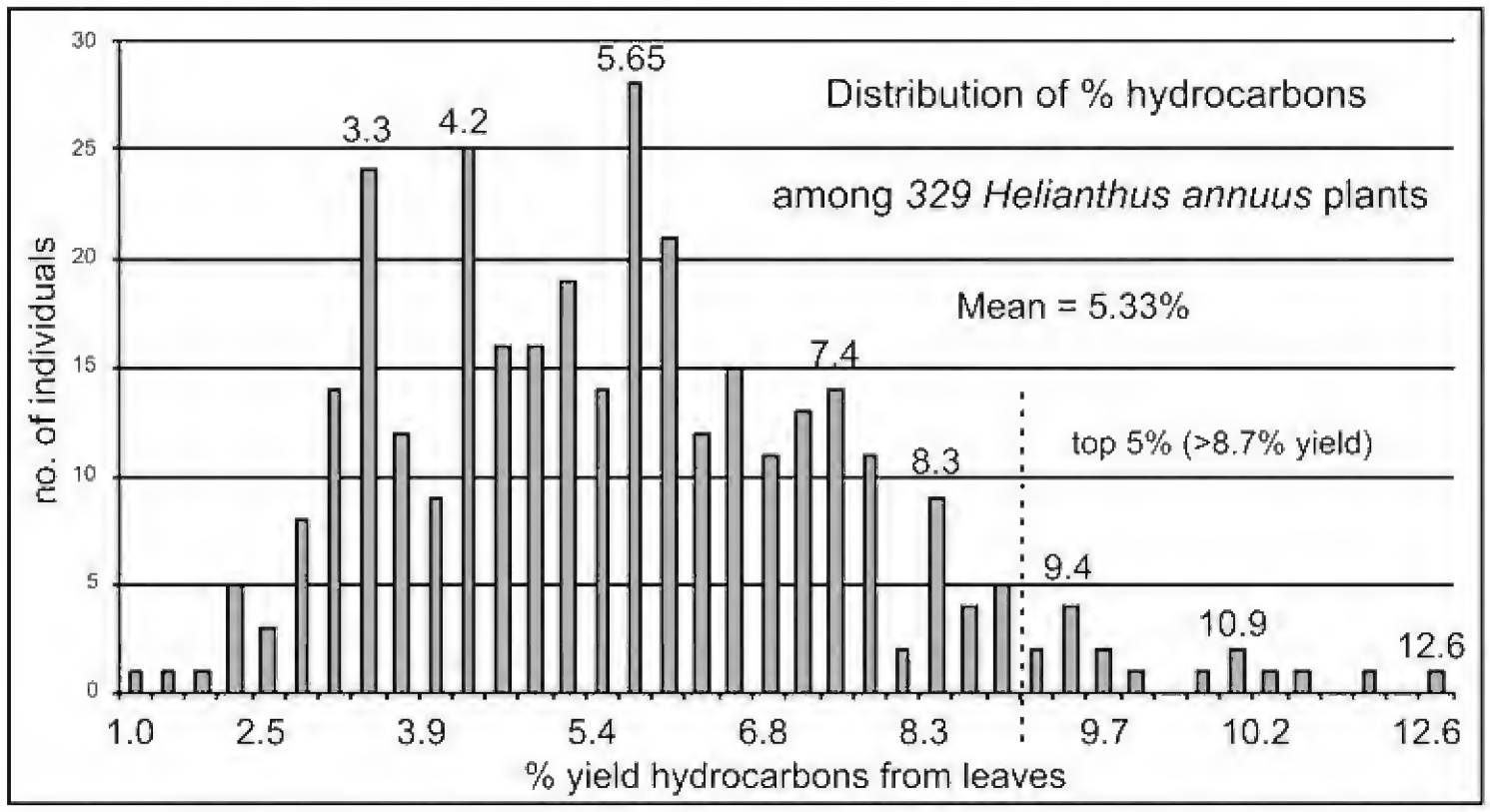

Figure 5. Frequency distribution of HC yields for 329 *H. annuus* plants. See text for discussion.

This study revealed the range of variation in native sunflowers is quite large, from 1.0 to 12.63%. It is remarkable to find such a wide range, but indicates the potential of *H. annuus* to produce copious amounts of hydrocarbons for use as fuel and in the petro-chemical industry. Many of the highest yielding plants were severely eaten by grasshoppers and covered with black (sugar) ants, feeding on resin extruded from the stem, petioles and leaf bracts. It could be that the high yields were responses to insect damage that induced defense chemicals. The induction of chemical defenses will be examined in a subsequent study, along with study of the effects of genetics vs. the environment on the production of hydrocarbons.

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Table 1. Yields of hydrocarbons (HC) *H. annuus*, from natural populations. Coefficient of variation computed as standard deviation / mean.

popn id, sample ids	population sampled	weight 8 lvs	% yield corr'd*	Coef. of variation	Range of yields	yield g/8 lvs
PT P1 - P0	14935 Post, TX	8.83	4.36	0.317	(2.33,6.63)	0.385
QN Q1-Q0	14936 Quanah, TX	14.41	4.32	0.238	(2.88,5.62)	0.623
MK M1-M0	14939 Meade, KS	10.38	5.64	0.186	(3.91,7.21)	0.585
DK D1-D0	14940 Dodge City, KS	9.75	3.52	0.262	(2.68,5.49)	0.346
EK E1-E0	14941 Ellsworth, KS	18.74	5.38	0.240	(3.63,7.35)	1.008
TO T1-T0	14942 Tulsa, OK	13.64	4.56	0.236	3.16,6.04)	0.622
EO O1-OT	14943 Enid, OK	17.60	4.97	0.239	(3.23,7.55)	0.875
WO W1-W0	14944 Woodward, OK, very large, smooth leaves	19.64	2.62	0.155	(1.92-3.09)	0.515
ST S1-S0	14945 grown from seed, ex Sonora, TX, PI413168	11.47	5.19	0.329	(1.99-7.55)	0.595
OS O1-TO	14946 Oslo, TX, native in prairie grass	6.75	5.95	0.203	(4.19-8.17)	0.406
	14947 Lake Tanglewood, TX					
LT: L1-L0	7/12/16, 1st collection	16.65	8.47	0.222	(6.73-12.15)	1.410
L2: LA-LJ	7-20-16, 2nd collection	13.92	**7.31**	0.163	(5.63-9.06)	1.018
			7.89 avg	0.195 avg	(5.63-12.15)	1.214
ID I1-I9	14948 grown from seed, ex Idaho, PI 531028	2.77	3.23	0.432	(1.0-6.14)	0.089
SS SA-SJ	14950 San Diego, CA, small leaves	5.83	4.59	0.292	(2.75-7.51)	0.268
SL SK-ST	14951 San Diego, CA, large leaves	9.04	4.68	0.218	(2.75-6.11)	0.432
	14952 Gruver, TX					
GT1:G1-G0	GT1 1-10 1 mi south	16.93	7.26	0.244	(5.01-11.06)	1.229
GT2:GA-GJ	GT2 11-20 1mi south	18.03	7.92	0.198	(6.25-10.78)	1.428
GT3:GKGT	GT3 21-30, 2 mi E, Rodeo	12.50	8.16	0.164	(7.00-10.51)	1.020
GT4:A1-AT	GT4 31-40, 1 mi south	14.50	8.60	0.235	(6.52-12.63)	1.247
			7.99 avg	0.201 avg	(5.01-12.63)	1.231
SC 1O-6O	14953 cultivated sunflower crop, Slough Farm, Oslo, TX	12.41	3.20	0.134	(2.75-3.85)	0.397
MC 1M-0M	14976 McLennan Co., TX *Holmes 16654*	11.95	6.18	0.144	(4.74-7.76)	0.739
FC 1F-0F	14977 Falls Co., Satin, TX *Holmes 16656*	9.49	5.29	0.142	(4.19-6.59)	0.502
LC 1L-0L	14978 Limestone Co. Mt. Calm, TX Holmes *16658*	6.13	3.58	0.313	(2.61-6.25)	0.219
HC 1H-0H	14979 Hill Co., TX *Holmes 16661*	5.21	4.92	0.372	(2.61-8.65)	0.263
EN 1E-0E	14980 Eagle Nest, NM	4.34	2.62	0.326	(1.17-3.85)	0.114
LU U1-U0	15023 Logan, UT	7.57	5.44	0.257	(3.98-8.67)	0.412
PI 1P-0P	15024 Preston, IT	4.29	6.30	0.278	(3.91-9.34)	0.270
POI 1I-0I	15025 Pocatello, ID	7.99	5.71	0.160	(4.46-7.55)	0.456
SLC 1U-0U	15026 Mill Creek, Salt Lake City, UT	9.54	5.74	0.266	(3.91-8.22)	0.548
RO R1-R0	15027 Redmond, OR	5.74	4.06	0.226	(3.37-6.28)	0.233
CN 1C-0C	14981 Capulin, NM	3.51	5.29	0.280	(3.16-8.34)	0.186
MT MA-MC	14982 grown from seed ex Montrose, KS, PI 413033	8.44	4.91	0.089	(4.65-5.42)	0.414

popn id, sample ids	population sampled	weight 8 lvs	% yield corr'd*	Coef. of variation	Range of yields	yield g/8 lvs
AZ Z1-Z0	15021 Camp Verde, AZ	4.48	3.79	0.332	(1.72-5.56)	0.170
BU B1-B0	15022 Brigham City, UT	5.70	5.90	0.312	(2.90-8.31)	0.336
RN R1-R0	15029 Reno, NV	2.87	5.11	0.299	(2.89-7.90)	0.142

*correction factor = soxhlet, 6hr extraction/ pentane 18 hr shaker yield = 2.06

Appendix I Population locations.

Helianthus petiolaris

common along roadside in sandy soil. flowering. 8.3 mi SW of Fritch, TX on TX 136, 35° 31' 53" N, 101° 38' 31" W. 3360 ft, Date: 4 June 2016, County: Potter; State: TX
Coll. Robert P. Adams No. 14937

Helianthus annuus L. below:

common along railroad and roadside in sandy soil. flowering. 5.3 mi SE of Post TX on US 84, 33° 01' 53" N, 101° 11' 25" W, 2300 ft, Date: 4 June 2016 County: Garza; State: TX
Coll. Robert P. Adams No. 14935

common along fence row and roadside in sandy soil. flowering.7 mi SE of Quanah, TX on US 287,34° 15' 57" N, 99° 36' 46" W, 1450 ft, Date: 5 June 2016 County: Hardeman; State: TX
Coll. Robert P. Adams No. 14936

1.5 mi s of Meade, on KS23, low area in edge of wheat field, 100s of plants in population, but generally uncommon. ~5% flowering. 37° 15' 49" N, 100° 20' 40" W, 2433 ft, Date: 7 July 2016; County: Meade; State: KS
Coll. Robert P. Adams No. 14939

8.5 mi NE of Dodge City, US 50, several on dirt piles of highway dept., but generally uncommon. ~5% flowering, 37° 47' 06" N, 99° 53' 14" W. 2534 ft. Date: 7 July 2016, County: Ford; State: KS
Coll. Robert P. Adams No. 14940

1.6 mi e of Ellsworth on KS140, on fence row on s side of wheat field, 20 plants, but generally uncommon. ~10% flowering. 38° 44' 24" N, 98° 11' 53" W, 1600 ft, Date: 7 July 2016, County: Ellsworth; State: KS
Coll. Robert P. Adams No. 14941

15 plants on disturbed area next to South Ash St. (just south of OK364), but generally uncommon, Jenks, OK (sw suburb of Tulsa). ~5% flowering. 36° 00' 57.85" N, 95° 58' 07.61" W, 613 ft, Date: 9 July 2016, County: Tulsa; State: OK
Coll. Robert P. Adams No. 14942

5.5 mi e of Enid on OK412,on fence row, side of wheat field, few plants but generally uncommon. ca 5% flowering, ~5% flowering. 36° 23' 51" N, 97° 46' 51" W, 1160 ft., Date: 9 July 2016, County: Garfield; State: OK
Coll. Robert P. Adams No. 14943

smooth leaves! 2.8 mi e of Woodward on OK412,on fence row, side of grass field, few plants but generally uncommon. ca 5% flowering mostly pre-flowering. 36° 25' 53" N, 99° 20' 28" W, 1880 ft., Date: 9 July 2016, County: Woodward; State: OK
Coll. Robert P. Adams No. 14944

cultivated at Oslo, TX, from seed (USDA PI413168-NC7) ex Sonora, TX. 80% flowering, 36° 25' 12.3" N, 101° 31' 54.6" W, 3239 ft, Date: 12 July 2016, County: cult in Hansford; State: TX.
Coll. Robert P. Adams No. 14945

native in grassland, JP & Amy TeBeest farm, 1 mi. s of Oslo Lutheran Church. ~5% flowering. 36° 25' 12.3" N, 101° 31' 54.6" W, 3239 ft., Date: 12 July 2016, County: Hansford; State: TX
Coll. Robert P. Adams No. 14946

2- 3 ft plants, lots of resin on petioles and leaf veins, many sugar (black) ants, most with wilted leaves, very dry in July, common in native grass and on disturbed roadside, brush dump area, Lake Tanglewood, ~50% flowering, 35° 04' 23.7" N, 101° 47' 29.0" W, 3239 ft., Date: 12 July 2016, County: Randall; State: TX
Coll. Robert P. Adams No. 14947

cultivated at Oslo, TX, from seed (USDA PI 531028) ex Idaho, 80% flowering.36° 25' 12.3" N, 101° 31' 54.6" W, 3239 ft, Date: 12 July 2016, County: cult in Hansford; State: TX
Coll. Robert P. Adams No. 14948

plants 2' tall, with small leaves, along San Pasqual Rd, 33° 05' 08.2" N, 117° 01' 46.2" W, 353 ft.
Date: 6 July 2016, County: San Diego; State: CA, Coll. Jim A. Bartel 1636
Lab Acc. Robert P. Adams No. 14950

plants to 8' tall, with large leaves, along San Pasqual Rd, 33° 05' 08.2" N, 117° 01' 46.2" W, 353 ft/ Date: 8 July 2016, County: San Diego; State: CA, Coll. Jim. Bartel 1636
Lab Acc. Robert P. Adams No. 14951

2-3' tall, 10% flowering, lots of damage to leaves by grasshoppers, etc., some with many black (sugar) ants, copious resin at base of leaves, along fence row, on TX 206, 1-5:1.2 mi s, 6-10: 1.3 mi. s of Gruver, TX. 36° 14' 52" N, 101° 24' 52" W, 3161 ft, Date: 16 July 2016, County: Hansford; State: TX
Coll. Robert P. Adams No. 14952

cultivated, irrigated near Oslo, TX, on Slough farm. at R-5.1 stage. 36° 22' 42.17" N, 101° 37' 21.4" W, 3350 ft., leaves mostly smooth. Date: 17 July 2016, County: cult in Hansford; State: TX
Coll. Robert P. Adams No. 14953

Coll. Walter Holmes
(WCH16654) McLennan Co. 12th Street at Flat Creek, Robinson (Waco), 27 July 2016 , Walter Holmes
Lab Acc. Robert P. Adams 14976
(WCH 16656) Falls Co. near Satin on FR 434, prairie roadside, 28 July 2016, Walter Holmes
Lab Acc. Robert P. Adams 14977
(WCH 16658) Limestone Co. near jct of Limestone Co roads 102 and 106, south of Mt. Calm, prairie, 29 July 2016, Walter Holmes
Lab Acc. Robert P. Adams 14978
(WCH 16661) Hill Co. US Hwy 84, West of Mt. Calm near jct with West Somers Lane, 29 July 2016, Walter Holmes
Lab Acc. Robert P. Adams 14979

roadside waste area, Eagle Nest, NM, 36° 33.650' N, 105° 15.969' W, 8260 ft, Date: 8 Aug 2016, County: Colfax; State: New Mexico, Coll. Amy TeBeest
Lab acc. Robert P. Adams 14980

roadside waste area, Capulin (city), NM, some grasshopper damage, 36° 44.527' N, 104° 00.178' W, 6820 ft, Date: 8 Aug 2016, County: Union: State: New Mexico, Coll. Amy TeBeest
Lab acc. Robert P. Adams 14981

cultivated at Oslo, TX, from seed (USDA PI1413033), ex Montrose, KS. Date: 2 Aug 2016, Coll. Amy TeBeest,
Lab acc. Robert P. Adams 14982

along roadsides. 16-18 mi east of Camp Verde on AZ 260. 34.489° N, 111.597° W, 5900 ft, Date: Aug. 27, 2016, County: Yavapai; State: AZ, Coll. David Thornburg ns,

Lab. acc. Robert P. Adams No. 15021
vacant lot behind new WalMart on disturbed soil. flowering and seeding, multiple branches. W1500S, 775W, Brigham City, UT, 41° 28' 57" N, 112° 01' 40" W, 4250 ft, Date: Sept. 2, 2016, County: Boxelder; State: UT
Coll. Robert P. Adams No. 15022

vacant lot behind new stores on disturbed soil on US 91 and E2000N, flowering and seeding, multiple branches. Logan, UT, 41° 46' 09" N, 111° 49' 59" W, 4506 ft, Date: Sept. 2, 2016, County: Cache; State: Utah
Coll. Robert P. Adams No. 15023

vacant lot in new subdivision on disturbed soil off of OR hwy 34/36 & just on n edge of Preston, flowering and seeding, multiple branches. 42° 06' 40" N, 111° 52' 01" W, 4703 ft, Date: Sept. 2, 2016, County: Franklin; State: Idaho
Coll. Robert P. Adams No. 15024

next to sidewalk, on slope, next to freeway (I15) access south, flowering and seeding, multiple branches, Pocatello, ID. 42° 52' 49" N, 112°' 25' 35" W, 4625 ft, Date: Sept. 2, 2016, County: Bannock; State: Idaho
Coll. Robert P. Adams No. 15025

next to sidewalk, flowering and seeding, multiple branches. common along sidewalks, Mill Creek, UT. s side of I80 on 2000 E, east side of 2000E. 42° 52' 49" N, 112°' 25' 35" W, 4625 ft, Date: Sept. 3, 2016, County: Salt Lake; State: Utah
Coll. Robert P. Adams No. 15026

disturbed area, vacant on SW Airport Way, ~373m sse of jct SW Airport Way & Veterans Way. Redmond, OR, 44° 15' 30" N, 121°' 09' 54" W, 3035 ft, Date: Sept. 3, 2016, County: Redmond; State: Oregon
Coll. Mark R. Corbet, ns, Lab Acc. Robert P. Adams No. 15027

disturbed area, Neil Rd and west frontage road on I580, s of Reno, NV. 39° 28' 11.6" N, 119°' 47' 20.4" W, 4485 ft, Date: Sept. 5, 2016, County: Washoe; State: Nevada
Coll. Chauncey Parker, ns, Lab Acc. Robert P. Adams No. 15029.

Seedling growth and leaf photosynthesis of *Acer grandidentatum* (Bigtooth maple, Sapindaceae) from isolated central Texas populations

Terri L. Nelson Dickinson
Department of Biology, University of Texas at San Antonio, One UTSA Circle,
San Antonio, TX 78249, USA terri.nelson.dickinson@gmail.com
and
O. W. Van Auken
Department of Biology, University of Texas at San Antonio, One UTSA Circle,
San Antonio, TX 78249, USA oscar.vanauken@utsa.edu

ABSTRACT

Two experiments were completed to address issues concerning apparent recruitment failure of native relict populations of *Acer grandidentatum* Nutt. (Bigtooth maple) found in the Edwards Plateau Region of Central Texas. In the first experiment seedlings were grown in pots at 20, 40, 60 and 100% (open or full sun, 1615 ± 8 μmol/m^2/s). In the second experiment leaf photosynthetic rates of seedlings growing in sun or shade below a canopy were examined. Growth of seedlings was greatest at 40% of the maximum light treatment or at a light level of 705 ± 22 μmol/m^2/s. Mortality was zero in the 40% light treatment and 100% at the highest light level tested. Light response curves were generated using photosynthetic rates of five leaves of separate juvenile maples growing in full sun or understory canopy shade. Rates were measured in the field at light levels from 0-2000 μmol/m^2/s. From these measurements a number of photosynthetic parameters were calculated and compared. No significant differences were seen between the curves for sun and shade leaves of *A. grandidentatum*. The only significantly different photosynthetic parameter measured was the maximum photosynthetic rate (A_{max}). The A_{max} was low at 3.89 ± 0.36 μmol CO_2/m^2/s for shade leaves and 5.23 ± 0.36 μmol CO_2/m^2/s for sun leaves. The light saturation point, the light compensation point and other calculated factors were low as well, but not significantly different. *Acer grandidentatum* is a shade tolerant species with a low photosynthetic rate which seems to be part of the reason it can persist in isolated Central Texas canyon woodland populations. Published on-line www.phytologia.org *Phytologia 99(1): 11-21 (Jan 19, 2017).* ISSN 030319430.

KEY WORDS: light levels, CO_2 uptake, gas exchange rates, shade plants

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It has been challenging to understand factors controlling growth and recruitment of woody species in woodland and forest communities, although many research papers have dealt with the topic (Baker et al. 2005). This is true for native relict populations of *Acer grandidentatum* Nutt. and other woody species that are present in the Edwards Plateau Physiographic Region of Central Texas (Van Auken 1988; Russell and Fowler 2004; Nelson Dickerson and Van Auken 2016). *Acer grandidentatum* is a small, deciduous, hardwood tree commonly known as bigtooth maple, but it has many other common names such as canyon maple, western sugar maple and others (Correll and Johnston 1970; Tollefson 2006). There has been some debate over the systematics of *A. grandidentatum,* but most current papers refer to it simply as *A. grandidentatum,* which is the convention that we will follow (Cronquist et al. 1997; Stevens 2001; Atha et al. 2011). The 129 *Acer* species have been traditionally grouped into the family Aceraceae, but more recently they have been considered as members of the subfamily Hippocastanoideae within the family Sapindaceae (Buerki et al. 2009; Watson and Dallwitz 2011).

Anecdotal reports suggest a decline of recruitment of juvenile *A. grandidentatum* in central Texas (Riskind 1979; McCorkle 2007; Adams 2010; BCNPSOT 2010; Heidemann 2011). These

same reports suggest the decline in recruitment is caused by browsing by large herbivores specifically by *Odocoileus virginianus* (white-tailed deer). A recent study confirmed that *A. grandidentatum* seedlings protected from large herbivores had a greater rate of survival than unprotected seedlings (Nelson Dickinson and Van Auken 2016). With leaf removal, a reduction in plant net photosynthesis occurs. This leaf loss compromises the ability of the plant to replace lost biomass (Ellsworth et al. 1994), which is most readily shown in juveniles. A plant's photosynthetic parameters affect its inherent growth rate and thus its biomass (Jones and McLeod 1989). Thus, understanding a plant's photosynthetic characteristics can help explain how an individual plant is able to compensate for episodes of herbivory and how it adapts to an environment that's been altered by herbivory (Crowley 1997). Nevertheless, until now, there have been no studies that we could find concerning photosynthetic rates of *A. grandidentatum.*

PURPOSES

The purposes of the present study were to determine the light requirements of juvenile *A. grandidentatum* plants and to compare differences in gas exchange rates at various light levels for leaves of sun and canopy shade *A. grandidentatum* plants.

METHODS

STUDY AREAS-There were two study areas. The plant growth experiment was carried out on a non-shaded roof patio of the science building of the University of Texas San Antonio (98°34'26"W and 29°37'19"N). The gas exchange measurements were made at Lost Maples State Natural Area, in Bandera and Real counties about 114 mi west of San Antonio, Texas (29°49'11"N and 99°34'59"W).

SEEDLING GROWTH-On March 29, 2010, a total of twenty first-year seedlings were obtained from a commercial source (Janzow, Boerne, TX), transplanted and randomly placed into one of four light treatments (five plants/treatment). Plants were randomly placed (one each) into 15.0 cm diameter x 14.5 cm tall pots lined with 3.79 L Ziploc® plastic bags containing 1350 g of soil. Additional nutrients were added as 5.5 g Osmocote per pot (14/14/14 NPK equivalent to 436 kg/ha nitrogen, 436 kg/ha phosphorous, and 436 kg/ha potassium). Plants were watered with deionized water as needed, usually 150 mL every day (Janzow 2007). They were placed on a non-shaded roof-top patio on UTSA's Science building within each of the light treatments.

Plants' sunlight exposure was limited using shade boxes measuring 0.5 m wide, 0.5 m long, and 1.0 m high. They were constructed with 1.3 cm diameter PVC pipe covered with zero to three layers of commercial black polyethylene shade cloth on five sides secured with plastic zip ties (Rainbow Gardens and Lowe's Home Improvement Stores, San Antonio, TX) to adjust light levels in each treatment.

Light levels were measured in each plant location, in each shade box on a clear day in May and October 2010 within ± 30 minutes of solar noon using a Li-COR® LI-188 integrating quantum sensor (Li-COR, Inc. Lincoln, NE). Each shade box contained five *A. grandidentatum* seedlings (one/pot). Light levels were 100 % or maximum (1615 ± 8 $\mu mol/m^2/s$, no shade cloth), 60 % (977 ± 42 $\mu mol/m^2/s$), 40 % (705 ± 22 $\mu mol/m^2/s$), and 20 % (281 ± 1 $\mu mol/m^2/s$). Boxes were affixed to railings with zip ties and weighted with sand bags to prevent movement. Pots were covered with clear plastic during rainy weather to prevent flooding, nutrient and soil loss.

Survival, aboveground, belowground and total dry mass were determined and recorded. Other plant responses were measured but not reported here (Nelson Dickinson 2011). Plants were harvested on October 14, 2010, dried to a constant mass at 80°C, and weighed. Data were tested for normality using the Shapiro-Wilks test and for homogeneity of variance using Bartlett's test (Sall et al. 2005). If probability

values fell below 0.05 on either test, data were transformed and retested. Aboveground dry mass, belowground dry mass, and total dry mass were log transformed, and then analyzed using a one-way ANOVA followed by Tukey-Kramer HSD. ANOM for proportions was used with a probability level of 0.05 to determine if there were differences in mortality across treatments (McKinley and Van Auken 2005).

GAS EXCHANGE MEASUREMENTS-Ten *Acer grandidentatum* saplings were randomly selected in and adjacent to a deer exclosure at Lost Maples State Natural Area, in Sabinal Canyon, Texas. One fully expanded, complete leaf was selected on each plant; five leaves from the shaded canopy understory plants and five from the open no canopy, full sun plants. The Li-Cor 6400 portable photosynthetic meter was used to measure gas exchange as a function of light level, or photosynthetically active radiation (PAR), for each leaf. Measurements were made with plants fully leafed out in May 2011, within ± three hours of solar noon using a gas flow rate of 400 µmol/s and a CO_2 concentration of 390 µmol/mol at PARs of 2000, 1600, 1200, 1000, 800, 600, 400, 200, 100, 50, 25, 10, 5, and 0 µmol/m^2/s. Each leaf used covered the entire chamber.

Two light response curves were generated, one for sun leaves and one for shade leaves. Photosynthetic rates along each curve were tested for normality using the Shapiro-Wilks test and for homogeneity of variance using Bartlett's test (Sall et al. 2005). A repeated measures ANOVA was completed to determine if there were significant differences between the two leaf types. A one-way ANOVA with Tukey's HSD was used to determine differences in photosynthetic rates at different light levels. For the sun and shade treatments, the maximum rate of photosynthesis (A_{max}) was determined, along with transpiration and conductance at the A_{max}. The initial slope of the curve, or quantum yield efficiency, was also measured. The PAR value at which this line reached A_{max} was the light saturation point (L_{sp}). Other factors measured were the dark respiration (R_d), the curve's y-intercept and the light compensation point (L_{cp}), the line's x-intercept. These values were also tested for normality using the Shapiro-Wilks test and for homogeneity of variance using Bartlett's test, then compared using a one-way ANOVA (Sall et al. 2005).

RESULTS

SEEDLING GROWTH-Total dry mass was significantly different across the four light treatments (One-way ANOVA; $F = 4.6639$, $P = 0.0159$) (Figure 1). The mean total dry mass in the 100 % sunlight treatment was 0.52 ± 0.11 g/plant. This was significantly different from the 40 % treatment, but was not significantly different from 20 % or 60 % treatments ($P = 0.01290$, 0.8802, 0.5003 respectively) (Tukey- Kramer HSD). The mean total dry mass in the 40 % sunlight treatment was greatest at 1.40 ± 0.67 g/plant. This was significantly different from the 100 % treatment ($P = 0.0129$), marginally different from the 20 % treatment ($P = 0.0555$), but not significantly different from the 60 % treatment ($P = 0.1335$).

Aboveground dry mass was significantly different across the four treatment levels (one-way ANOVA, $P = 0.0156$), but only slightly different and only significantly different between the 20 and 100 % treatments (Figure 1). Belowground dry mass was significantly different across all four light treatment levels (one-way ANOVA, $P = 0.0492$), but we could not determine where the differences were with the Tukey-Kramer HSD (multiple range test; Figure 1), thus the letters in the figure are all the same across all light levels.

Mortality was complete in the full sunlight treatment (100 %), while at the 40 % light level, there was zero mortality (Figure 2). Both of these values were significantly different from the mean at the 0.05 level (ANOM for Proportions; LDL = 0, UDL = 0.868). Twenty percent mortality occurred in the 60 %

light level and 40 % mortality in the 20 % light level, neither of which were statistically significant (ANOM for Proportions; LDL = 0, UDL = 0.868).

GAS EXCHANGE RATES-Photosynthetic light response curves for full sun and shade leaves of A. grandidentatum were not significantly different from each other (repeated measures ANOVA, P = 0.0709, Figure 3A and B). Mean photosynthetic rate for shade leaves of A. grandidentatum was 2.27 ± 0.23 µmol $CO_2/m^2/s$, which was not significantly different from the mean photosynthetic rate for sun leaves that was 2.94 ± 0.36 µmol $CO_2/m^2/s$ (one-way ANOVA, $P = 0.0709$).

Mean maximum photosynthetic rate (A_{max}) for shade leaves of *A. grandidentatum* was 3.89 ± 0.36 µmol $CO_2/m^2/s$ at a PAR of 880 µmol/m^2/s, while the A_{max} for sun leaves of was 5.23 ± 0.36 µmol $CO_2/m^2/s$ at a PAR at of 1200 µmol/m^2/s (Table 1). A_{max} values were significantly different from each other (one-way ANOVA, $P = 0.0296$), while the PARs at the A_{max} values were not significantly different between treatments (one-way ANOVA, $P = 0.2861$).

The quantum yield efficiency or initial slope (ϕ or IS) for shade leaves (0.030 ± 0.010 µmol CO_2/(µmol quanta) was not significantly different from that of sun leaves (0.032 ± 0.010 µmol CO_2/(µmol quanta) (one-way ANOVA, $P = 0.0677$, Table 1). The light compensation point (L_{cp}), the light saturation point (L_{sp}) and dark respiration (R_d) for shade leaves were not significantly different from the sun leaves (one-way ANOVA, $P = 0.1431$, 0.2618 and 0.0758 respectively, Table 1).

There were no overall significant differences in mean transpiration rate between sun and shade leaves (repeated-measures ANOVA; $P = 0.2274$) (Table 1). However, the transpiration rate for sun leaves increased from 0.34 ± 0.12 mmol $H_2O/m^2/s$ at the lowest light level to 1.47 ± 0.12 mmol $H_2O/m^2/s$ at the highest light level tested with a few significant differences. Usually significant differences were between the lowest light level and the highest (one-way ANOVA; $P < 0.0001$; Tukey - Kramer HSD; $P < 0.05$) (data not shown). The mean transpiration rate for shade leaves increased from 0.36 ± 0.08 mmol $H_2O/m^2/s$ to 1.04 ± 0.08 mmol $H_2O/m^2/s$ with a similar trend in significant differences (one-way ANOVA; $P < 0.0001$; Tukey - Kramer HSD; $P < 0.05$) (data not shown).

There were no overall significant differences in mean stomatal conductance between sun and shade leaves (repeated-measures ANOVA; $P = 0.9305$) (Table 1). However, the conductance for sun leaves increased from 0.01 ± 0.01 mol $H_2O/m^2/s$ to 0.05 ± 0.01 mol $H_2O/m^2/s$ with few significant differences. Usually significant differences were between the lowest light level and the highest (one-way ANOVA; $P < 0.0001$; Tukey - Kramer HSD; $P < 0.05$) (data not shown). The conductance for shade leaves increased from 0.02 ± 0.05 mol $H_2O/m^2/s$ to 0.04 ± 0.05 mol $H_2O/m^2/s$ with few significant differences and trends similar to transpiration (one-way ANOVA; $P < 0.0001$; Tukey - Kramer HSD; $P < 0.01$) (data not shown).

DISCUSSION

Acer grandidentatum seedlings grew best in the 40 % light treatment, which was 705 ± 22 µmol/m/s, and most closely matches the light levels found below an *A. grandidentatum* canopy at Lost Maples State Natural Area. This was where there were higher numbers of *A. grandidentatum* saplings and mature trees and suggests better seedling survival (Nelson Dickerson and Van Auken 2016). All of the plants survived in the 40 % light treatment, whereas none of the *A. grandidentatum* seedlings in the highest light exposure survived, supporting the hypothesis that this species is a shade and not a sun species. Light levels far above a plant's light saturation point that do not cause an increase in

photosynthesis, may cause damage to leaf tissues and the photosynthetic apparatus or increase water loss to the point of wilting and possibly mortality (Crawley 1997).

Table 1. Mean ± one standard error for the maximum net photosynthetic rates (A_{max}), light level (PAR) at the A_{max} light saturation (L_{sat}), light compensation points (L_{cp}), dark respiration rates (R_d), initial slope or quantum yield efficiency (*IS*), mean stomatal conductance (g_s) and mean transpiration rate (E) of *A. grandidentatum* leaves found in full sun and shade. Stars indicate a significant difference between values for the two treatments (one-way ANOVA; $P < 0.05$).

Parameter	Shade leaves	Sun leaves
A_{max} (µmol CO_2/ m/s)	3.89 ± 0.36*	5.23 ± 0.36*
PAR at A_{max} (µmol/m/s)	880 ±198	1200 ± 198
Mean photosynthetic rate	2.27 ± 0.23	2.94 ± 0.23
L_{sat} (µmol/m/s)	139.11 ± 18.48	170.67 ± 18.48
L_{cp} (µmol/m/s)	9.74± 2.22	14.83± 2.22
R_d (µmol CO_2/m^2s)	0.32 ± 0.05	0.47 ± 0.05
IS (µmol CO_2/(µmol quanta)	0.032 ± 0.010	0.030 ± 0.010
g_s (mol H_2O/m/s)	0.04 ± 0.01	0.04 ± 0.01
E (mmol H_2O/m/s)	0.83 ± 0.08	0.99 ± 0.12

When plants are grown with insufficient light, they may have decreased leaf area, basal diameter and dry mass (Jones and McLeod 1989), but may increase their shoot height or decrease their leaf to shoot ratios (Holt 1995). *Acer grandidentatum* seedlings in the present study that received 20 % of ambient sunlight had high mortality and low growth (Figure 2). Similar deep shading of *A. saccharum* and *Aesculus glabra* seedlings in early spring led to 80 % mortality after three years, compared to 27 % mortality in control plants (Augspurger 2008). Though *A. grandidentatum* seems to be best characterized as a shade plant, it still requires approximate 40 % sunlight to compensate for its respiration to allow growth and survival. The majority of growth for all experimental seedlings in the current study occurred in spring, during the first half of the experiment (not presented). Growth for all plants slowed through the intense heat and light of summer, and most mortality was observed late in the experiment (Nelson Dickerson 2011).

The current results differ somewhat from similar studies done on the closely related *A. saccharum*. First-year *A. saccharum* seedlings grown at 13, 25, 45 and 100 % sunlight for one year increased their number of leaves and dry mass as light level increased, with maximum values found at 100 % sunlight (Logan and Krotkov 1968). The study was conducted in Ontario, Canada, and does not disclose the actual light levels used in the experiment. It is quite possible that the amount of sunlight received by these plants was lower than the 100 % suggested. In addition, ambient temperature was significantly different than used in the current experiment due to the higher latitude and shorter day length or possibly other factors that were not the same as in the present study. However, *A. saccharum* is considered to be a shade plant (Logan and Krotkov 1968; Ellsworth et al. 1994; Kwit et al. 2010).

Gas exchange rates of members of the genus *Acer* indicates the genus includes both shade tolerant and shade intolerant species (Morrison and Mauck 2007; Verdu and Climent 2007). While no information on the photosynthetic parameters of A. grandidentatum have been identified in the literature, it is assumed to be at least moderately shade tolerant because of its distribution in protected canyon bottoms and as an understory late succession species (Correll and Johnston 1970; Bazzaz and Carlson 1982; Nelson Dickerson and Van Auken 2016). The slow growth of A. grandidentatum also suggests shade tolerance, as photosynthetic parameters are closely tied to relative growth rates (Coley et al. 1985; Poorter 1990; Tollefson 2006; Van Auken et al. 2016).

Acer grandidentatum maximum photosynthetic rates of 3.89 ± 0.36 µmol $CO_2/m^2/s$ for shade leaves and 5.23 ± 0.36 µmol $CO_2/m^2/s$ reported for sun leaves in the present study are consistent with classification of as a shade plant. *Acer grandidentatum* sun plants found at higher elevations, higher rainfall and lower temperatures appear to have higher A_{max} values in full sun (Van Auken and Bush, in preparation, unpublished), but plants in shade had similar low A_{max} values. Succession in many cases is driven by temporal differences in resource availability and particularly by changes in available nutrients, especially nitrogen, and light levels (Tilman 1985; Van Auken and Bush 2013).

Early succession sites usually have high light levels and low soil nitrogen. Usually early successional species are shade intolerant and late successional species are shade tolerant (Boardman 1977; Tilman 1985; Mooney and Ehleringer 1997; Valladares and Niinements 2008; Van Auken and Bush 2013). As successional time passes and communities mature, increased canopy shading decreases available light at the soil surface and shade tolerant and higher soil nitrogen requiring plants become more common (Tilman 1985; Bush and Van Auken 1986; Van Auken and Bush 2013). Early successional species exhibit higher rates of photosynthesis, transpiration, and conductance than late successional species, while late successional or climax community species are more likely to be shade tolerant and reach their light saturation points at much lower light levels (Horn 1974; Furuya and Van Auken 2009, 2010; Wayne and Van Auken 2009; Van Auken and Bush 2011). Early succession sites also have greater variability in abiotic conditions, such as swinging between environmental extremes, so early successional plants frequently have greater plasticity in their adaptive responses than late successional species (Horn 1974; Bazzaz and Carlson 1982; Holt 1995; Hull 2002; Van Auken and Bush 2011).

Bazzaz and Carlson (1982) measured photosynthetic rates of full sun and shade leaves of fourteen species. They found that the difference between initial slope, light compensation point, and dark respiration for full sun and shade leaves was much greater for herbaceous early succession species than for late succession hardwood species. The values they reported for late successional hardwoods are similar to the values obtained for A. grandidentatum in the present study (Table 1). The present study found no significant differences between most variables for sun and shade leaves, consistent with observations that A. grandidentatum is a late succession species (Bazzaz and Carlson 1982; Hull 2002).

Light response curves for A. saccharum seedlings in clearings had A_{max} values of 3.32 µmol $CO_2/m^2/s$, while understory individuals had A_{max} values of 1.81 µmol $CO_2/m^2/s$, the only factors that were significantly different between locations (Ellsworth and Reich 1992). We found similar values for A. grandidentatum in the present study. These two species may be able to increase their photosynthetic rate to take advantage of sunflecks, short term increases in light availability, but limited data is available. Acer grandidentatum seems to exhibit lower plasticity in photosynthetic rate than other woody species, which may affect its growth as well as its ability to become a dominant member of the canopy (Hull 2002). Photosynthetic response curves below a forest canopy measured at four different times in a single growing season found that most light response parameters decreased during the growing season and affirmed that A. saccharum is shade tolerant, and seedlings must survive most of their first year in a densely shaded forest canopy (Kwit et al. 2010).

Measured transpiration rates (E) and stomatal conductance (g_s) rates for *A. grandidentatum* were low compared to values for shade intolerant species (Boardman 1977; Bsoul et al. 2007). These rates indicated the stomates were open and CO_2 uptake was probably normal. Rates are consistent with values for shade tolerant species but not shade intolerant species (Horn 1974; Boardman 1977; Bazzaz and Carlson 1982; Tilman 1985; Holt 1995; Mooney and Ehleringer 1997; Valladares and Niinements 2008; Hull 2002; Van Auken and Bush 2013).

Photosynthetic rates of *A. grandidentatum* were lower at high light levels than those of most other dominant plant species in the community at Lost Maples State Natural Area, but not at low light sub-canopy conditions (Furuya 2007; Grunstra 2011; Grunstra and Van Auken 2015). Differing light requirements have been shown to affect succession and plant community composition (Bush and Van Auken 1986; Wayne and Van Auken 2009; Van Auken and Bush 2011). Plants with low photosynthetic rates may experience difficulty growing below the canopy and then through the canopy without a disturbance to canopy plants. When this lowered potential is combined with browsing pressure, the effect can become even stronger (Van Auken and Bush 2009). Community composition in Sabinal Canyon in Lost Maples State Natural Area and other central Texas communities are likely affected by complex interaction of inherent photosynthetic capacities and abiotic requirements of the species present. This would including preferential feeding of large herbivores, and the effect of that herbivory on the biotic and abiotic conditions present in the environment. There are a number of species found in these central Texas communities that can grow at high light levels, but most cannot grow in deep shade below a closed canopy (Furuya 2007; Grunstra 2011; Grunstra and Van Auken 2015).

THE FUTURE

Populations of *A. grandidentatum* in central Texas are relatively rare and are really outlier populations. Management of these populations in the past has mostly been hap-hazard at best and dependent on the whims of owners of properties where they have been found. Understanding that they are understory/sub-canopy species or shade species was unknown until the present study. Sensitivity to native and domestic herbivores has been suspected for many years but not demonstrated until very recently. What will happen to these populations in the future? This is uncertain and difficult to predict. If their reproductive cycle is continually disrupted, they will become extinct in central Texas. If herbivory by native and domestic species is not reduced the same thing will happen. What is the timeline of the potential extinction of these isolated native populations? This is uncertain at this time. It is hard to say because apparently individuals can live for hundreds of years and the death rate of adults is unknown and the rate of recruitment of juveniles into these populations is not known either.

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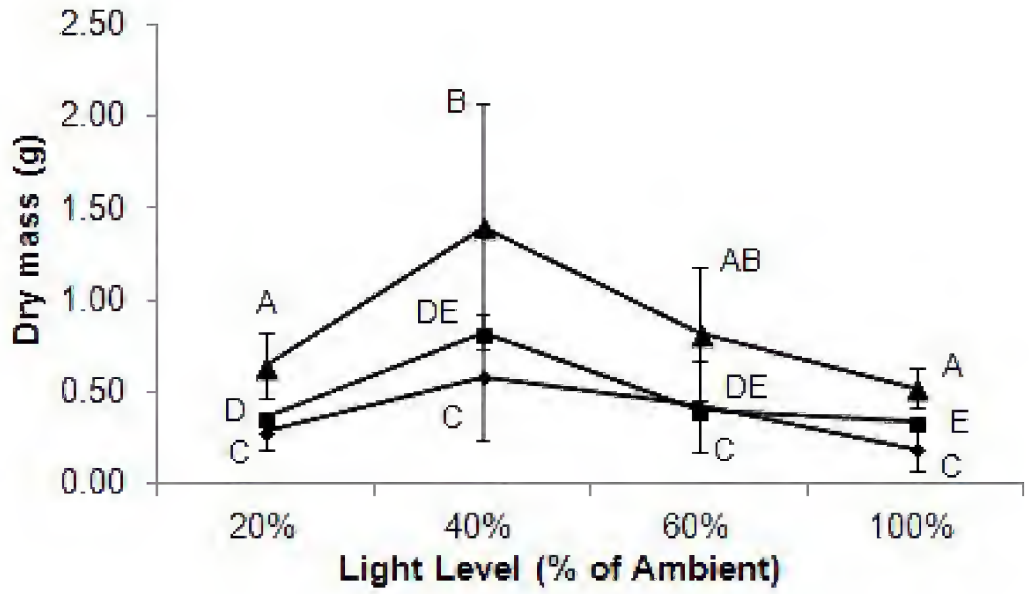

Figure 1. Mean aboveground (■), belowground (◆), and total dry (▲) mass of *Acer grandidentatum* at varying light levels as a percentage of ambient sunlight. Bars indicate ± one standard deviation of the mean. Different letters on the same line indicate significant differences for that factor (one-way *ANOVA;* $P < 0.05$, Tukey - Kramer HSD; $P < 0.05$).

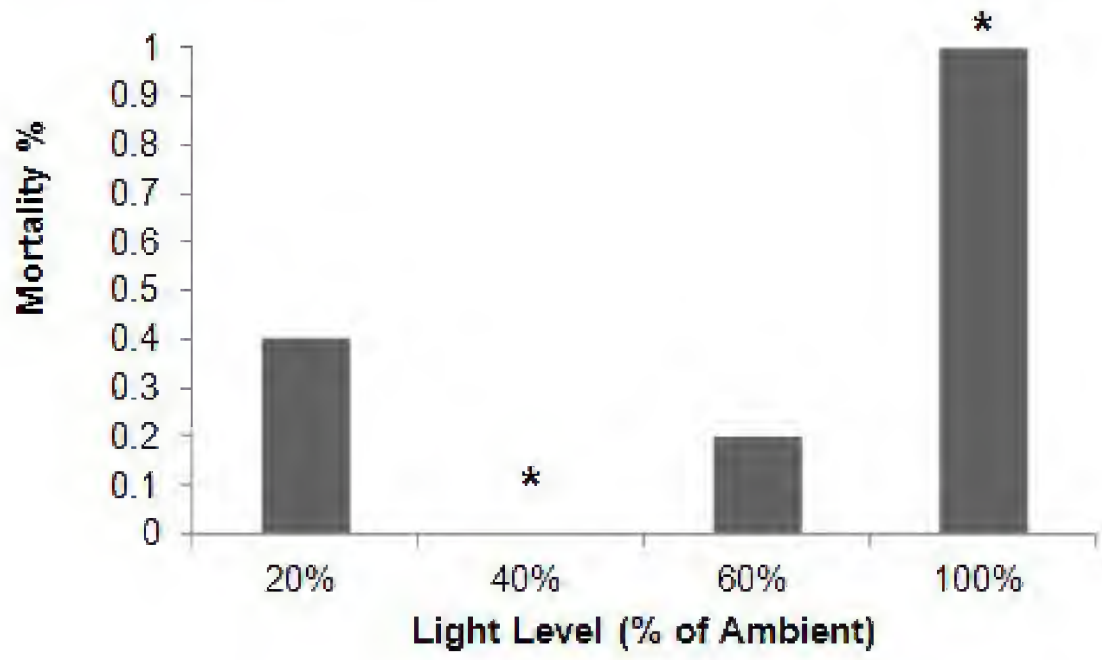

Figure 2. Relative mortality (1=100%) of *Acer grandidentatum* seedlings grown at varying light levels is presented as a percentage of ambient sunlight. The * indicates values which were significantly different from the mean (ANOM for Proportions, $\alpha = 0.05$, LDL [lower detection limit] = 0, UDL [upper detection limit] = 0.868). There were no mortalities in the 40% light treatment.

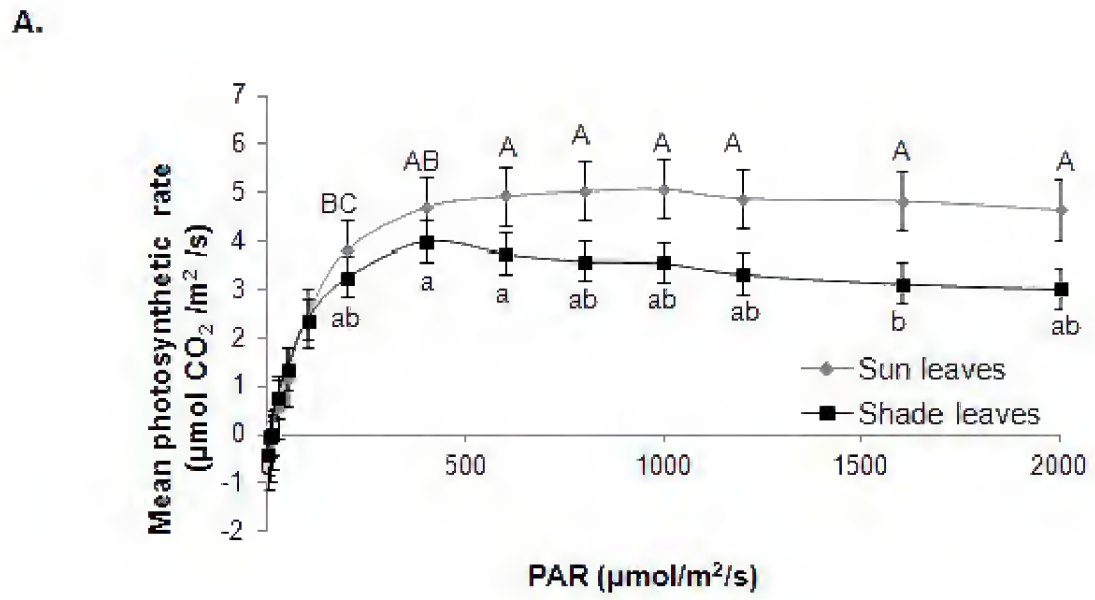

Figure 3. **A.** Mean photosynthetic rate of full sun and shaded leaves of *A. grandidentatum* as a function of light level (PAR). **B.** The lower portion of graph A between 0 and 100 PAR. Uppercase letters represent values for sun leaves, while lowercase letters represent values for shaded leaves. Different upper or lowercase letters indicate significant differences within the curve (one-way *ANOVA; P* < 0.0001; Tukey-Kramer HSD; *P* < 0.05). Error bars represent ± one standard error.

Discovery of *Juniperus sabina* var. *balkanensis* R. P. Adams and A. N. Tashev in western Turkey (Anatolia)

Robert P. Adams
Biology Department, Baylor University, Gruver Lab, Gruver, TX 79040, USA
Robert_Adams@baylor.edu

Adam Boratynski
Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 52-035, Kornik, Poland

Tuğrul Mataraci
Eskidji Müz. AŞ., Sanayi Cad. Vadi Sokak No:2, Yenibosna-Bahçelievler, İstanbul, Turkey

Alexander N. Tashev
University of Forestry, Dept. of Dendrology, 10, Kliment Ochridsky Blvd., 1756 Sofia, Bulgaria
and

Andrea E. Schwarzbach
Department of Health and Biomedical Sciences, University of Texas - Rio Grande Valley,
Brownsville, TX 78520, USA.

ABSTRACT

Additional analyses of trnS-trnG and nrDNA from herbarium specimens from Europe revealed the presence of *J. sabina* var. *balkanensis* in western Turkey near Izmir and expands the range previously known only from Bulgaria and adjacent mountains in Greece. A more detailed map of the taxon's distribution is presented. Published on-line www.phytologia.org *Phytologia 99(1): 22-31 (Jan 19, 2017).* ISSN 030319430.

KEY WORDS: *Juniperus sabina var. balkanensis, J. sabina*, distribution, nrDNA, trnS-trnG, chloroplast capture.

Recently, Adams et al. (2016) reported on the capture of *J. thurifera* (or an ancestor) chloroplast by *J. sabina* var. *balkanensis*. Chloroplast capture has been rarely reported in conifers. In *Pinus* and other conifers, Hipkins et al. (1994) concluded that "past hybridization and associated 'chloroplast capture' can confuse the phylogenies of conifers." Bouille et al. (2011) found significant topological differences in phylogenetic trees based on cpDNA (vs. mtDNA sequences) in *Picea* that suggested organelle capture.

In *Juniperus*, Terry et al. (2000) suggested that chloroplast capture was involved in the distribution of cp haplotypes in *J. osteosperma* in western North America. More recently, Adams (2015a, b) found widespread hybridization and introgression between *J. maritima* and *J. scopulorum* in the Pacific northwest, with introgression from *J. maritima* into *J. scopulorum* eastward into Montana. The disparity between cpDNA and nuclear markers (nrDNA and maldehy) suggested that cp capture had occurred.

The *Juniperus* of section *Sabina*, of the eastern hemisphere, can be divided into two groups based on the number of seeds per female cone (often called berries) and female cone shape. The single seed/cone (single-seeded) *Juniperus* of the eastern hemisphere have cones that are ovoid with a noticeable pointed tip, whereas the multi-seeded *Juniperus* are generally globose and often have an

irregular surface (Adams 2014). *Juniperus sabina* L. is a smooth leaf-margined, multi-seeded juniper of the eastern hemisphere. It is very widely distributed from Spain through Europe to Kazakhstan, western China, Mongolia and Siberia (Fig. 1). *Juniperus sabina* has a range that is discontinuous between Europe and central Asia; the species is generally a shrub less than 1 m tall and ranges up to 1-2 m wide. But in the Sierra Nevada of Spain, it forms a horizontal shrub.

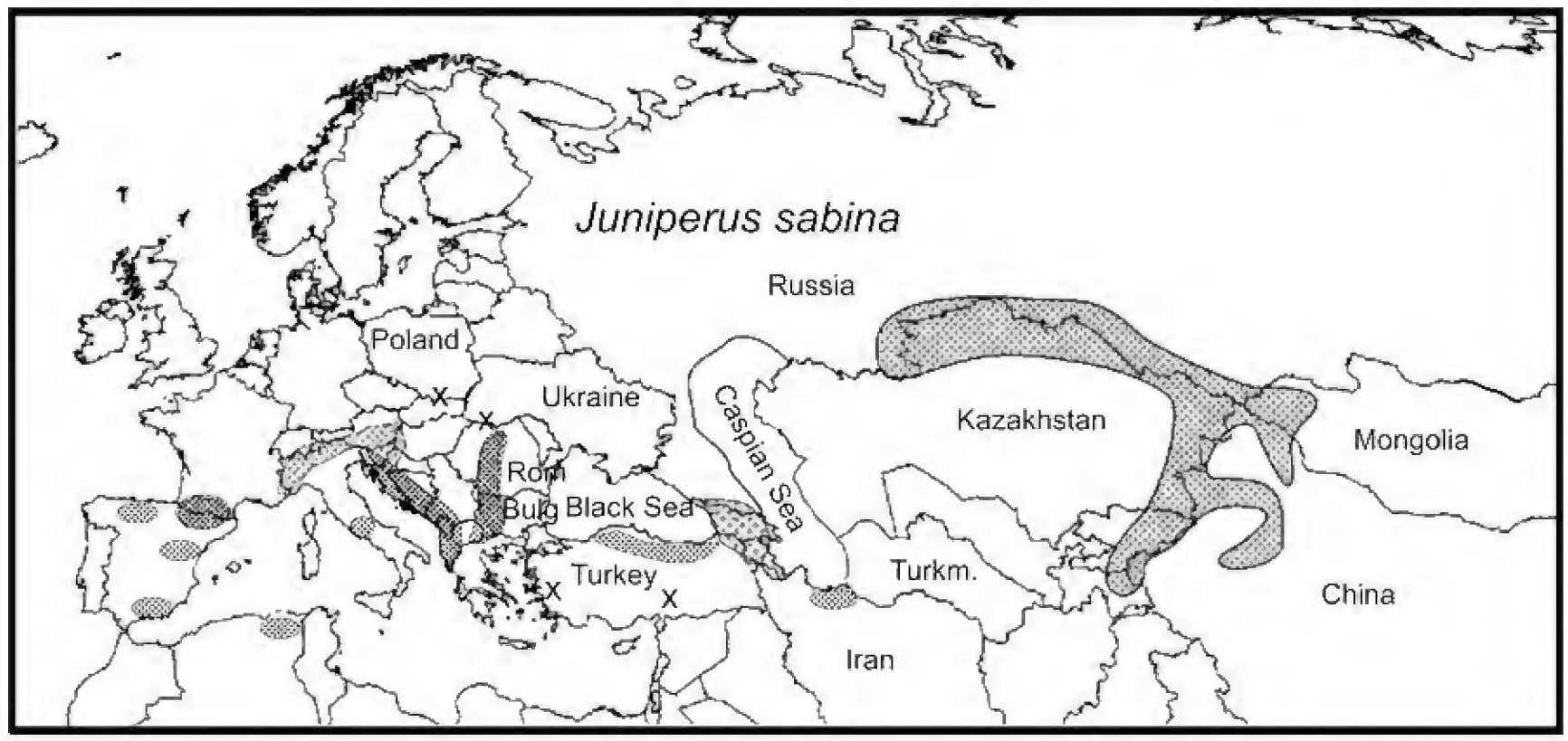

Fig. 1. Distribution (shaded areas) of *J. sabina*. x = outlying populations of *J. sabina*.

Adams et al. (2016) showed that nrDNA (ITS) did not resolve *J. sabina* populations due to the lack of sequence variation.

However, their analyses (Adams et al., 2016) of cp DNA (petN-psbM, trnSG, trnDt, trnLF) revealed that *J. sabina* contained two kinds of cpDNA: typical *J. sabina* and *J. sabina* var. *balkanensis* cpDNA in a clade with *J. thurifera* (Fig. 2).

It might be noted that *J. sabina* from Kazakhstan and Xinjiang form a clade (Fig. 2). The use of four cp regions resulted in a clade of the junipers from the western hemisphere (box, Fig. 2).

In order to investigate the amount of divergence of the 'balkanensis' chloroplast from that of present day *J. thurifera*, a minimum spanning network was computed using both SNPs and indels, herein called mutations. This analysis found 52 mutations within the set: *J. sabina* (*sensu stricto*), *J. sabina J. s.* var. *balkanensis* and *J. thurifera*. The minimum spanning network (Fig. 3) shows that all the 'balkanensis' plants differ by only 6-8 mutations from *J. thurifera* chloroplast. However, the nearest link connecting 'balkanensis' to *J. sabina* (*sensu stricto*) is 36 mutations!

Notice (Fig. 3) that Azerbaijan/ Mongolia accessions group with Kazakhstan/ Xinjiang and this group differs by 7 mutations from the Europe/ Algeria group. This suggests that *J. sabina* in central Asia may be a different variety of *J. sabina*. That needs to be examined in more detail (in progress).

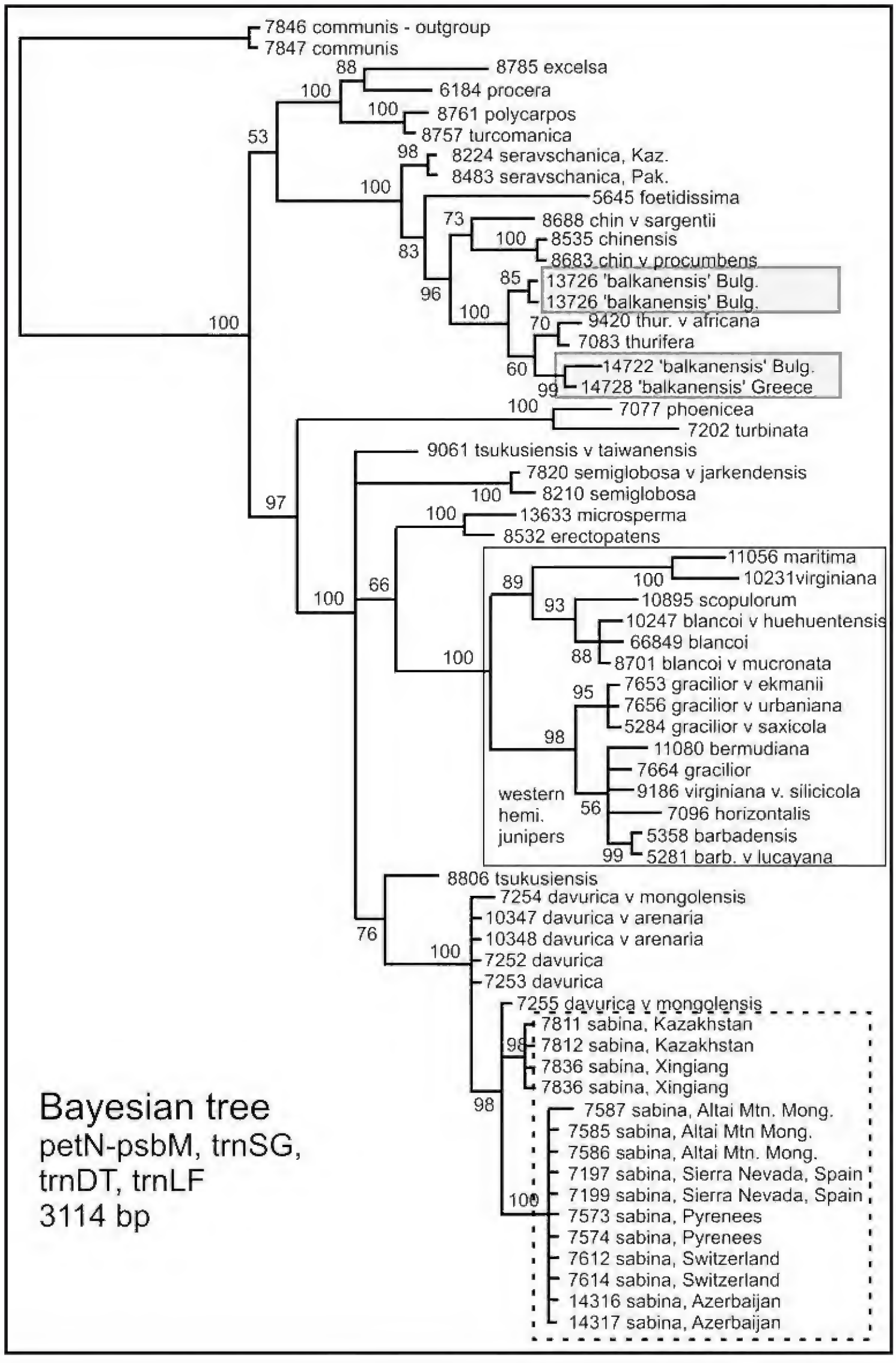

Figure 2. Bayesian analysis based on four cp regions (adapted from Adams et al., 2016).

Adams et al. (2016) concluded that *J. sabina* var. *balkanensis* captured the chloroplast of an ancestor of the *thurifera* lineage during an ancient hybridization event at a time when species distributions overlapped. Because var. *balkanensis* has morphology almost identical to *J. sabina* (*sensu stricto*), this hybridization event was likely followed by successive backcrosses to *J. sabina* after the

hybridization event, resulting in a nuclear genome, including morphology, that is nearly identical to *J. sabina* (*sensu stricto*). In fact, Adams et al., 2016 found in the nrDNA analysis that *J. s.* var *balkanensis* was clearly interspersed in a clade with other *J. sabina*. So it is not surprising that a comparison of the morphology of *J. sabina and J. s.* var. *balkanensis,* has, to date, revealed only a few quantitative differences (Adams et al. 2016, Table 1).

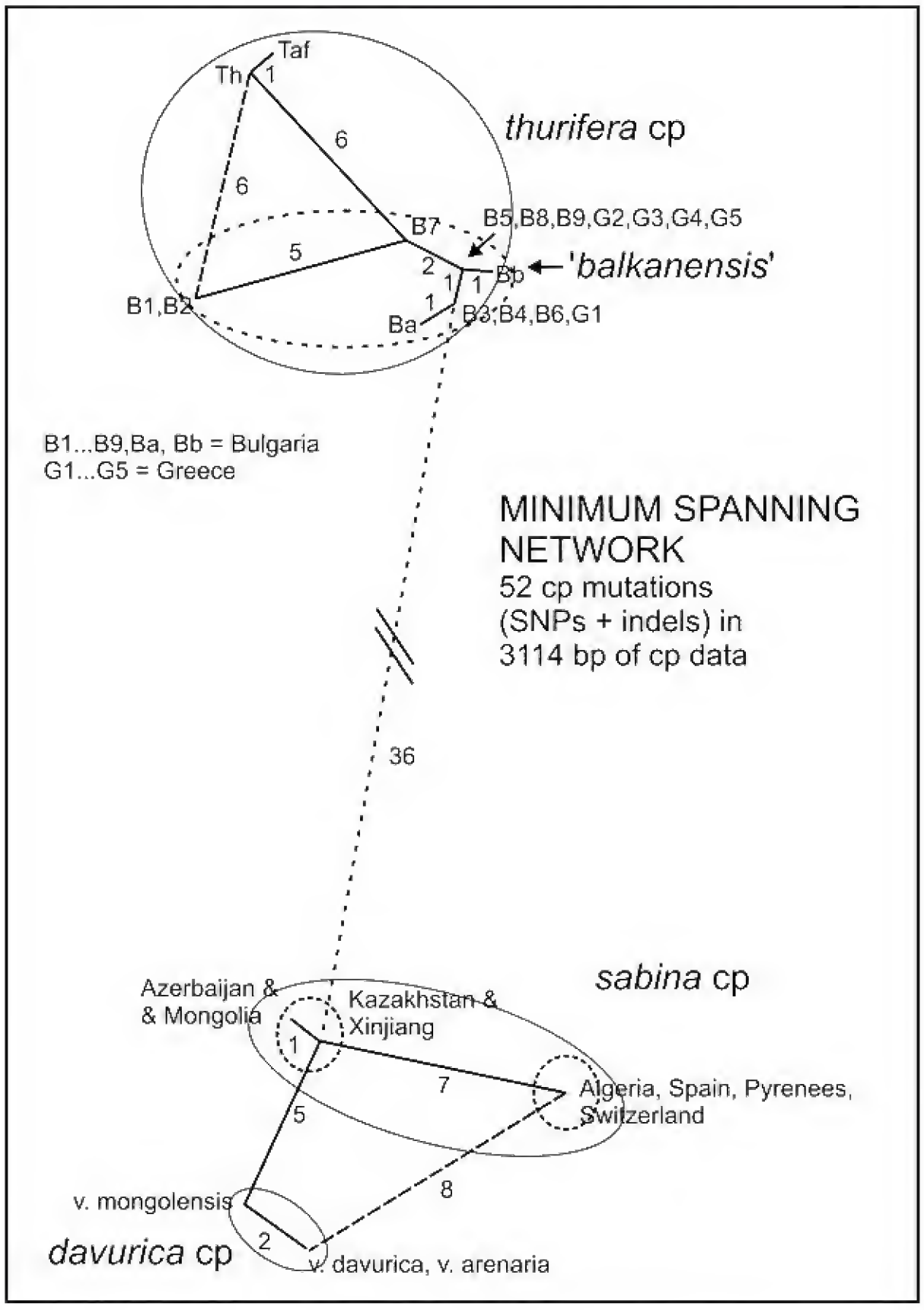

Figure 3. Minimum spanning network based on 52 mutations (SNPs + indels) in 4 cp markers (3114 bp). The numbers next to the lines are the number of mutations for that link. The dotted line connects the *thurifera* cp taxa to the *sabina* cp taxa by 36 mutations. The dashed line is the second nearest neighbor of *J. sabina* to *J. davurica* cp type. (8 mutations).

Juniperus sabina var. *balkanensis* is known only from sloping rocky limestone, at 1240 - 1630m, in the mountains of Bulgaria and northern Greece (Fig. 4). Adams et al. (2016) postulated that it may occur northward into Romania, westward into Macedonia and/ or eastward into northern Turkey.

The purpose of the present paper is to report on a broader sampling of *J. sabina* from herbarium specimens to more precisely determine the distribution of *J. sabina* var. *balkanensis*.

Fig. 4. Habit and habitat of *J. s.* var. *balkanensis* in the eastern Rhodopes mountains, Bulgaria. *Juniperus communis*, columnar trees, are in the background.

MATERIAL AND METHODS

Specimens used in this study (species, popn. id., location, collection numbers): *J. chinensis*, CH, Lanzhou, Gansu, China, *Adams 6765-6767*; *J. sabina*: (SN), Sierra Nevada, Spain, *Adams 7197, 7199, 7200*; (PY), Pyrenees Mtns., Spain/ France border, *Adams 7573-7577*; (SW), Switzerland, *Adams 7611, 7612, 7614, 7615*; TS, Tian Shan Mtns., Xinjiang, China, *Adams 7836-7838*; Mongolia, Altai Mtns., *Adams 7585-7587*; Kazakhstan, Paniflor, *Adams 7811-7812*; Azerbaijan: *Adams 14316-14320*;
J. davurica (DV), 15 km se Ulan Bator, Mongolia, *Adams 7252, 7253, 7601*; *J. davurica* var. *arenaria* (AR) sand dunes, Lake Qinghai, Qinghai, China, *Adams 10347-10352*; river bank, Gansu, *J-Q. Liu and Adams 10354-10356*; *J. davurica* var. *mongolensis* (MS) sand dunes, 80 km sw Ulan Bator, Mongolia, *Adams 7254-7256*;
Collections of taxon with non-J. sabina cpDNA in Adams, Schwarzbach and Tashev (2016): (acronyms used in Fig. 7)
Bulgaria and Greece
B1-B5 Eastern Rhodopes, Bulgaria, *Adams 13725-13729 (A. Tashev 2012-1-5*);
B6 Central Stara Plania, Sokolna reserve, Bulgaria, *Adams 14721 (A. Tashev 2015 Balkan 1*;
B7-B9, Ba, Bb Rila Mountain, Bulgaria, *Adams 14722-14726 (A. Tashev 2015 Rila 1.1-1.3, 2.1-2.2)*;
G1-G5 Mt. Tsena, Greece, *Adams 14727-14731* (*A. Tashev 2015 So. 1-5 Tsena*);

Samples new for this study: (with Lab Acc. ID = Adams xxxxx)
Austria
14872 Austria, Alps, Ötztal, Zwiselstein, N 46.935°, E11.039°, 1650-1700m alt., leg. K. Boratyńska, A.Boratyński, 2015, 15.001, KOR 51592, female
14873 Austria, Alps, Ötztal, Below Sőlden, N 46.994°, E11.012°, 1300 alt., leg. K. Boratyńska, A.Boratyński, 2015, 15.005, KOR 51596, male
14874 Austria, Alps, Ötztal, Below Sőlden, N 46.994°, E11.012°, 1300 alt., leg. K. Boratyńska, A.Boratyński, 2015, 15.005, KOR 51595, female
France
14863 France, Alps de Dauphiné, St. Crépin, N 44.71°, E 6.61°, ca 1000m alt, leg. A. Boratyński, K. Boratyńska 2003, 03.19.116, KOR 43778, female

Italy

14870 Italy, Alps, Val d'Aosta, Introd, Les Combes, N 45.689°, E 7.166°, 1250 m alt. Lag. K. Boratyńska, A. Boratyński, 15.014. KOR 51590, female

14871 Italy, Alps, Val d'Aosta. Introd, Les Combes, N 45.689°, E 7.166°, 1250 m alt. Lag. K. Boratyńska, A. Boratyński, 15.013. KOR 51589, male

Poland

14858 Poland, Carpathians, Pieniny National Park, Facimiech, N 49.40°, E 20.43°, ca 600m alt. From specimen propagated vegetatively about 2005 and planted in dendrological garden of Forest Botany Chair, Forest Faculty, Poznań University of Life Sciences

Russia

14865 Russia, Altay, Aktru Valley, SWW of Bielucha Mt., ca. N 49.80°, E 86.40°, 2500m alt., leg. Fałtynowicz W., 2010. KOR 4796, female.

Spain

14860 Spain, Cuenca, Serraña de Cuenca, between Tragacete and La Cueva (Vega de Cordorno), N 40.433°, W 1.905°, ca 1450 m alt., lg. Boratyńska K., Boratyński A., 2006, SP.06.026, KOR 44733, female

14862 Spain, Teruel, Puerto de Cabigordo near Cedrillas E of Teruel, N 40.41°, W0.95°, ca 1500m alt., Leg. A. Boratyński, K. Boratyńska, HS_01.03.17, KOR 43212,

14864 Spain, Sierra Nevada, Veleta Mt., above Alberque Universitario, N 37.09°, W 3.38°, ca 2500m alt., leg. A. Boratyński 1991, KOR 25299

14866 Spain, Sierra Nevada, Monte Ahí de Cara, N 37.13°, W3.43°, 1900-2000m alt., leg. A.Boratyński, Ja. Didukh., D.Tomaszewski, Z. Boratyński, KOR 46220, female

14869 Spain, Leon, Los Barios de Luna, N 42.88°, W 5.87°, 1150-1200m alt., leg. K.Boratyńska, A. Boratyński, 2015, KOR 51542, female

14875 Spain, Sierra de Albarracin, S of Brochales, N 40.50°, W 1.57°, ca 1600m alt., leg. A. Boratyński, K. Boratyńska, 2006. female

14876 Spain, Aragon, Moncayo, N 41.77°, W 1.80°, ca 1900-2000m alt., leg. D. Gomez, 2004, female

Switzerland

14867 Switzerland, Alps, Visp, Aussenberg, N 46.31°, E 7.87°, ca 950-1000m alt., leg. K.Boratyńska, A.Boratyński, 2015, 15.016, KOR 51570, female

14868 Switzerland, Alps, Visp, Aussenberg, N 46.31°, E 7.87°, ca 950-1000m alt., leg. K.Boratyńska, A.Boratyński, 2015, 15.017, KOR 51581, male

Turkey

14861 Turkey, Manisa. Spil Dağı Milli Parki (National Park) (Tas Suret), N38.55°, E 27.42°, ca 1250 m alt., leg. A. Boratyński, K. Boratyńska, 2005, TU_05/55, KOR 44573, female

14934 Turkey, Manisa, Spil Dağı Milli Parki (National Park), N38°, 57', E 27° 41', 1024 m., *Tuğrul Mataraci 2016-1*

14938 Turkey, Gümüshane, Kürtün, Aktas village, Karakaya (Northeast Anatolia), 40° 36' 03" N, 38° 53' 21" E., 2376 m. Coll. *A. Kandemir 10745.*

Ukraine

14859 Ukraine, Crimea, Chatyr Dag, N 44.773°, E 34.313°, 1100-1200m alt. Lg. A. Boratyński, G. Iszkuło, A. Lewandowski, 2006. UA06.007, KOR 45572

Voucher specimens for all collections are deposited at Baylor University Herbarium (BAYLU) and Herbarium (University of Forestry, Sofia, Bulgaria).

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN, trnD-T, trnL-F, trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary

enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010). The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.) or Sequencher v. 5 (genecodes.com). Sequence datasets were analyzed using Geneious v. R7 (Biomatters. Available from http://www.geneious.com/), the MAFFT alignment program. Further analyses utilized the Bayesian analysis software Mr. Bayes v.3.1 (Ronquist and Huelsenbeck 2003). For phylogenetic analyses, appropriate nucleotide substitution models were selected using Modeltest v3.7 (Posada and Crandall 1998) and Akaike's information criterion. Minimum spanning networks were constructed from mutational events (ME) data using PCODNA software (Adams, Bartel and Price, 2009; Adams, 1975; Veldman, 1967).

RESULTS

The results of this study (and the previous, Adams et al. , 2016 study) are given in Table 1. The distribution of *J. sabina* var. *balkanensis* and *J. sabina* is shown in Fig. 5. The distribution of *J. thurifera*

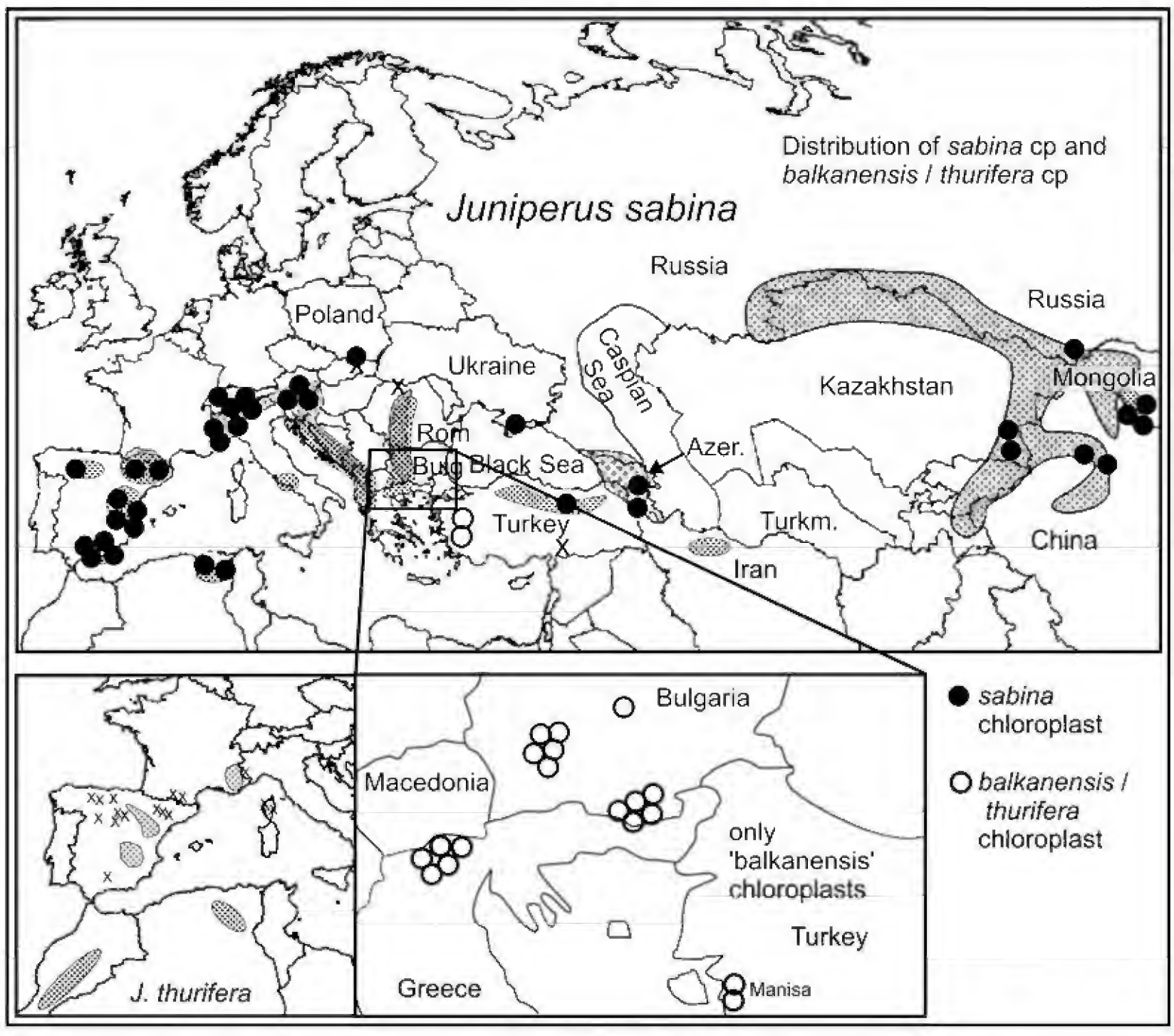

Figure 5. Distribution of *J. sabina* var. *balkanensis* and typical *J. sabina* chloroplast. The present day distributions of *J. thurifera* and var. *africana* (in north Africa) are shown in the insert on the lower left.

is presented in the insert, lower left (Fig. 5). It appears that *J. s.* var. *balkanensis* has a quite restricted range. Additional samples are needed from Romania, Turkey and northwesterly from Albania/ Macedonia northwesterly to Slovenia to determine the distribution more precisely.

At present level of understanding, the distributions of *J. s.* var. *balkanensis* and *J. thurifera* do not appear to overlap, negating modern hybridization. However, there were large changes in plant distributions in the Pleistocene and earlier, it seem probable that *J. thurifera*-like ancestors were sympatric with *J. sabina,* and presenting opportunities for chloroplast capture from *J. thurifera*.

ACKNOWLEDGEMENTS

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Table 1. Classification of *J. sabina* specimens based on trnS-trnG (plus petN-psbM, trnDT, trnLF) and nrDNA (ITS).

Lab Acc. #, Location	trnSG (cp genome) classification	nrDNA classification
13725 Bulgaria, eastern Rhodopes	v. *balkanensis*	v. *sabina*
13726 Bulgaria, eastern Rhodopes	v. *balkanensis*	v. *sabina*
13727 Bulgaria, eastern Rhodopes	v. *balkanensis*	v. *sabina*
13728 Bulgaria, eastern Rhodopes	v. *balkanensis*	v. *sabina*
13729 Bulgaria, eastern Rhodopes	v. *balkanensis*	v. *sabina*
14721 Bulgaria, Sokolna reserve	v. *balkanensis*	v. *sabina*
14722 Bulgaria, Rila Mtn.	v. *balkanensis*	v. *sabina*
14723 Bulgaria, Rila Mtn.	v. *balkanensis*	v. *sabina*
14724 Bulgaria, Rila Mtn.	v. *balkanensis*	v. *sabina*
14725 Bulgaria, Rila Mtn.	v. *balkanensis*	v. *sabina*
14726 Bulgaria, Rila Mtn.	v. *balkanensis*	v. *sabina*
14727 Greece, Tsena Mt.	v. *balkanensis*	v. *sabina*
14728 Greece, Tsena Mt.	v. *balkanensis*	v. *sabina*
14729 Greece, Tsena Mt.	v. *balkanensis*	v. *sabina*
14730 Greece, Tsena Mt.	v. *balkanensis*	v. *sabina*
14731 Greece., Tsena Mt.	v. *balkanensis*	v. *sabina*
14934 w Turkey, Spil Dağı Milli Parki	v. *balkanensis*	v. *sabina*
14861 w Turkey, Spil Dağı Milli Parki	v. *balkanensis*	v. *sabina*
13167 Algeria	v. *sabina*	v. *sabina*
13168 Algeria	v. *sabina*	v. *sabina*
14872 Austria, Otztal, Zwiselstein	v. *sabina*	v. *sabina*
14873 Austria, Ötztal, Below Sölden,	v. *sabina*	v. *sabina*
14874 Austria, Ötztal, Below Sölden,	v. *sabina*	v. *sabina*
14316 Azerbaijan	v. *sabina*	v. *sabina*
14317 Azerbaijan	v. *sabina*	v. *sabina*
7836 China, Heaven Lake, Xinjiang	v. *sabina*	v. *sabina*
7837 China, Heaven Lake, Xinjiang	v. *sabina*	v. *sabina*
14863 France, Alps de Dauphine	v. *sabina*	v. *sabina*
7573 France, Pyrennes Mtns	v. *sabina*	v. *sabina*
7574 France, Pyrennes Mtns	v. *sabina*	v. *sabina*
14870 Italy, Val d'Aosta, Alps	v. *sabina*	v. *sabina*
14871 Italy, Val d'Aosta, Alps	v. *sabina*	v. *sabina*
7811 Kazakhstan, Paniflor	v. *sabina*	v. *sabina*
7812 Kazakhstan, Paniflor	v. *sabina*	v. *sabina*
7585 Mongolia, Altair Mtns	v. *sabina*	v. *sabina*
7586 Mongolia, Altair Mtns	v. *sabina*	v. *sabina*
7587 Mongolia, Altair Mtns	v. *sabina*	v. *sabina*
14858 Poland, Pieniny N.P.,	v. *sabina*	v. *sabina*
14865 Russia, Altay Mtn.	v. *sabina*	v. *sabina*
7197 Spain, Sierra Nevada	v. *sabina*	v. *sabina*
7199 Spain, Sierra Nevada	v. *sabina*	v. *sabina*
14860 Spain, Serrana de Cuenca	v. *sabina*	v. *sabina*
14862 Spain, Teruel	v. *sabina*	v. *sabina*
14864 Spain, Sierra Nevada	v. *sabina*	v. *sabina*
14866 Spain, Sierra Nevada	v. *sabina*	v. *sabina*
14869 Spain, Los Barios de Luna	v. *sabina*	v. *sabina*
14875 Spain, Sierra de Albarracin	v. *sabina*	v. *sabina*
14876 Spain, Aragon, Moncayo	v. *sabina*	v. *sabina*

7611 Switzerland, Alps	v. *sabina*	v. *sabina*
7612 Switzerland, Alps	v. *sabina*	v. *sabina*
7614 Switzerland , Alps	v. *sabina*	v. *sabina*
14867 Switzerland, Aussenberg	v. *sabina*	v. *sabina*
14868 Switzerland, Aussenberg	v. *sabina*	v. *sabina*
14938 northeast Turkey	v. *sabina*	v. *sabina*
14859 Ukraine, Crimea, Chatry Dag	v. *sabina*	v. *sabina*

The effects of different concentrations of gibberellic acid (GA3) on seed germination of *Helianthus annuus* and *H. petiolaris*

Robert P. Adams and Amy K. TeBeest
Biology Department, Gruver Lab, Baylor University, Gruver, TX 79040, USA
robert_adams@baylor.edu

ABSTRACT

Germination tests were conducted by soaking native, wild *Helianthus annuus* and *H. petiolaris* seeds in various concentrations of gibberellic acid (GA3) for 1 week, 4°C. For *H. annuus*, the most effective concentrations of GA3 were 1000 ppm (61.7%) and 500 ppm (58.3%). Lower concentrations of GA3 were less effective. For *H. petiolaris*, the most effective concentrations of GA3 were 1000 ppm (56.1%), 500 ppm (65.0%), and 250 ppm (62.2%) and, again, lower concentrations of GA3 were less effective. Transplanting the germinated seeds of *H. annuus* to soil in pots, resulted in nearly 100% success, indicating no apparent long-term effects from the GA3 treatment. Published on-line www.phytologia.org *Phytologia 99(1): 32-35 (Jan 19, 2017).* ISSN 030319430.

KEY WORDS: *Helianthus annuus*, *H. petiolaris*, seed germination, dormancy, gibberellic acid (GA3).

Native, wild sunflowers (Helianthus spp.) are known to be difficult to germinate (Seiler, 1993). Recently, Adams and TeBeest (2016) reported on various stratification treatment effects on germination of *Helianthus petiolaris*. We found a moderate concentration of GA3 (500 ppm) with one week stratification at 4°C was very effective in increasing the germination rate of recalcitrant native sunflower seeds (80% vs. 30% control). Stratification (1 wk at 4°C) increased germination, regardless of the seed treatment. Ethrel (25 ppm) treatment was effective, but not as much as GA3 (500 ppm). Soaking sunflower seed in water for 12 or 16 hr resulted in no seed germination.

The literature on pre-treatment methods for sunflower seed germination has been recently reviewed (Adams and TeBeest, 2016).

The purpose of the present paper is extend the tests on pre-treatment using GA3 at different concentrations to determine the concentration of GA3 that produces highest seed germination in *H. annuus* and *H. petiolaris*, native, wild collected seed.

MATERIALS AND METHODS

Seeds of *H. petiolaris*, PI451978-NC7, Ellsworth, KS were obtained from GRIN (Germplasm Resources Information Network), USDA.
Seeds of *H. annuus*: were collected 16 July 2016, from a natural population, 1 mi. south of Gruver, TX (*Adams 14952*).

All seeds were surface sterilized by:
1. Washing with soap/tap water;
2. Dipping in 70% ethanol, 30 sec;
3. Sterilizing by soaking in 20% Chlorox (8.25% sodium hypochlorite) for 30 min.;
4. Thoroughly rinsing in sterilized ddwater (Protocol from Singhung Park, Kansas State University).

Germination tests: Effects of various concentrations of gibberellic acid (GA3, PlantHarmones.net, 90%) stored at 4°C, 1 week (7 days) in GA3 solutions.
1000 ppm GA3 stock solution: dissolved 1.0 g GA3 in 5 ml ethanol, added to 995 ml DI water to produce 1000 ppm stock. Diluted with DI water to make: 500 ppm, 250 ppm, 125 ppm, and 62.5 ppm stocks.
Control: soaked in DI water, 4°C, 1 week.
20 seeds were used in each of 3 replicates (60 seeds total). The seeds were soaked in DI, or various GA3 solutions in beakers, 4°C, 1 week. In addition, for both *H. annuus* and *H. petiolaris*, 60 seeds were placed in sterilized filter paper, pre-wetted with 500 ppm GA3, then placed in sealed plastic bags at 4°C, 1 week. Seeds were germinated at RT (21°C), in normal lab fluorescent lighting. Seeds were examined for fungal contamination daily and contaminated seeds removed. After 14 days, the seeds with emergent roots were scored as germinated.

RESULTS

Table 1 shows that for *H. annuus*, the most effective concentrations of GA3 were 1000 ppm (61.7%) and 500 ppm (58.3%). Lower concentrations of GA3 were less effective. This is shown in figure 1, where 1000 and 500 ppm were much more effective than lower concentration of GA3.

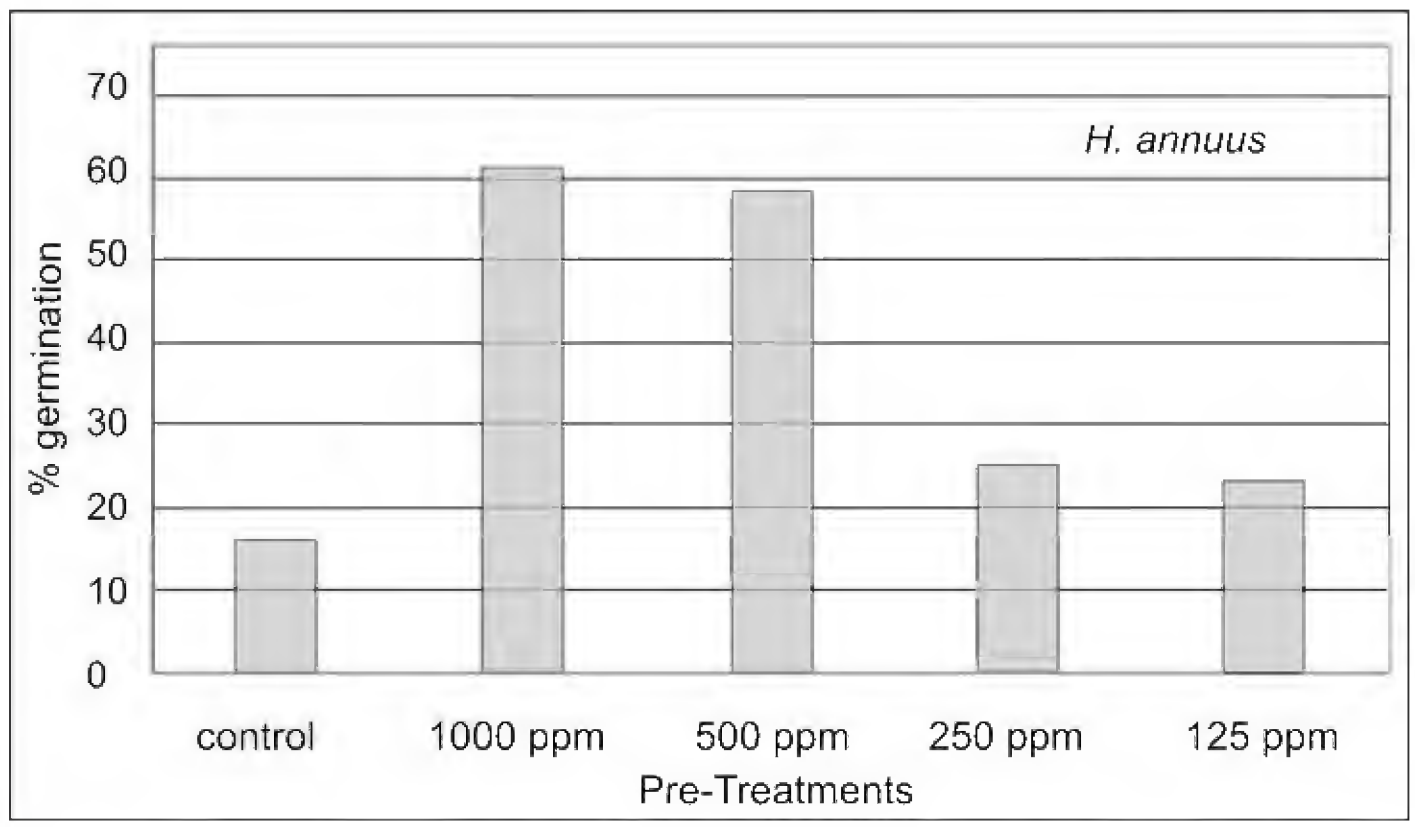

Figure 1. Germination of *H. annuus* at various concentrations of GA3, soaked 1 week, 4°C. Control: soaked in DI water, 1 week, 4°C.

For *H. petiolaris*, the most effective concentrations of GA3 were 1000 ppm (56.1%), 500 ppm (65.0%), and 250 ppm (62.2%).

In contrast to the results for *H. annuus*, lower concentration of GA3 were somewhat effective in seed germination for *H. petiolaris* (Fig. 2), with considerable enhanced germination at 250 and 125 ppm GA3.

Comparing soaking seeds in a beaker of 500 ppm GA3 vs. storage in filter paper saturated with 500 ppm GA3 resulted 58.3% vs 51.7% (*H. annuus*, Table 1) and 65.0% vs. 44.8% (*H. petiolaris*, Table 2). This seems to indicate that there is a small advantage in soaking the seeds in a beaker.

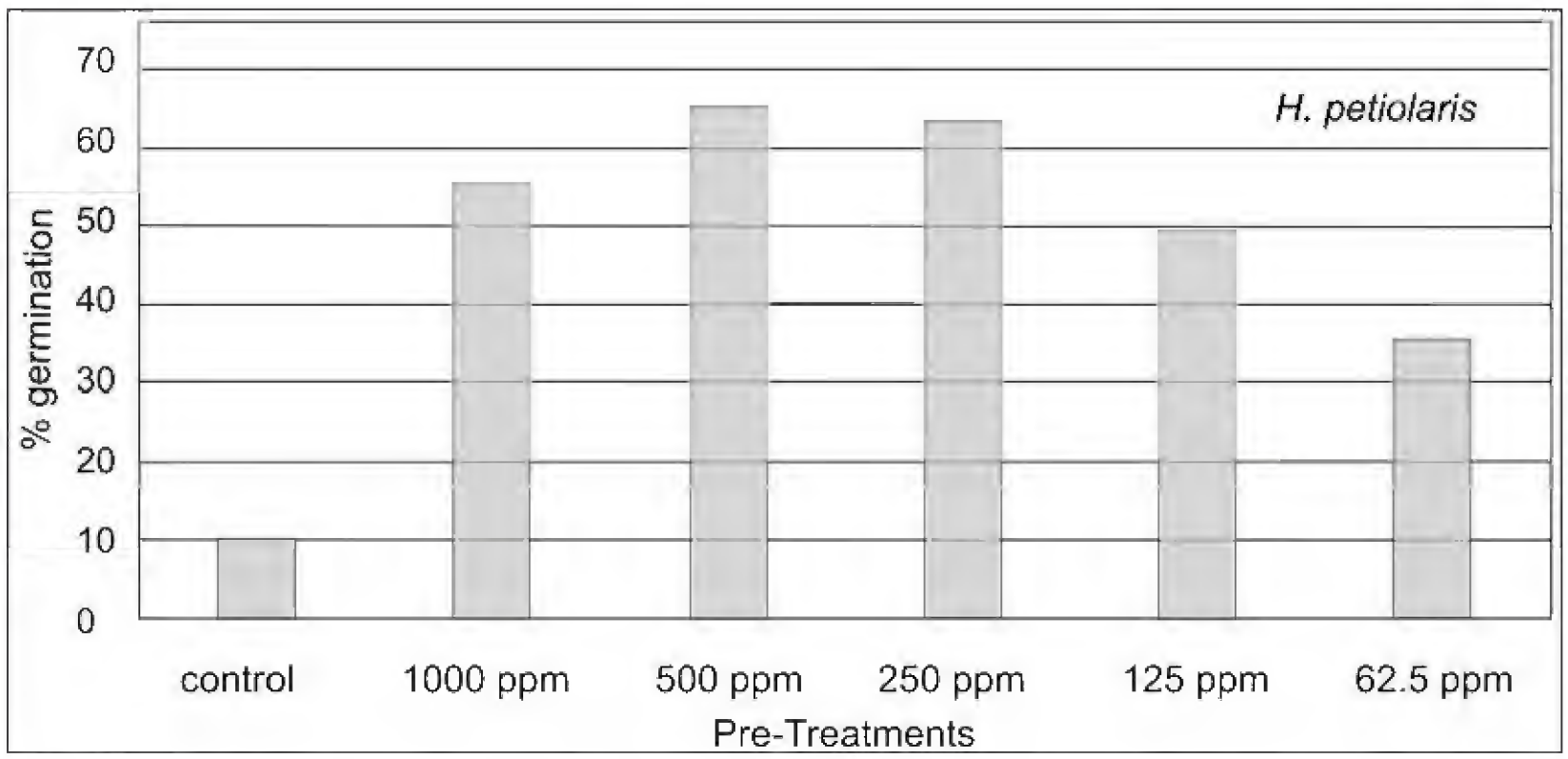

Figure 1. Germination of *H. petiolaris* at various concentrations of GA3, soaked 1 week, 4°C. Control soaked in DI water, 1 week, 4°C.

In summary, this study found an effective pre-treatment to enhance seed germination of *H. annuus* and *H. petiolaris* is soaking in GA3 for 1 week, 4°C. It should be noted that transplanting germinated seeds of *H. annuus* to soil in pots, resulted in nearly 100% success, indicating no apparent long-term effects from the GA3 treatment.

ACKNOWLEDGEMENTS

This research funded by Baylor University. Thanks to Laura Marek and Lisa Pfiffner, GRIN, USDA, for helpful discussions

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Seiler, G. J. 1993. Wild sunflower species germination. Helia 16: 15-20.

Table 1. Germination tests of *H. annuus*, native, Gruver, TX. References: Kumari and Singh (2000) Maiti et al. 2006.

Pre-Treatment, all soaked 1 wk, 4°C	germination rates
1. control: seeds soaked in DI water	9/60 = 16.7%
2. 1000 ppm GA3	37/60 = 61.7%
3. 500 ppm GA3	35/60 = 58.3%
3a. 500 ppm GA3 on filter paper	31/60 = 51.7%
4. 250ppm GA3	15/60 = 25.0%
5. 125ppm GA3	14/60 = 23.2%

Table 1. Germination tests of *H. petiolaris*, native, Ellsworth, KS. References: Kumari and Singh (2000) Maiti et al. 2006.

Pre-Treatment, all soaked 1 wk, 4°C	germination rates
1. control: seeds soaked in DI water	6/59 = 10.2%
2. 1000 ppm GA3	32/57 = 56.1%
3. 500 ppm GA3	39/60 = 65.0%
3a. 500 ppm GA3 on filter paper	26/58 = 44.8%
4. 250 ppm GA3	33/53 = 62.8%
5. 125 ppm GA3	28/57 = 49.1%
6. 62.5 ppm GA3	21/58 = 36.2%

Legitimacy of the name *Croton bigbendensis* (Euphorbiaceae)

Billie L. Turner
Plant Resources Center, The University of Texas, Austin, TX 78712
billie.turner@austin.utexas.edu

ABSTRACT

The legitimacy of the name *Croton bigbendensis* is discussed and the circumstances concerning the issuance of a Holotype based on pistillate and staminate plants explained. Published on-line www.phytologia.org *Phytologia 99(1): 36-37 (Jan 19, 2017).* ISSN 030319430.

KEY WORDS: *Croton bigbendensis,* nomenclature, holotype.

Turner (2004) published the name **Croton bigbendensis** B.l. Turner, this largely confined to the southern Big Bend region of western Texas. The taxon was typified by a single collection (composed of several plants) at the same place at the same time. Because the population was composed of both pistillate and staminate plants, I provided the number *Turner 22-204A* for the pistillate plants and *Turner 22-204b* for the staminate plants. The plants concerned clearly belonged to the same collection, all bearing the same number, although I did designate a pistillate plant from the population as the Holotype, however, my intent was to treat *Turner 22-204* (both A and b) as holotype material, this clearly stated and so pictured in my figures 1 and 2. But some purists (cf. discussion provided by Wiersema 2016) view such typification as contrary to the Code, contending that only a single plant number should have been applied to the Holotype, thus invalidating the name, although my application of such was quite clear, this discussed further in more detail by my archrival, Henrickson (2010), who would recognize my novelty as but a variety, at best, this after a lengthy digression into my systematic mores.

Strangely, W. van Ee and Berry (2016), did not account for the name **C. bigbendensis** in their treatment of *Croton* for the Flora of North America, nor did they mention the work of Henrickson. I would like to place on record here that I believe the name **C. bigbendensis** B.L. Turner is properly typified and deserves recognition, as justified in the above. As to the taxonomic criticism of the taxon posited by Henrickson, I leave such evaluation to future workers having not the bias Henrickson and I both possess.

An up to date distributional map of **C. bigbendensis** is provided in the present account (Fig. 1), this part of my Atlas of the Vascular Plants of Texas (Turner 2017, in prep.).

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Ee, W. van and P.E Berry. 2016. *Croton*, in N. Amer. Fl. 12: 206-224.

Henrickson, J. 2010. *Croton bigbendensis* Turner (Euphorbiaceae) Revisited. J. Bot. Res. Inst. Texas 4: 295-301.

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Wiersema, J.H. 2016. Proposal to provide a more direct definition of the term “gathering". Taxon 65: 1186.

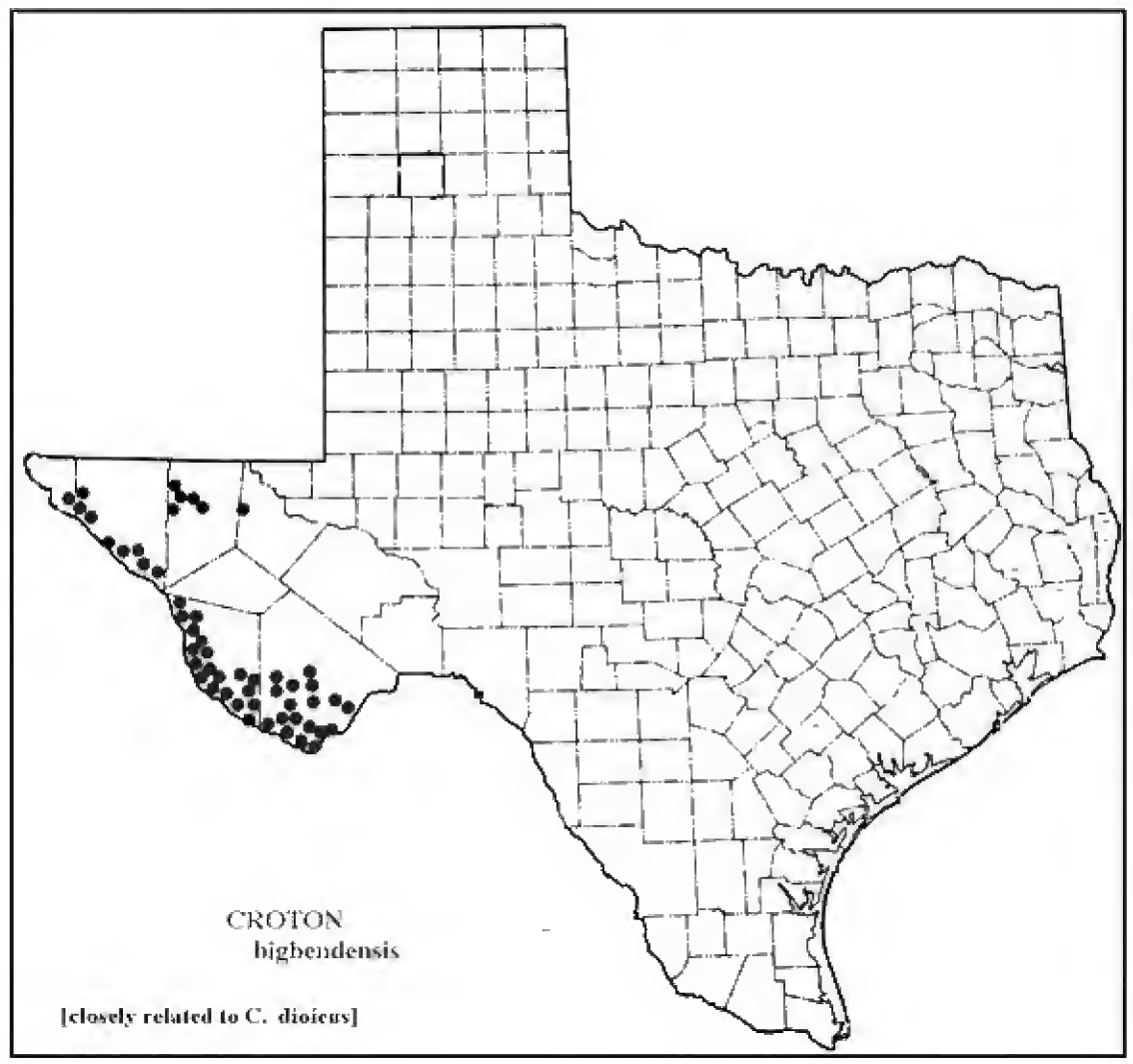

Figure 1. Distribution of *Croton bigbendensis* in Texas.

Multiple evidences of past evolution are hidden in nrDNA of *Juniperus arizonica* and *J. coahuilensis* populations in the trans-Pecos, Texas region

Robert P. Adams
Biology Department, Baylor University, Box 97388, Waco, TX 76798, USA, Robert_Adams@baylor.edu

ABSTRACT

Geographical analysis of variation in nrDNA polymorphisms of *J. arizonica* and *J. coahuilensis* in the trans-Pecos, TX region showed multiple patterns of hybridization, both modern and relictual (Pleistocene) introgression, incomplete lineage sorting and relictual hybridization. The concept that nrDNA from a single plant could harbor multiple evidences of past evolution appears to be novel. Total nrDNA polymorphisms were maximal in the Ft. Davis, Alpine, Marfa trans-Pecos area and on the granitic rocks at Hueco Tanks State Park, TX. Published on-line www.phytologia.org *Phytologia 99(1): 38-47 (Jan 19, 2017).* ISSN 030319430.

KEY WORDS: *Juniperus arizonica, J. coahuilensis,* Cupressaceae, hybridization, introgression, incomplete lineage sorting, nrDNA polymorphisms, petN-psbM DNA.

Recently, Adams (2016) found (by petN-psbM sequencing) that *Juniperus arizonica*, previously known only from Arizona and New Mexico, occurs in trans-Pecos Texas in the Franklin Mtns., Hueco Mtns., Hueco Tanks State Park, Quitman Mtns., Eagle Mtns. and Sierra Vieja Mtns., primarily on igneous material (Figs. 1, 2). These trans-Pecos juniper populations have previously been identified as *J. coahuilensis* . These taxa have very distinct differences in their DNA and are in separate clades (Adams, 2014, Adams and Schwarzbach, 2011, 2013). The cp region petN-psbM is especially efficient in separating these taxa, as 5 SNPs occur in the 794 bp region.

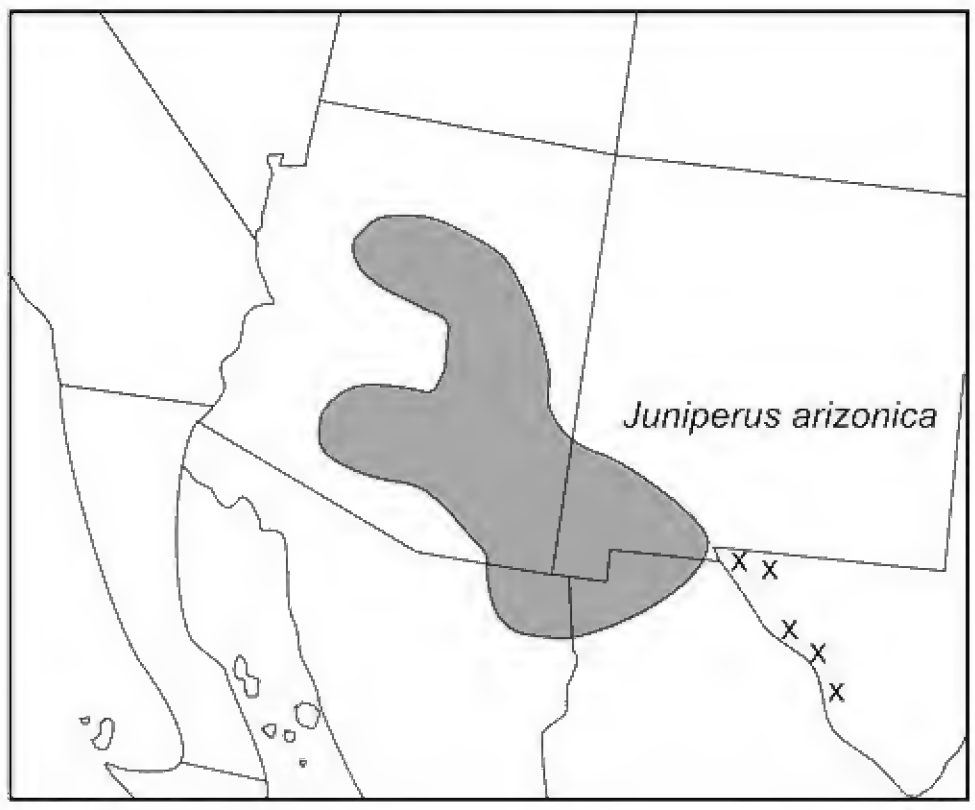

Figure 1. Distribution of *J. arizonica* (Adams 2016).

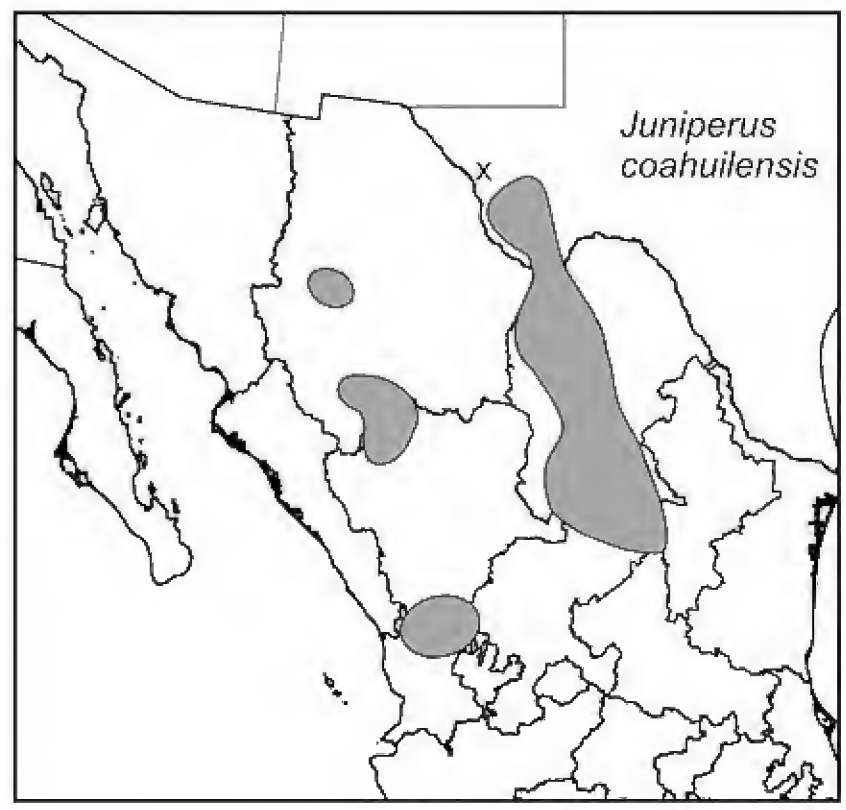

Figure 2. Distribution of *J. coahuilensis*

Detailed mapping of plants by their cp DNA (petN-psbM) showed that all the plants (or specimens) in New Mexico and northern Mexico, as well as plants examined from the Franklin Mts., Hueco Tanks SP, Quitman Mts., Eagle Mts., and one plant from Sierra Vieja Mtns. contained the *J. arizonica* cp DNA (Fig. 3). Junipers from Ft. Davis, Alpine, Marfa and Big Bend (1) all had the *J. coahuilensis* cp DNA (Fig. 3). The occurrence and extent of hybridization and introgression in that

region is not known, except for a study of hybridization between *J. coahuilensis* and *J. pinchotii* in the Chisos Mtns. (Adams and Kistler, 1991).

Recently, Adams et al. (2016) have reported that in the sister genus, *Hesperocyparis*, artificial hybrids between *Hesperocyparis* (= *Cupressus* in part) *arizonica* and *H. macrocarpa*, nrDNA was inherited as heterozygous for diagnostically different sites. They concluded that, at least in *Hesperocyparis* (and likely in the Cupressaceae, including *Juniperus*), analysis of heterozygous nrDNA (ITS) could be used for the detection and analysis of hybridization. Because F_2 progeny and backcrosses were not analyzed, they could not comment on the amount and/ or speed of lineage sorting in *Hesperocyparis*.

The purpose of this paper is to report on the composition of nrDNA in populations in the trans-Pecos, TX region and the investigation of hybridization, introgression and incomplete lineage sorting.

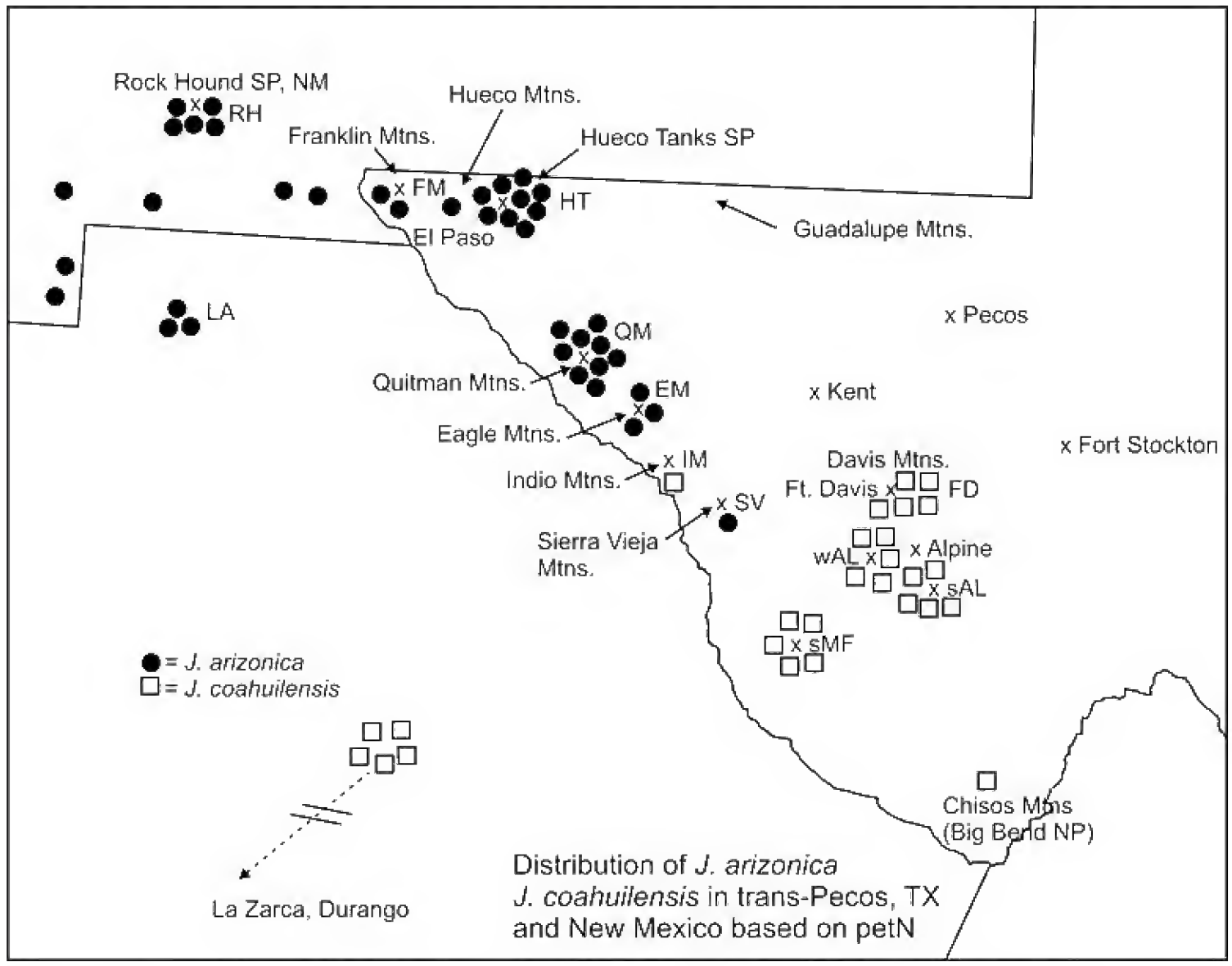

Figure 3. Distribution of *J. arizonica* and *J. coahuilensis* based on petN-psbM cp data.

MATERIALS AND METHODS

Plant material and populations studied:

Sedona, AZ

J. arizonica by petN DNA, common in grassland. tree 6 m tall. female, with *J. osteosperma* on alluvial soil. On AZ highway 179, between Sedona and I17. 34° 42.431'N, 111° 46.369' W, 1150 m, 13 March, 2005, Yavapai Co., AZ, *Robert P. Adams 10634-10636*,

Cottonwood, AZ

J. arizonica by petN DNA, abundant, on alluvial fan, 3 mi. SW of Cottonwood, AZ, on D. Thornburg's property, 34° 41' 17.4" N, 112° 03' 05.46" W, 4060 ft., 13 Jan., 2010, Yavapai, Co., AZ, Coll. David Thornburg ns, Lab Acc. *Robert P. Adams 14908-14913*,

Southern New Mexico:
J. arizonica by petN DNA, Hidalgo Co., NM, Animas Mtns, 31.61176° N, 108.7791° W, 5750', Seinet Cat # 57778, *Wagner 1283*, 22 Jul 1975, Lab Acc. *Robert P. Adams 14697*,
J. arizonica by petN DNA, Luna Co., NM, Tres Hermanos Mtns, 31.9010° N, 107.7794° W, 4250', Seinet Cat # 85666, *J L Carter 1246*, 14 Aug 1993, Lab Acc. *Robert P. Adams 14698*,
J. arizonica by petN DNA, Hidalgo Co., NM. Animas Mtns, 31.5938° N, 108.7684° W, 6000', Seinet Cat # 57776, *Wagner 1005*, 17 Jun 1975, Lab Acc. *Robert P. Adams 14701*,
J. arizonica by petN DNA, no cones, Hidalgo Co., NM, Animas Peak, Animas Mtns., 31.5813° N, 108.7843° W, 8452' (Google Earth), Seinet Cat # 25131, *WC Martin 4678*, 29 Oct 1960, Lab Acc. *Robert P. Adams 14705*,
J. arizonica by petN DNA, Hidalgo Co., NM, Big Hatchet Mtns.,with Quercus, Parthenium, Ocotillo, Mesquite, Agave 31.6249° N, 108.36425° W, 5350', *Ken Heil 9254*, 28 May 2010, Lab Acc. *Robert P. Adams 14716*,
J. arizonica by petN DNA, Grant Co., NM, ca 1.5 mi. s of NM hwy 9, near 'Old Hachiti' townsite. Chihuahuan desert scrub - creosote, Lycium koberlina and Dalea formosa. 31.9139° N, 108.41472° W, 4745', *Ken Heil 32357*, 29 Apr 2010, Lab Acc. *Robert P. Adams 14717*,
Rock Hound State Park, NM (type locality, *J. arizonica*)
J. arizonica by petN DNA, multi-stemmed shrubs to 4m, in Bouteloua grassland. Pollen shed in Mar-April?, Fruit rose color. Rock Hound State Park. 17km S, and 8 south of Deming, NM, 32° 11.161'N, 107° 36.651' W, 1420 m, 6 Feb., 1996, Luna Co., NM,, *Robert P. Adams 7635-7637*
J. arizonica by petN DNA, common in *Bouteloua* grassland. shrub-trees to 3-5 m., Rock Hound State Park., 32° 11.161'N, 107° 36.651' W, 1420 m, 12 Mar, 2005, Luna Co., NM, *Robert P. Adams 10630*,
Quitman Mtns.
J. arizonica by petN, Hudspeth Co., TX, common on degraded granite, north face of Quitman Mtns., with desert-scrub. On south side of I10, ~6.3 mi. w of Sierra Blanca, TX, 31°12' 25" N; 105° 27' 51" W, 4629', *Robert P. Adams 14798-14806,* 12 March 2016,
Hueco Tanks St. Park, TX
J. arizonica by petN El Paso Co., TX, uncommon, 50- 100 trees seen, on granite, Hueco Tanks St. Park, 31° 54' 49.7" N; 106° 02' 6.8" W, 4560', *Robert P. Adams 14827-14835*, 18 March 2016. *Robert P. Adams 14718.*
11.2 s of Alpine, TX on Tex 118
J. coahuilensis, by petN, Brewster Co, TX, abundant in grassland, 11.2 s of Alpine, TX on Tex 118. 30° 14' 08" N; 103° 34' 00" W, 5222', *Robert P. Adams 14807-14811,* 15 March 2016,
11.0 mi w of Alpine, TX on US 90
J. coahuilensis, by petN, Brewster Co, TX, 11.0 mi w of Alpine on US 90, abundant in grassland, in Paisano Mtns., 30° 17' 42" N; 103° 48' 02" W, 4967', *Robert P. Adams 14812-14816,* 15 March 2016,
4.2 mi se of Ft. Davis, TX on Tex 118, CDRI
J. coahuilensis, Jeff Davis Co., TX, common locally, in grassland. 4.2 mi se of Ft. Davis, on Tex 118, e 1.0 mi into Chi. Desert Res. Inst., 39° 09' 27.54" N; 86° 18' 23.31" W, 5050', *Robert P. Adams 14817-14821*, 16 March 2016,
19.4 mi. s of Marfa, TX on US 67
J. coahuilensis, by petN, Presidio Co., TX, common in grassland, 19.4 mi. s of Marfa, on US 67, 30° 04' 07" N; 104° 10' 19" W, 5137', *Robert P. Adams 14822-14826*, 16 March 2016,
La Zarca, Mexico
J. coahuilensis, large population with thousands of trees. 85 km N. of La Zarca on Mex. 45, 1740m, 10 Dec, 1991, Durango, Mexico, *Robert P. Adams 6829-6831*,
Voucher specimens for new collections 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° 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 petN-psbM primers utilized.

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.).

RESULTS AND DISCUSSION

Sequencing petN-psbM yielded 794 bp with 5 SNPs separating *J. arizonica* and *J. coahuilensis*. In addition, nrDNA was sequenced yielding 1270 bp with only 1 SNP (at site 533) separating *J. arizonica* and *J. coahuilensis*. Using these data, samples were classified accordingly (Table 1). Based on heterozygous peaks at site 533, 11 samples were classified as hybrids (AxC, Table 1). According to nrDNA, hybrids occur mostly in the Animas Mts., NM, Hueco Tanks SP, TX and Quitman Mtns., TX (Fig. 4.). Note one hybrid in the Marfa, TX population. The nrDNA data, indicates that populations of *J. coahuilensis* in the Alpine - Ft. Davis - Marfa area are nearly pure. It should be noted that the soils of Hueco Tanks and Quitman Mtns. are granitic, whereas the Alpine - Ft. Davis - Marfa area soils are volcanic.

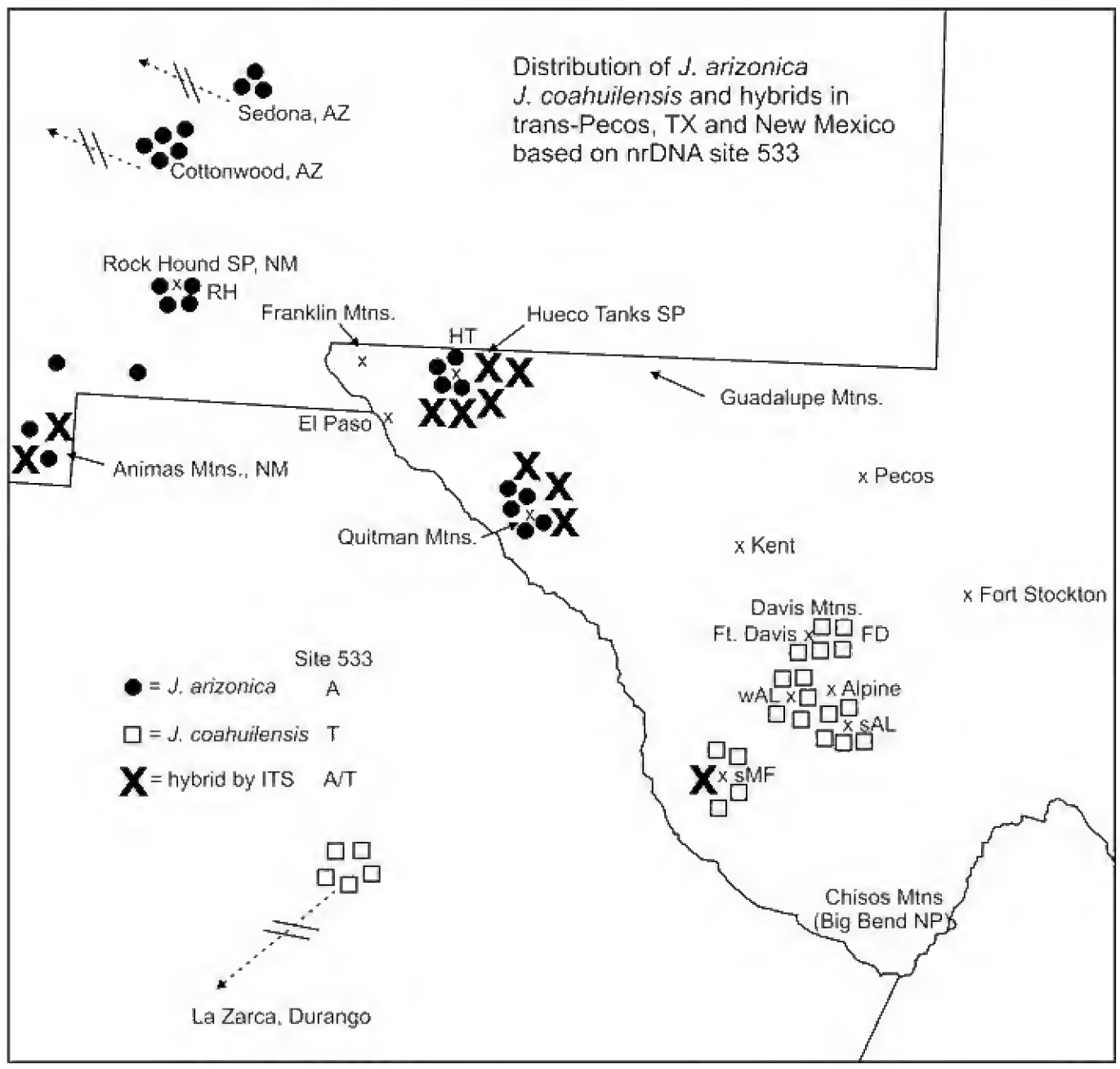

Fig. 4. Distribution of *J. arizonica* x *J. coahuilensis* hybrids based on nrDNA.

In order to visualize the correlation of nrDNA and cp (petN) classifications, each plant was scored for species or hybrid for nrDNA and cpDNA. Mapping this classification shows a relatively sharp demarcation between *J. arizonica* and *J. coahuilensis* (Fig. 5). The zone of hybridization is in Hueco Tanks State Park, Quitman Mtns., and Anima Mtns. and this appears to be a region of introgression northward from *J. coahuilensis* (Fig. 5).

The Hueco Tanks State Park, Quitman Mtns., and Anima Mtns. populations are on granitic soil and the *J. coahuilensis* populations in the Ft. Davis, Alpine, Marfa region are on volcanic soil. Soil differences may be the factor that determines the northern range of *J. coahuilensis* and could present a barrier for additional introgression northward into *J. arizonica*.

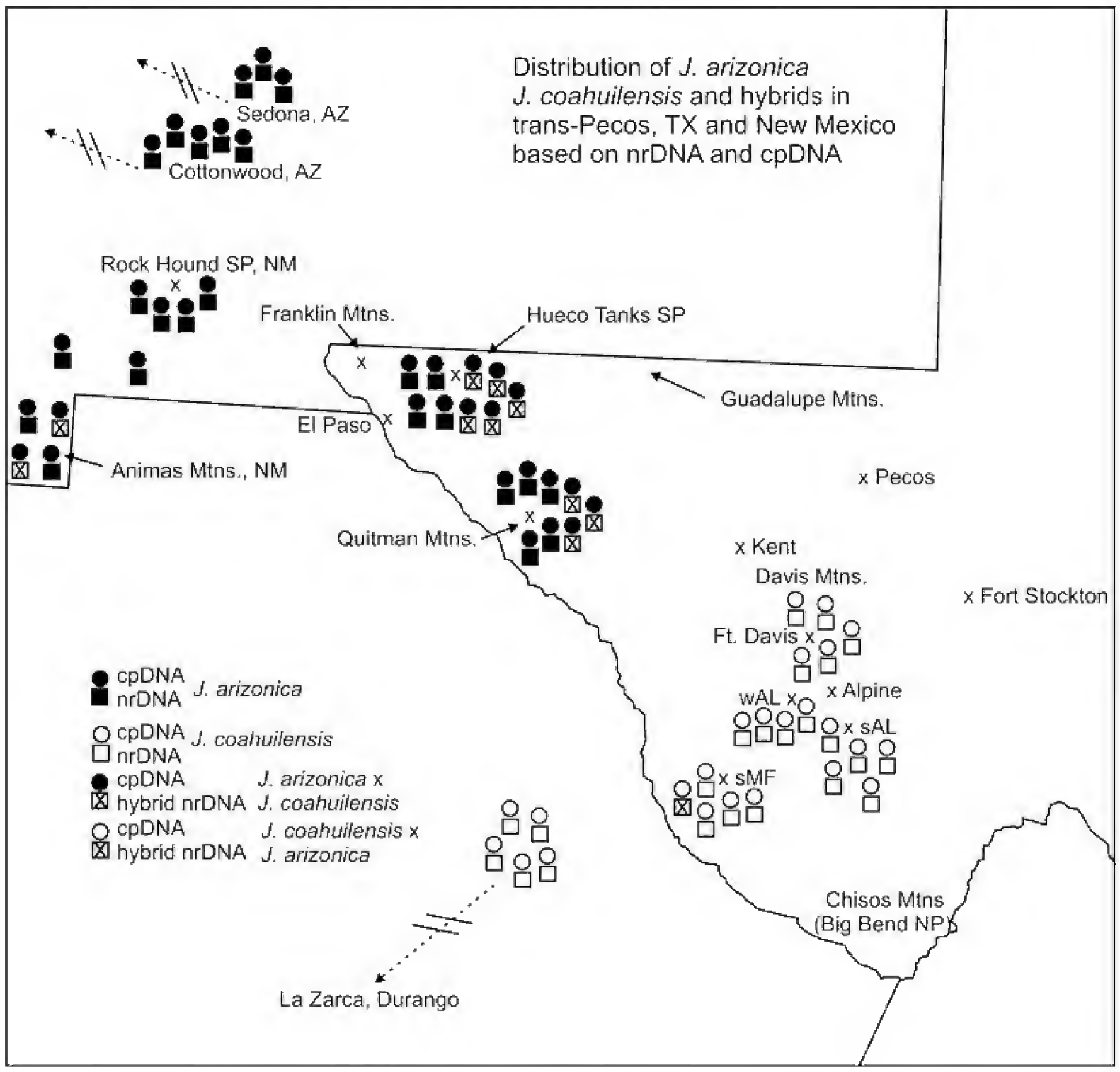

Figure 5. Mapping plants showing their classification as *J. arizonica, J. coahuilensis*, or hybrids for both nrDNA and cpDNA.

Mapping the number of nrDNA polymorphic sites per plant shows very low polymorphic sites in the normal range of *J. arizonica* (NM and AZ, Fig. 6). However, where *J. arizonica* and *J. coahuilensis* hybridize and thence southward, there are several populations with plants having 1 to 6 polymorphic sites (excluding site 533). Hueco Tanks is very variable: 3 plants with 0 polymorphisms; 3 with 1; 2 with 5; and 1 with 6 polymorphisms (Fig. 6). The Davis Mtns - Alpine area is also a region with lots of polymorphisms (Fig. 6). In contrast to the more mountainous sites, the Marfa population (19.3 mi sw of Marfa, in a *Bouteloua* grassland) had low polymorphisms in its nrDNA.

The trans-Pecos region likely experienced a mixing of southern Rockies flora to move southward and the flora of the Sierra Madre Oriental flora to move northward during cooling and heating eras in the Pleistocene. This provided opportunities for many *Juniperus* species, now spatially separated, to hybridize in the past.

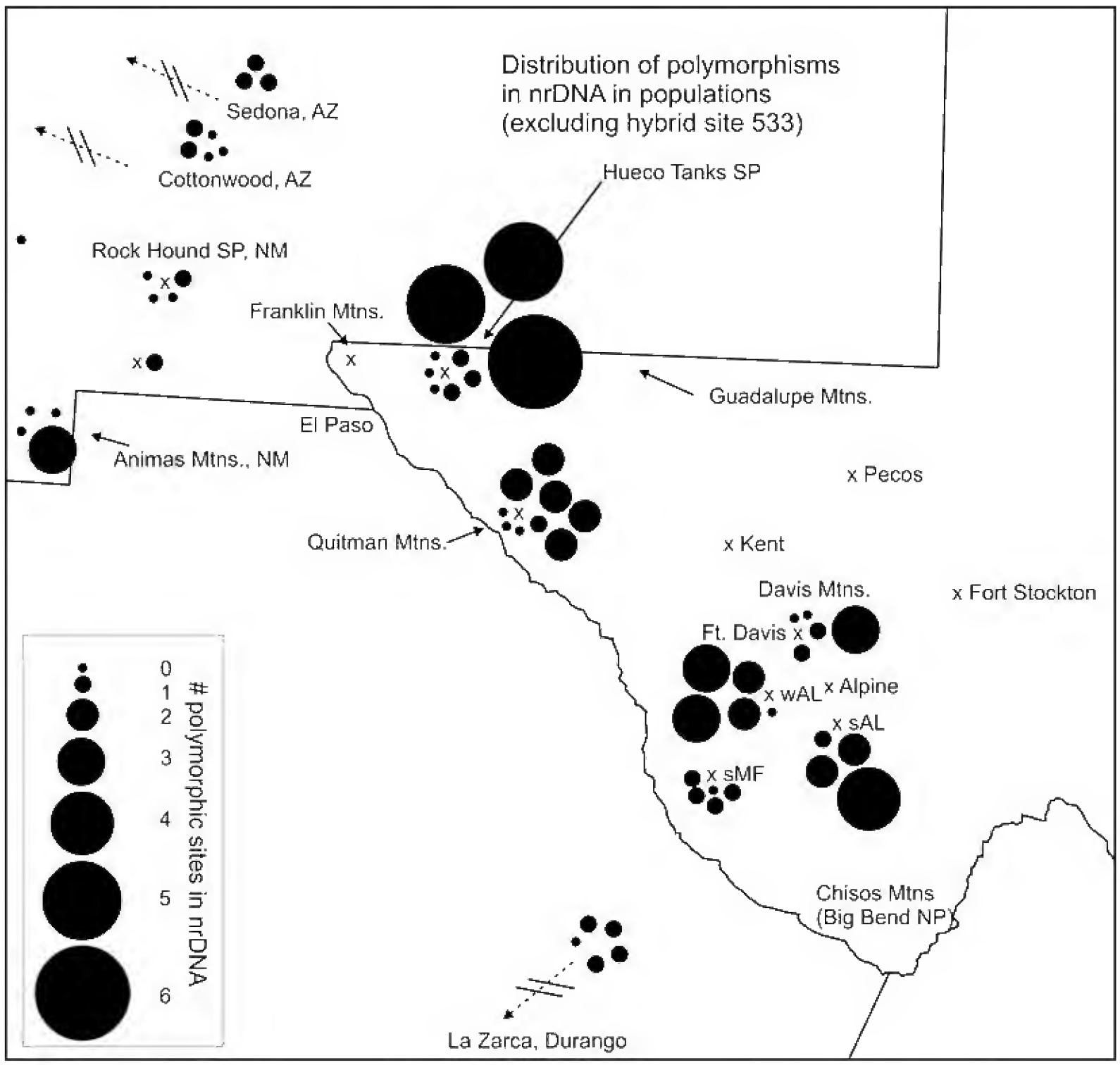

Fig. 6. Distribution of the number of nrDNA polymorphisms/ plant.

A closer examination of individual plant nrDNA site polymorphisms revealed that nrDNA harbors several evolutionary patterns that vary by region. For site 543, 11 plants contained (C/G) and these range from the Quitman Mtns., northwest to Cottonwood and Sedona, AZ (Fig. 7). Only one plant was G/G, and that was in the Cottonwood, AZ population. This is near the northwestern limit of *J. arizonica*. Site 543 might be an indicator of introgression from *J. coahuilensis* into *J. arizonica*.

In addition, another polymorphism occurs (C/T, Fig. 7), but only in the La Zarca, MX population. Additional research is needed to determine if the T comes from hybridization with another Mexican juniper, from incomplete lineage sorting or just a local mutation.

The distribution of variation in site 173 (A/G) is centered between *J. arizonica* and *J. coahuilensis* in the Animas Mtns., NM, Hueco Tanks SP, and Quitman Mtns. (Fig. 8.) This may to be either relictual hybridization, or incomplete lineage sorting.

nrDNA site 304 contains two geographical patterns. One (A/T, Fig. 9) is similar to that for site 173 (Fig. 8) in the Animas Mtns., NM, Hueco Tanks SP, and Quitman Mtns. The second pattern (C/T, Fig. 9) is found in only the south Alpine, TX population. The C/T site might be due to introgression from the east or south from Mexico, perhaps from mixing of taxa during the Pleistocene. Or it may be just a local mutation in that population.

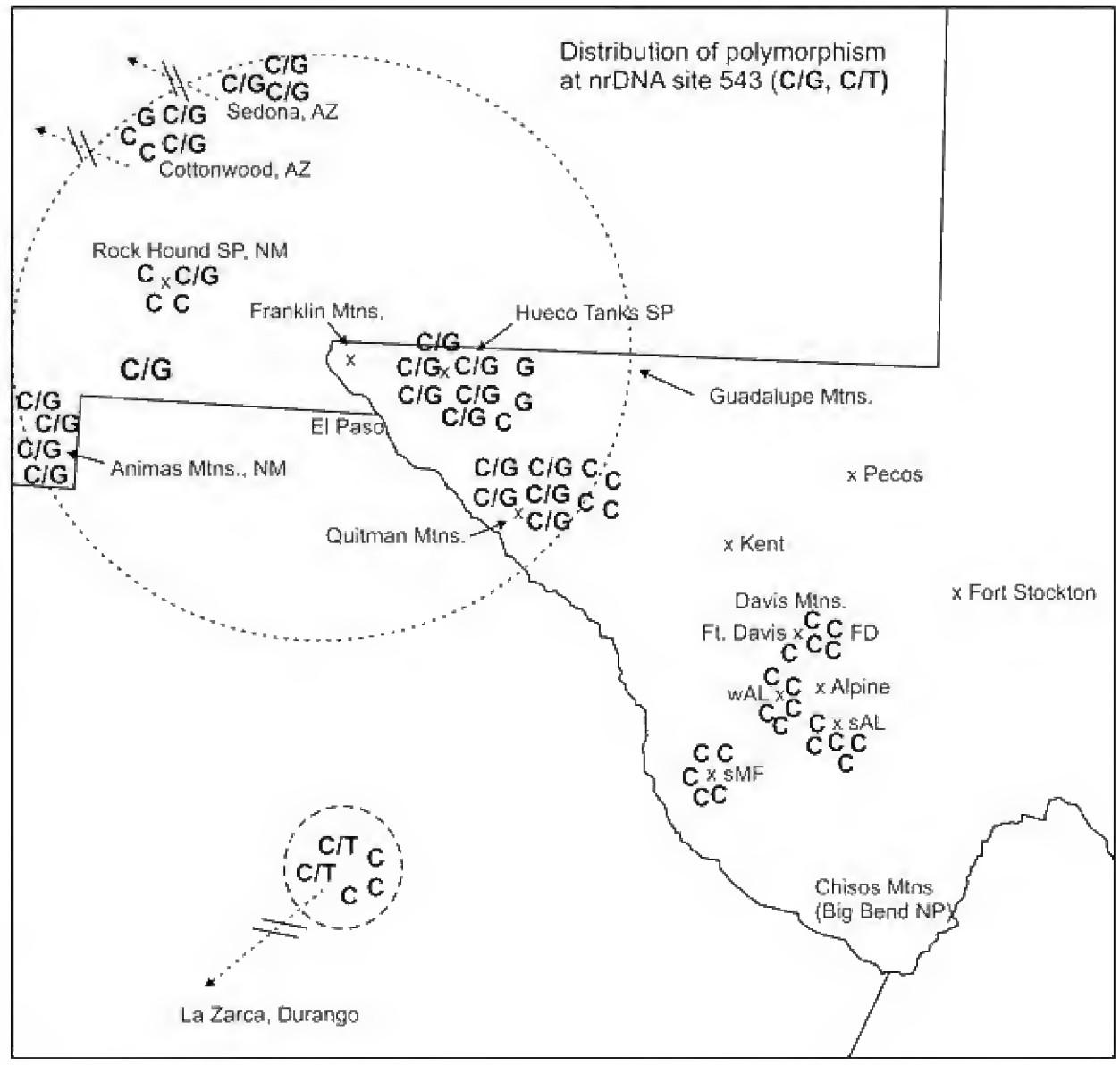

Figure. 7. Distribution of polymorphisms at nrDNA site 543.

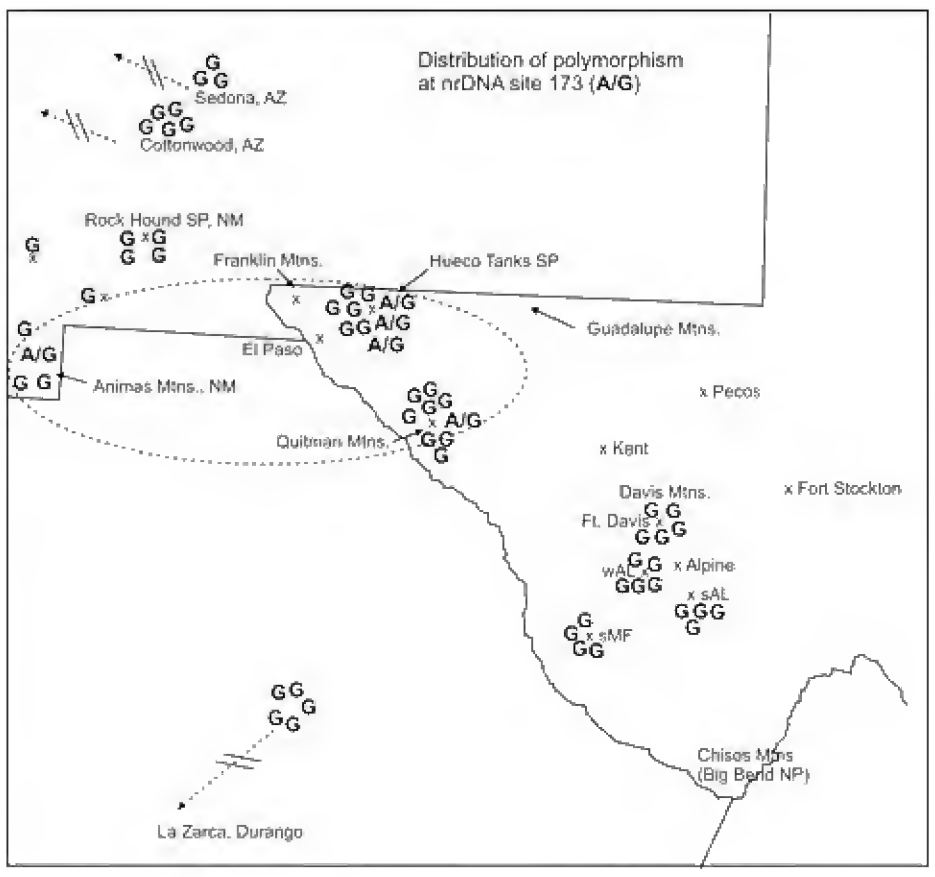

Figure 8. Dist. of nrDNA site 173 polymorphism.

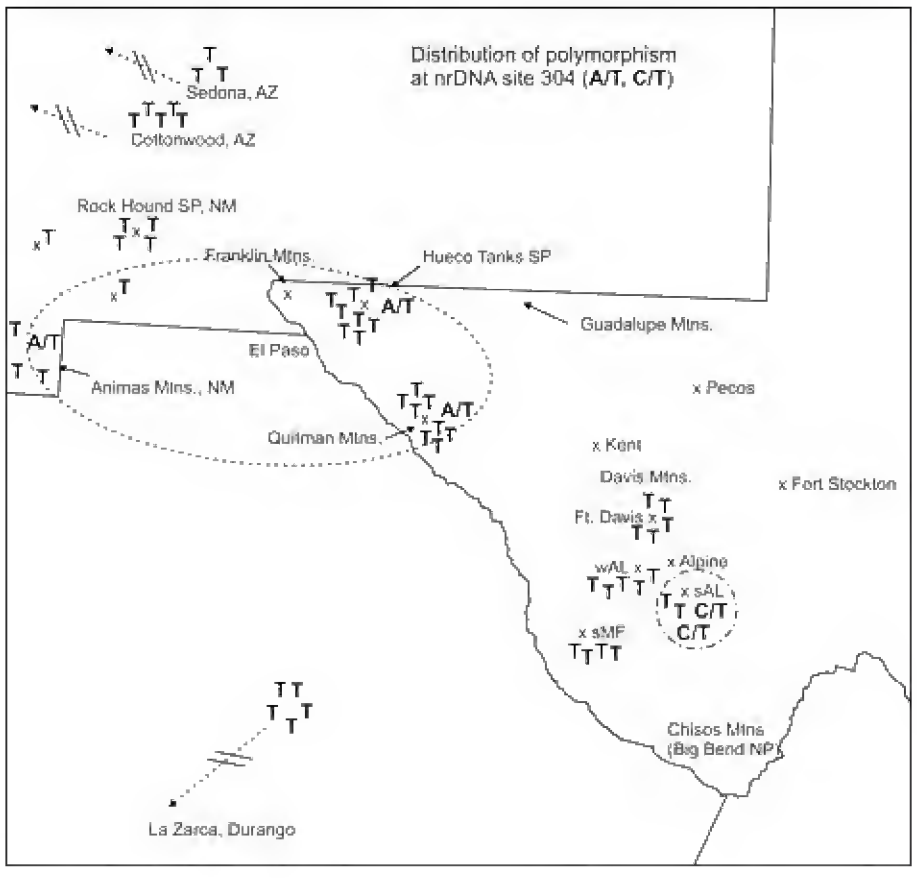

Figure 9. Dist. of nrDNA site 302 variation.

Variation in site 318 (C/T, Fig. 10) spans the *J. arizonica* - *J. coahuilensis* range junction and seems likely to be from relictual hybridization. There appears no source of the C allele in any population of *J. arizonica or J. coahuilensis* examined. Alternatively, it could be incomplete lineage sorting.

Finally, two sites show very similar patterns: both sets of polymorphisms are confined to the Ft. Davis - Alpine - Marfa area and both sites have plants with mixed bases as well as plants with homozygous bases. Site 302 (A/G) was found in all four populations, plants homozygous for A are in all 4 populations, but only one plant homozygous for G was found (in the Ft. Davis population, Fig. 11).

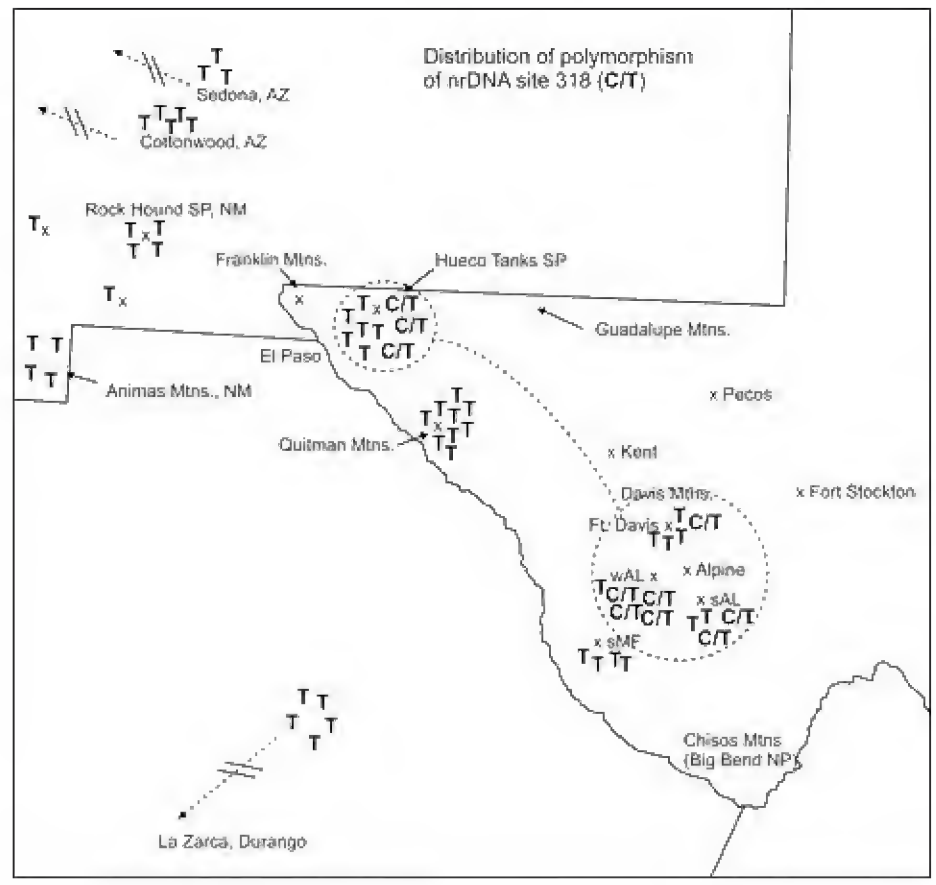

Figure 10. Distribution of site 318 (C/T) polymorphisms.

A similar pattern was found for site 303 (C/T). C/T was present in all four populations, plants homozygous for C were in all 4 populations, but only 2 plants homozygous for T occurred in the Ft. Davis and Marfa populations (Fig. 12). These two sites are difficult to explain. It almost appears that an unknown (to the author) species is present that has (G,C) at 302, 303 and is hybridizing with *J. coahuilensis* (A, C) at 302,303. Other juniper species in the area are *J. pinchotii* (Kent, and Fort Stockton), *J. monosperma* (near Kent), and *J. deppeana* (higher elevations in the area). Of course, it might be Pleistocene relictual hybridization with a species (or its ancestor) now growing in Mexico.

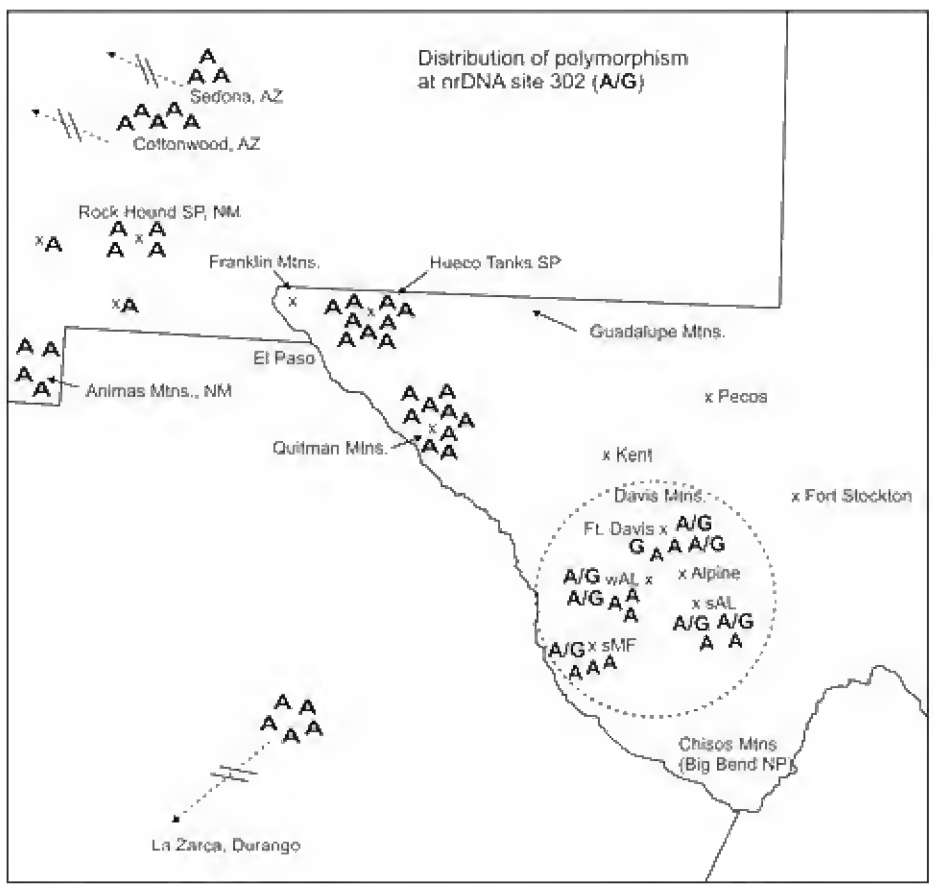

Figure 11. Geographical variation in variation at nrDNA site 302.

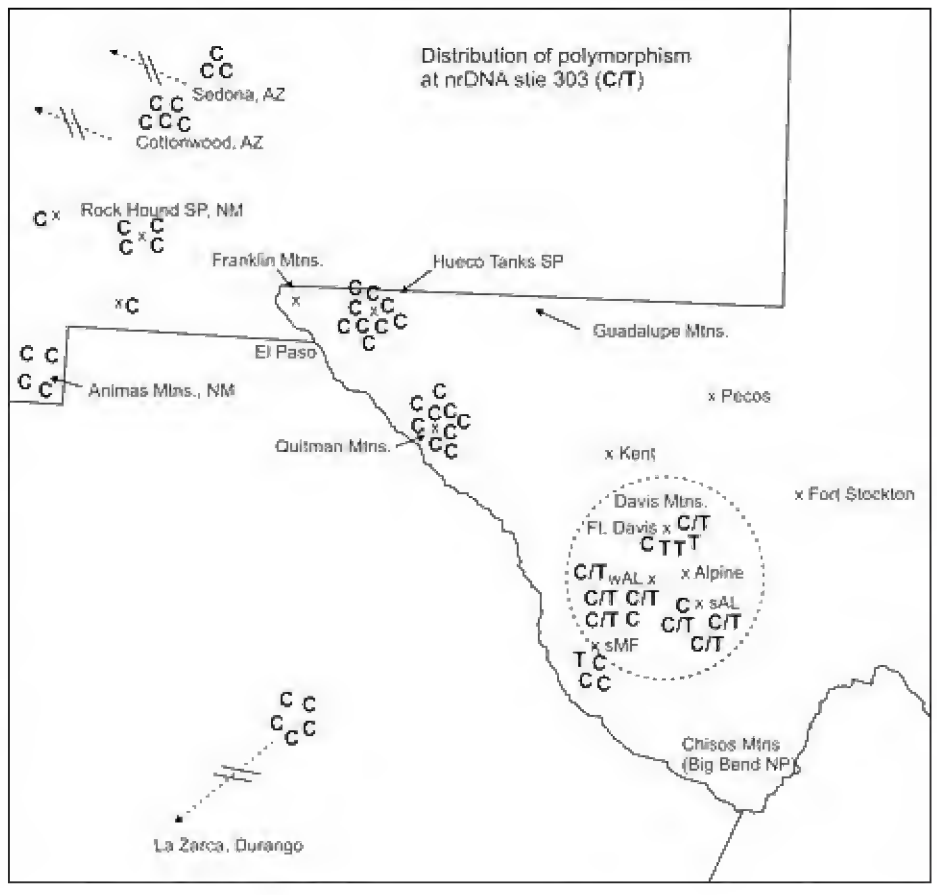

Figure 12. Variation in nrDNA site 303.

SUMMARY

Geographical analysis of variation in nrDNA polymorphisms of *J. arizonica* and *J. coahuilensis* in the trans-Pecos, TX region showed multiple patterns of hybridization, both modern and relictual (Pleistocene) introgression, incomplete lineage sorting and relictual hybridization.

The concept that nrDNA from a single plant could harbor multiple evidences of past evolution appears to be novel. The pre-occupation of evolutionary systematists with phylogeny has resulted a lack of critical variation in nrDNA. Heretofore, the standard procedure is to sequence nrDNA (as the sole proxy of the nuclear DNA), then add in a few cpDNA sequences, then ran the data in a phylogenetics software and publish 'the Phylogeny', and then move on to another genus. That may satisfy a need for a broad evolutionary framework of a group (genus). But, as shown in this report, there may be considerable evidence of past evolutionary events in nrDNA that would be completely ignored (and missed) by only running a phylogenetic analysis.

Total nrDNA polymorphisms were maximal in the Ft. Davis, Alpine, Marfa trans-Pecos area and on the granitic rocks at Hueco Tanks State Park, TX. Additional research using Single Copy Nuclear Genes (SCNG) is needed to further address the variation found in this region.

ACKNOWLEDGEMENTS

Thanks to George M. Ferguson (UA), Ken Heil (SJNM), Tim Lowrey (UNM), Mike Powell (SRSC) and Richard Worthington (UTEP) for letting me sample (or sending small fragments) herbarium specimens. This research was supported in part with funds from Baylor University. Thanks to Amy TeBeest for lab assistance and Andrea Schwarzbach for helpful suggestions on the manuscript.

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Table 1. Classification of samples based on petN and nrDNA. **Bold** are putative hybrids between *J. arizonica* and *J. coahuilensis* by ITS site **533**, A in *arizonica*, T in *coahuilensis*, (A/T in 533) were scored as hybrids (AxC). The more common polymorphic sites are shown. A few rarer, polymorphic sites are listed next to the sample name.

Sample	petN	ITS	173	191	196	302	303	304	318	**533**	543	605	708	# poly
az10634Sedona 181M	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	A	A	1
az10635Sedona 681M	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	A	A	1
az10636Sedona	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	A	A	1
az14908Cottonwood	ariz	ariz	G	G	T	A	C	T	T	A	**G**	A	A	0
az14909Cottonwood	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	A	A	1
az14910Cottonwood	ariz	ariz	G	G	T	A	C	T	T	A	C	A	A	0
az14912Cottonwood	ariz	ariz	G	G	T	A	C	T	T	A	C	A	A	0
az14913Cottonwood 121Y	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	A	A	1
az14717GrantCoNM 181M	ariz	ariz	G	G	T	A	C	T	T	A	C	A	A	0
az7635RockHoundSP	ariz	ariz	G	G	T	A	C	T	T	A	C	A	A	0
az7636RockHoundSP	ariz	ariz	G	G	T	A	C	T	T	A	C	A	A	0
az7637RockHoundSP	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	A	A	1
az10630RockHoundSP	ariz	ariz	G	G	T	A	C	T	T	A	C	A	A	0
az14698LunaCoNM	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	A	A	1
az14697HidalgoCoNM	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	A	A	1
az14701HidalgoCoNM	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	A	A	1
az14705HdalgoCoNM	**ariz**	**AxC**	**G**	**G**	**T**	**A**	**C**	**T**	**T**	**A/T**	**C/G**	**A**	**A**	**2**
az14716HidalgoCoNM	**ariz**	**AxC**	**A/G**	**G**	**T**	**A**	**C**	**A/T**	**T**	**A/T**	**C/G**	**A**	**A**	**4**
coa14827HuecoTanks	ariz	coah	G	G	T	A	C	T	T	T	**C/G**	A	A	2
coa14828HuecoTanks	**ariz**	**AxC**	**G**	**G**	**T**	**A**	**C**	**T**	**T**	**A/T**	**C**	**A**	**A**	**1**
coa14829HuecoTanks	**ariz**	**AxC**	**A/G**	**G**	**C/T**	**A**	**C**	**T**	**C/T**	**A/T**	**C/G**	**A**	**A/G**	**6**
coa14830HuecoTanks	**ariz**	**AxC**	**A/G**	**G**	**C/T**	**A**	**C**	**T**	**C/T**	**A/T**	**C/G**	**A**	**A/G**	**6**
coa14831HuecoTanks	**ariz**	**AxC**	**A/G**	**G**	**C/T**	**A**	**C**	**A/T**	**C/T**	**A/T**	**C/G**	**A**	**A/G**	**7**
coa14832HuecoTanks	ariz	coah	G	G	T	A	C	T	T	A	**C/G**	A	A	1
coa14833HuecoTanks	**ariz**	**AxC**	**G**	**G**	**T**	**A**	**C**	**T**	**T**	**A/T**	**C/G**	**A**	**A**	**2**
coa14834HuecoTanks	ariz	ariz	G	G	T	A	C	T	T	A	G	A	A	0
coa14835HuecoTanks	ariz	ariz	G	G	T	A	C	T	T	A	G	A	A	0
coa14798 Quitman Mtns.	**ariz**	**AxC**	**A/G**	**G**	**T**	**A**	**C**	**A/T**	**T**	**A/T**	**C**	**A**	**A**	**3**
coa14799 Quitman Mtns.	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	A	A	1
coa14800 Quitman Mtns.	ariz	ariz	G	G	T	A	C	T	T	A	C	A	A	0
coa14801 Quitman Mtns.	**ariz**	**AxC**	**G**	**G**	**T**	**A**	**C**	**T**	**T**	**A/T**	**C**	**A**	**A**	**1**
coa14802 Quitman Mtns.	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	**A/C**	A	2
coa14803QuitmanMtns.804R	**ariz**	**AxC**	**G**	**G**	**T**	**A**	**C**	**T**	**T**	**A/T**	**C/G**	**A/C**	**A**	**3**
coa14804Quitman Mtns. 804R	ariz	coah	G	G	T	A	C	T	T	T	C	A	A	0
coa14805Quitman Mtns.	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	**A/C**	A	2
coa14806Quitman Mtns.	ariz	ariz	G	G	T	A	C	T	T	A	**C/G**	**A/C**	A	2
coa14807sofAlpine	coah	coah	G	G	del	**A/G**	**C/T**	**C/T**	**C/T**	T	C	A	A	4
coa14808sofAlpine	coah	coah	G	G	T	A	C	T	**C/T**	T	C	A	A	1
coa14810sofAlpine	coah	coah	G	G	del	A	**C/T**	**C/T**	T	T	C	A	A	2
coa14811sofAlpine	coah	coah	G	A	del	**A/G**	**C/T**	T	T	T	C	A	A	2
coa14812wofAlpine	coah	coah	G	G	del	**A/G**	**C/T**	T	**C/T**	T	C	A	A	3
coa14813wofAlpine 313R	coah	coah	G	G	T	A	**C/T**	T	**C/T**	T	C	A	A	2
coa14814wofAlpine	coah	coah	G	G	T	A	C	T	T	T	C	A	A	0
coa14815wofAlpine	coah	coah	G	G	del	**A/G**	**C/T**	T	**C/T**	T	C	A	A	3
coa14816wofAlpine	coah	coah	G	G	del	A	**C/T**	T	**C/T**	T	C	A	A	2
coa14817FtDavis 1000Y	coah	coah	G	G	T	A	C	T	**C/T**	T	C	A	A	1
coa14818FtDavis	coah	coah	G	G	del	A	T	T	T	T	C	A	A	0
coa14819FtDavis	coah	coah	G	G	del	G	T	T	T	T	C	A	A	0
coa14820FtDavis 689K	coah	coah	G	**A/G**	del	**A/G**	**C/T**	T	T	T	C	A	A	3
coa14821FtDavis 1100Y	coah	coah	G	G	del	**A/G**	T	T	T	T	C	A	A	1
coa14822sofMarfa	coah	coah	G	**A/G**	T	A	C	T	T	T	C	A	A	1
coa14823sofMarfa1100Y	coah	coah	G	G	T	A	C	T	T	T	C	A	A	0
coa14824sofMarfa	coah	coah	G	G	del	**A/G**	T	T	T	T	C	A	A	1
coa14825sofMarfa	**coah**	**AxC**	**G**	**A/G**	**T**	**A**	**C**	**T**	**T**	**A/T**	**C**	**A**	**A**	**2**
coa14826sofMarfaY1100	coah	coah	G	**A/G**	del	na	na	na	na	T	C	A	A	1
coa6829LaZarca	coah	coah	G	**A/G**	T	A	C	T	T	T	C	A	A	1
coa6830LaZarca	coah	coah	G	A	T	A	C	T	T	T	C	A	A	0
coa6831LaZarca	coah	coah	G	**A/G**	T	A	C	T	T	T	C	A	A	**1**
coa10241km45nDgo	coah	coah	G	G	T	A	C	T	T	T	**C/T**	A	A	**1**
coa10242km45nDgo,503Y	coah	coah	G	G	T	A	C	T	T	T	**C/T**	A	A	**1**
number of polymorphic			**5**	**6**	**3**	**7**	**8**	**5**	**10**	**11**	**24**	**4**	**3**	

Comparison of leaf essential oils of fastigiate (strict) and horizontal forms of *Cupressus sempervirens* from Cyprus, Montenegro, Turkey, and United States.

Robert P. Adams
Biology Department, Baylor University, Box 97388, Waco, TX
76798, USA,
Robert_Adams@baylor.edu

Tuğrul Mataraci
Eskidji Müz. AŞ., Sanayi Cad. Vadi Sokak No:2, Tarabya, İstanbul, Turkey

Salih Gücel
Environmental Research Institute, Near East University, North Nicosia, Cyprus

and

Jim A. Bartel
San Diego Botanic Garden, P. O. Box 230005, Encinitas, CA 92023

ABSTRACT

The volatile leaf oils of the horizontal form of *C. sempervirens* from natural populations in Cyprus and Turkey were very uniform and dominated by α-pinene (36.2, 26.0%), myrcene (2.4, 2.4%), δ-3-carene (18.3, 16.0%), terpinolene (3.2, 3.8%), α-terpinyl acetate (4.7, 3.5%), cedrol (4.4, 3.3%), manoyl oxide (0.7, 3.8%), iso-pimara-7,15-diene (0.4, 2.6%), isoabienol (2.4, 4.0%), and trans-totarol (1.5, 5.7%). Overall, the major terpenes compositions were very uniform for the sampled natural populations (Cyprus, Turkey) and fastigiate (strict) forms from California and Istanbul. But they were very variable for the oils from other fastigiate forms (Turkey and Montenegro). The fastigiate forms of *Cupressus sempervirens* from California and Istanbul (14674) have oils that are similar to natural populations. Variation in the composition of oils from cultivated fastigiate forms in Turkey and Montenegro suggests that these cultivars arose from multiple selections of fastigiate (strict) trees, rather than cloning and widespread cultivation. The volatile leaf oil composition does not support the recognition of the two growth forms of *C. sempervirens* as distinct taxa. Published on-line www.phytologia.org *Phytologia 99(1): 48-53 (Jan 19, 2017).* ISSN 030319430.

KEY WORDS: *Cupressus sempervirens, C. horizontalis, C. fastigiata,* terpenoids, geographic variation, taxonomy.

Cupressus sempervirens L. ranges naturally from the eastern Mediterranean, Crete, Cyprus, eastern Aegean Islands, Iran, Israel, Jordan, Lebanon, Syria, Turkey, and possibly Libya (Sękiewicz et al. 2016). The species has been widely cultivated within and outside its range throughout the warm temperate world (More and White 2002). Farjon (2005, 2010) noted that *C. sempervirens* has traditionally been separated into two "elements": pyramidal trees with horizontal branches (horizontal form) (= *C. horizontalis* Mill.) and fastigiate trees with strict branching (fastigiate form) (= *C. fastigiata* DC.). The fastigiate trees are often called Italian, cemetery, graveyard, and Tuscan cypress in the Old World, while in the New World, the widely cultivated fastigiate cultivars are called Italian and cemetery cypress. Farjon (2005) concluded that the fastigiate (strict) form of *C. sempervirens*, widely cultivated all over the Mediterranean and beyond, was selected many centuries ago from natural populations, which likely were largely horizontal.

The volatile leaf essential oils of *Cupressus sempervirens* (both horizontal and fastigiate forms) have been analyzed based mostly on locally cultivated fastigiate trees. The report by Ulukanli et al. (2014) is typical reporting the major components being: α-pinene (35.6%), trans-pinocarveol (5.22%), α-phellandrene-8-ol (4.56%), β-pinene (3.1%), limonene (2.8%), borneol 2.3%) and camphene (2.2%). Chanegriha, et al. (1977) reported on the leaf oils of *C. sempervirens* from Algeria (cv. *stricta*?) as having α-pinene (44.9%), δ-3-carene (10.6%), limonene (4.5%), terpinolene (2.7%), terpin-4-ol (1.9%), α-terpinyl acetate (12.0%) and manoyl acetate (1.5%). Floreani et al. (1981) reported the essential oil of cv. *stricta* (Argentina) contained α-pinene (50.1%), camphene (1.4%), β-pinene (4.1%), δ-3-carene (30.5%), limonene (3.5%), terpinolene (1.3%) and α-terpineol (1.6%). Other reports are by Adams et al. (1997), Amri et al. (2013), Pauly et al. (1983), Floreani et al. (1982), and Gamero et al. (1978)

This paper compares the volatile leaf oil of the horizontal form of *C. sempervirens* from natural populations in Cyprus and Turkey to that of cultivated fastigiate trees from Montenegro, Turkey, and California, USA.

MATERIALS AND METHODS

Plant materials:

***Cupressus sempervirens* L. (horizontal form):**

Cyprus: 35° 16' 34.58" N, 33° 23' 14.12" E, 361 m, 3 June 2015, Salih Gucel ns, Lab Acc. *Robert P. Adams 4560-14564,*

Turkey: pyramidal trees, branches horizontal,

Vicinity of Beskonak village, Serik, Antalya, 37° 17' N, 31° 18' E, elev. 180 m, 23 May 2015, Coll. Tuğrul *Mataraci, 2015-14,* Lab Acc: *Robert P. Adams 14565,*

In Köprülü Kanyon National Park, on the road of Ancient city of Selge, Beskonak village, Serik, Antalya, 37° 21' N, 31° 53' E, elev. 708 m, 23 May 2015, Coll. *Tuğrul Mataraci, 2015-15*, Lab. Acc: *Robert P. Adams 14566.*

In Köprülü Kanyon National Park, on the road of Ancient city of Selge, Beskonak village, Serik, Antalya, 37° 22' N, 31° 13' E, elev. 817 m, 23 May 2015, Coll. *Tuğrul Mataraci, 2015-16*, Lab. Acc: *Robert P. Adams 14567.*

In Köprülü Kanyon National Park, on the road of Ancient city of Selge, Beskonak village, Serik, Antalya, 37° 21' N, 31° 14' E, elev. 764 m, 23 May 2015, Coll. *Tuğrul Mataraci, 2015-17*, Lab. Acc: *Robert P. Adams 14568*

***Cupressus sempervirens* (fastigiate form):**

Montenegro:

fastigiate (strict), columnar tree in maquis, appearing natural but likely an escaped cultivar, Komunal Budva, Petrovac, between coasts of Lucica and Buljarica, forest rd, ca. 42° 12' N, 18° 57' E, 30 m, 24 Aug 2015, Coll. *Tuğrul Mataraci, 2015-28*, Lab Acc: *Robert P. Adams 14672*,

cultivated, strict, columnar trees, in the park, Komunal Budva, Petrovac, Sv, Stefab coast, 42° 12' N, 18° 57' E, 2 m, 24 Aug 2015, Coll. *Tuğrul Mataraci, 2015-29*, Lab Acc: *Robert P. Adams 14673.*

Turkey:

cultivated, Ayvalik- Town cemetery, Balikesir Province, living hedge around the cemetery; up to 20m tall, strict habit, 39° 17' N, 26° 41' E, ca. 50 m, 18 July 2015, Coll. *Tuğrul Mataraci, 2015-24*, Lab Acc: *Robert P. Adams 14597*,

cultivated in park, Istanbul, Beyoğlu-Halicioğlu jct., strict habit, 41° 29' N, 28° 56' E , 34 m, 12 Aug 2015, Coll. *Tuğrul Mataraci, 2015-25*, Lab Acc: *Robert P. Adams 14647*,

cultivated on the highway between Izmit-Kocaeli, strict habit, 40° 46' N, 29° 39' E, 26m, 16 Aug 2015, Coll. *Tuğrul Mataraci, 2015-26*, Lab Acc: *Robert P. Adams 14648*,

cultivated, Emirgan Park. Istanbul,strict, columnar trees, 41° 11' N, 29° 05' E, 84 m, 6 Sept 2015, Coll. *Tuğrul* Mataraci, 2015-30, Lab Acc: *Robert P. Adams 14674*,

United States:
cultivated, Carlsbad, CA, approx. 33° 06' 56.6" N, 117° 18' 39.3" W., 151 ft, 17 July 2015, San Diego Co., Coll. *Jim A. Bartel, 1631-1635*, Lab Acc. *Robert P. Adams 14591-14595*. Dates trees *1631-1635* planted: 1985, 2005, 2000, 1980, 2010,
All specimens are deposited in the BAYLU herbarium.

Isolation of Oils - Fresh leaves (200 g) were steam distilled for 2 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored at -20°C until analyzed. The extracted leaves were oven dried (100°C, 48 h) for determination of oil yields.

Chemical Analyses - The oils were analyzed on a HP5971 MSD mass spectrometer, scan time 1 sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see 5 for operating details). Identifications were made by library searches of our volatile oil library (Adams, 2007), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

RESULTS AND DISCUSSION

The volatile leaf oils of the horizontal form of *C. sempervirens* from natural populations in Cyprus and Turkey were very uniform and dominated (Table 1) by α-pinene (36.2, 26.0%), myrcene (2.4, 2.4%), δ-3-carene (18.3, 16.0%), terpinolene (3.2, 3.8%), α-terpinyl acetate (4.7, 3.5%), cedrol (4.4, 3.3%), manoyl oxide (0.7, 3.8%), iso-pimara-7,15-diene (0.4, 2.6%), isoabienol (2.4, 4.0%), and trans-totarol (1.5, 5.7%).

The oils compositions of samples of *C. sempervirens* cv. 'Glauca Stricta' from near San Diego, CA, USA proved to very uniform, suggesting that these are likely clones. The average values of the components show its oil to be quite similar to the horizontal form of *C. sempervirens* from natural populations from Cyprus and Turkey (Table 1.) In contrast, the oils of the fastigiate forms from Turkey and Montenegro were quite variable (Table 1). Interestingly the oils from a cultivated tree and the 'wild' (escaped cultivar?) fastigiate tree in Montenegro had quite different oils (Table 1).

Jacobson (1996) elaborated on the introduction and cultivation of *Cupressus sempervirens* cultivars into the United States. He notes the introduction of the Italian cypress (cv. 'Stricta') into North America is unknown, but George Washington planted one at Mt. Vernon in 1786. It seems very probable that Italian cypress was introduced into Mexico by the Spaniards much earlier, as it is universally planted at churches and cemeteries in Mexico. Jacobson (1996) lists the introductions of known cultivars as:
cv. 'Glauca Stricta' ≤ 1934; cv. 'Stricta', date uncertain; cv. 'Swane's Golden' ≤1977-78 by Swane Bros. Nursery, Australia; cv. 'Totem' ≤1992, ex Duncan & Davies nursery, NZ; cv. 'Variegata' ≤1930s likely from England ca. 1848. The commonly cultivated Italian cypress around San Diego, CA appears to be cv. 'Glauca Stricta.'

It is interesting that three components characteristic of cedarwood oil (α-cedrene, β-cedrene, cedrol) are present in the leaf oils from Cyprus, Turkey, 'Stricta' from California, and 14674 and 14647 from Turkey, but only a trace or absent from the other oils from fastigiate trees (Table 2). Overall, the major terpenes compositions are very uniform for the horizontal form from natural populations (Cyprus, Turkey) and cultivated fastigiate trees in California and Istanbul, but very variable (Table 2) for the other cultivated fastigiate tree oils (Turkey and Montenegro).

α-pinene varies from 19.9% to 65.7% among the *stricta* oils (Table 2). In fact, the *stricta* Turkey 14597 is most unusual in having a high concentration of α-pinene, but very low concentrations of δ-3-carene (0.2%), linalool (trace), α-cedrene (none), β-cedrene (0.1%), cedrol (trace) and abietadiene (trace).

Cultivated fastigiate *Cupressus sempervirens* trees from California and Istanbul (14674) both have oils that are very similar to from natural populations from Cyprus and Turkey (Tables 1, 2). Variation in the composition of oils from cultivated trees in Turkey and Montenegro suggests that these cultivars arose from multiple selections of fastigiate trees, rather than cloning and subsequent widespread cultivation. The volatile leaf oil composition does not support the recognition of the two growth forms of *C. sempervirens* as distinct taxa. Similarly Farjon (2010) considered that the cultivated fastigiate form was not a taxonomic variety but a cultigen.

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Table 1. Leaf essential oil compositions for *Cupressus sempervirens*. Compounds in bold show large differences between samples. Table abbreviations: horiz. = horizontal form, fast. = fastigiate form, Turk. = Turkey, Calif. = California, Istan. = Istanbul, Mont. = Montenegro. Cyprus 15030 is the average of 5 samples (14560-14564); Turkey 15031 is the average of 4 samples (14564-14568); California is the average of 5 samples (14591-14595). In these three cases, because little variation existed among the samples, average oils are presented. All the other samples (Table 1) were collected from individual trees. Mont. c 14673 is from a cultivated tree in Montenegro, whereas, Mont. ec 14672 is from an escaped cultivar (?) tree in Montenegro.

KI	compound	horiz. Cyprus 15030	horiz. Turk. 15031	fast. Calif. 15032	fast. Istan. 14674	fast. Turk. 14647	fast. Turk. 14648	fast. Turk. 14597	fast. Mont. c 14673	fast. Mont. ec 14672
921	tricyclene	0.01	0.1	0.1	0.1	t	0.1	0.1	t	0.1
924	α-thujene	0.02	0.1	0.1	0.5	0.1	t	t	0.4	1.8
932	**α-pinene**	**36.2**	**26.0**	**39.1**	**34.4**	**28.5**	**35.2**	**65.7**	**19.9**	**29.6**
945	α-fenchene	0.6	0.4	0.6	0.6	0.8	0.9	0.1	0.9	0.3
946	camphene	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.1	0.2
969	sabinene	0.5	0.6	0.7	3.4	0.4	0.4	1.0	1.2	3.6
974	β-pinene	1.2	1.1	1.1	1.1	1.1	0.9	1.9	1.3	1.4
988	**myrcene**	**2.4**	**2.4**	**2.2**	**2.4**	**2.3**	**2.3**	**2.6**	**2.7**	**3.9**
1002	α-phellandrene	t	t	t	t	t	t	t	t	t
1008	**δ-3-carene**	**18.3**	**16.0**	**16.8**	**17.3**	**30.1**	**25.7**	**0.2**	**30.7**	**12.2**
1014	α-terpinene	0.2	0.2	0.1	0.2	0.1	0.1	t	0.2	0.5
1020	p-cymene	0.2	0.2	t	0.1	0.1	t	t	t	0.5
1023	sylvestrene	0.2	0.2	0.2	0.2	0.3	0.3	t	0.4	t
1024	limonene	1.4	1.2	2.2	1.0	1.0	0.8	1.4	1.7	2.3
1025	β-phellandrene	0.9	1.2	1.5	0.9	1.0	0.7	1.3	1.7	2.4
1044	(E)-β-ocimene	t	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
1054	γ-terpinene	0.4	0.3	0.3	0.4	0.2	0.3	0.2	0.4	1.3
1067	linalool oxide	t	0.1	t	t	t	t	t	t	0.1
1082	m-cymenene	t	t	t	t	t	t	t	t	t
1086	**terpinolene**	**3.2**	**3.8**	**4.1**	**3.2**	**3.2**	**4.4**	**1.4**	**4.8**	**1.3**
1099	**linalool**	**1.5**	**0.6**	**0.2**	**0.3**	**0.6**	**t**	**t**	**0.4**	**1.1**
1122	methyl octanoate	t	t	t	t	t	0.1	t	t	t
1123	α-camphenal	0.3	0.1	t	t	0.1	t	t	t	0.1
1133	cis-p-mentha-2,8-dien-1-ol	0.2	t	t	t	0.1	0.1	t	t	t
1135	trans-pinocarveol	0.2	t	t	t	t	0.1	t	t	t
1141	camphor	0.2	t	t	t	0.1	0.1	t	t	t
1154	karahanaenone	0.8	0.1	t	t	t	t	t	t	t
1159	p-mentha-1,5-diene-8-ol, isomer	0.3	0.1	t	t	0.2	0.1	t	t	t
1160	pinocarvone	0.2	t	t	t	t	t	t	t	t
1166	p-mentha-1,5-diene-8-ol	t	t	t	t	t	t	t	t	t
1067	umbellulone	0.1	0.3	t	t	t	t	t	t	t
1174	terpinen-4-ol	1.6	1.3	0.7	0.6	0.6	0.3	0.2	0.5	1.4
1176	m-cymen-8-ol	0.1	0.5	t	0.2	0.1	t	0.2	0.2	0.2
1179	p-cymen-8-ol	0.2	0.1	t	t	0.1	t	t	t	t
1186	α-terpineol	0.3	0.2	0.2	t	0.2	0.1	t	0.1	0.1
1204	myrtenol	t	0.2	t	t	t	t	t	t	t
1204	verbenone	0.3	0.1	t	t	0.2	0.1	t	t	t
1241	carvacrol, methyl ether	t	0.2	0.1	0.1	0.2	1.0	t	0.4	0.5
1254	linalool acetate	t	t	t	t	t	t	t	t	t
1287	bornyl acetate	0.2	0.2	0.1	0.4	0.2	0.1	t	0.8	1.5
1315	<2E,4E->decadienal	t	0.5	0.1	t	t	t	t	t	t
1323	methyl decanoate	t	t	t	t	t	t	t	t	t
1334	linalool propionate	0.6	0.7	0.4	0.4	1.3	0.7	t	1.0	0.4
1346	**α-terpinyl acetate**	**4.7**	**3.5**	**2.0**	**1.5**	**4.4**	**2.8**	**1.1**	**2.7**	**2.4**
1345	α-cubebene	t	t	t	t	t	t	t	t	t
1374	α-ylangene	t	t	t	t	t	t	t	t	t
1400	tetradecane	t	0.1	t	t	t	0.1	t	t	0.1
1410	**α-cedrene**	**0.3**	**0.1**	**0.1**	**0.1**	**t**	**-**	**-**	**t**	**-**
1411	2-epi-β-funebrene	t	0.1	0.1	0.1	t	-	-	t	-
1417	(E)-caryophyllene	0.1	0.2	0.1	0.2	0.3	t	0.1	0.4	0.8
1419	**β-cedrene**	**0.3**	**0.3**	**0.1**	**0.2**	**0.3**	**t**	**0.1**	**0.4**	**t**

KI	compound	horiz. Cyprus 15030	horiz. Turk. 15031	fast. Calif. 15032	fast. Istan. 14674	fast. Turk. 14647	fast. Turk. 14648	fast. Turk. 14597	fast. Mont c 14673	fast. Mont w 14672
1448	cis-muurola-3,5-diene	0.3	0.3	0.1	0.1	t	0.6	0.2	0.4	0.3
1452	α-humulene	0.2	0.5	0.1	0.2	0.3	t	0.2	0.3	0.4
1465	cis-muurola-4(14),5-diene	0.8	0.7	0.2	0.3	0.2	1.5	0.5	0.8	0.9
1478	γ-muurolene	0.2	0.1	t	t	0.1	t	0.2	0.2	0.5
1480	germacrene D	2.1	2.6	0.7	4.1	1.2	0.6	3.5	3.4	3.4
1499	epi-zonarene	0.2	0.2	t	t	t	0.6	t	0.2	0.3
1500	α-muurolene	0.1	0.1	t	t	t	t	0.3	0.1	0.1
1513	γ-cadinene	0.1	t	t	t	t	t	t	0.1	0.2
1521	trans-calamenene	0.3	0.2	t	0.1	0.1	0.3	0.2	0.2	0.4
1522	δ-cadinene	0.3	0.2	t	0.1	0.2	0.2	0.2	0.2	0.3
1600	**cedrol**	**4.4**	**3.1**	**4.5**	**6.2**	**1.6**	**-**	**t**	**t**	**0.1**
1652	α-cadinol	0.6	0.7	0.2	0.4	0.7	1.3	1.3	0.8	1.0
1685	germacra-4(15),5,10(14)-trien-1-al	0.2	0.3	0.1	0.1	1.0	0.3	0.3	0.4	0.5
1921	methyl hexadecanoate	0.2	0.2	t	t	t	t	t	t	0.1
1958	iso-pimara-8(14),15-diene	0.5	0.7	0.5	0.4	0.6	1.2	0.4	1.5	0.8
1987	**manoyl oxide**	**0.7**	**3.8**	**8.5**	**0.2**	**1.3**	**0.7**	**2.0**	**1.6**	**2.2**
1987	**iso-pimara-7,15-diene**	**0.4**	**2.6**	**1.7**	**0.2**	**1.4**	**0.4**	**1.3**	**1.5**	**1.5**
2055	abietatriene	1.5	3.4	1.4	0.5	0.9	1.6	2.5	1.2	1.1
2087	**abietadiene**	**0.6**	**t**	**0.1**	**3.0**	**t**	**5.4**	**t**	**4.2**	**t**
2103	6-octadecanoic acid, methyl ester	0.4	t	t	t	t	0.2	t	t	0.5
2105	**isoabienol**	**2.4**	**4.0**	**1.7**	**1.4**	**0.9**	**0.9**	**1.2**	**2.2**	**4.7**
2149	abienol	0.4	1.3	1.0	3.2	0.4	0.8	0.2	1.1	0.9
2269	sandaracopimarinol	-	0.2	0.1	0.2	t	0.2	t	t	0.1
2282	sempervirol	t	0.4	0.1	t	t	t	t	t	0.1
2314	**trans-totarol**	**1.5**	**5.7**	**3.1**	**5.5**	**1.9**	**1.4**	**0.8**	**3.8**	**4.2**
2331	trans-ferruginol	0.2	0.7	0.4	0.7	0.4	0.2.	t	0.5	0.6

KI = linear Kovats Index on DB-5 column. Compositional values less than 0.1% are denoted as traces (t). Unidentified components less than 0.5% are not reported.

Table 2. Comparison of the leaf oil compositions for the most variable compounds among samples.

KI	compound	horiz. Cyprus 15030	horiz. Turk. 15031	fast. Calif. 15032	fast. Istan. 14674	fast. Turk. 14647	fast. Turk. 14648	fast. Turk. 14597	fast. Mont c 14673	fast. Mont w 14672
932	**α-pinene**	**36.2**	**26.0**	**39.1**	**34.4**	**28.5**	**35.2**	**65.7**	**19.9**	**29.6**
1008	**δ-3-carene**	**18.3**	**16.0**	**16.8**	**17.3**	**30.1**	**25.7**	**0.2**	**30.7**	**12.2**
1086	**terpinolene**	**3.2**	**3.8**	**4.1**	**3.2**	**3.2**	**4.4**	**1.4**	**4.8**	**1.3**
1099	**linalool**	**1.5**	**0.6**	**0.2**	**0.3**	**0.6**	**t**	**t**	**0.4**	**1.1**
1410	**α-cedrene**	**0.3**	**0.1**	**0.1**	**0.1**	**t**	**-**	**-**	**t**	**-**
1419	**β-cedrene**	**0.3**	**0.3**	**0.1**	**0.2**	**0.3**	**t**	**0.1**	**0.4**	**t**
1600	**cedrol**	**4.4**	**3.1**	**4.5**	**6.2**	**1.6**	**-**	**t**	**t**	**0.1**
1987	**manoyl oxide**	**0.7**	**3.8**	**5.2**	**0.2**	**1.3**	**0.7**	**2.0**	**1.6**	**2.2**
1987	**iso-pimara-7,15-diene**	**0.4**	**2.6**	**5.2**	**0.2**	**1.4**	**0.4**	**1.3**	**1.5**	**1.5**
2087	**abietadiene**	**0.6**	**t**	**0.1**	**3.0**	**t**	**5.4**	**t**	**4.2**	**t**
2105	**isoabienol**	**2.4**	**4.0**	**1.7**	**1.4**	**0.9**	**0.9**	**1.2**	**2.2**	**4.7**
2314	**trans-totarol**	**1.5**	**5.7**	**3.1**	**5.5**	**1.9**	**1.4**	**0.8**	**3.8**	**4.2**

Survey of cotton (*Gossypium* sp.) for non-polar, extractable hydrocarbons for use as petrochemicals and liquid fuels

Robert P. Adams and **Amy K. TeBeest**
Baylor-Gruver Lab, Baylor University, 112 Main Ave., Gruver, TX 79040
robert_Adams@baylor.edu

James Frelichowski, Lori L. Hinze and **Richard G. Percy**
USDA-ARS, PA, SPARC, Crop Germplasm Research, 2881 F&B Road,
College Station, TX 77845

and

Mauricio Ulloa and **John Burke**
USDA-ARS, PA, CSRL, Plant Stress and Germplasm Development Research,
3810 4th Street, Lubbock, TX 79415

ABSTRACT

An ontogenetic study of a commercial cotton cultivar (FiberMax 1320), grown dryland, revealed that the dry weight (DW) of leaves reached a maximum at the 1st flower stage, and then declined as bolls opened. However, % pentane soluble hydrocarbon (HC) yield continued to increase throughout the growing season (due to the decline of leaf DW). It seems likely that as the bolls mature and seed are filled, carbohydrates from the leaves are catabolized and sugars are transported to the bolls for utilization. Per plant HC yields increased from square bud stage to 1st flower, remained constant until 1st boll set, then declined at 1st boll-opened stage. This seems to imply that most of the HC are not catabolized and converted to useable metabolites for filling bolls and seeds. A survey of arid land cotton accessions, grown under limited irrigation or similar to dryland at Lubbock, TX, found % HC yield ranged from a low of 2.88% to highs of 5.78 and 5.54% . Per plant HC yields ranged from 0.017 to 0.043 g/ g leaf DW . Correlation between % HC yield and avg. leaf DW was non-significant (-0.103). A survey of USDA germplasm cotton accessions, grown with supplemental underground drip irrigation to achieve best yields germinated by irrigation, thence grown dryland at College Station, TX, found % HC yields were very high, with four accessions yielding 11.34, 12.32, 13.23 and 13.73%. Per plant HC yields varied from 0.023 to 0.172 g/ g leaf DW. Hopi had a high % HC yield (10.03%), but it was the lowest per plant yield (0.023 g/ g leaf DW). In contrast, China 86-1 with the second highest % HC yield (13.23%) was the highest per plant yield (0.172 g). The correlation between % HC yield and avg. leaf DW was non-significant (0.092). Thus, as seen in the arid land accessions, it appears that one might breed for both % HC yield and leaf DW in cotton. Published on-line www.phytologia.org *Phytologia 99(1): 54-61 (Jan 19, 2017)*. ISSN 030319430.

KEY WORDS: Cotton, *Gossypium* sp., yields of pentane extractable leaf hydrocarbons, petrochemicals, liquid fuels.

There is a revived interest in sustainable, renewable sources of petrochemicals and fuels from arid and semi-arid land crops with the uncertainty of sustained crude oil production in the world. Adams et al. (1986) screened 614 taxa from the western US for their hexane soluble hydrocarbon (HC) and resin (methanol soluble) yields. They found the highest HC yielding species were from arid and semi-arid lands in the Asteraceae (11 species), Asclepiadaceae (1), Celastraceae (1), Clusiaceae (1) and Euphorbiaceae (1). The top 2% (12/614) had whole plant HC yields ranging from 10.4 to 16.4%.

Recently, Adams et al. (2017) surveyed native and cultivated sunflowers for their yields of leaf HC for use as a potential semi-arid land crop and found high yielding (pentane extractable HC) plants. The top 2% had HC yields (ex leaves) ranging from 10.9 to 12.6% (Fig. 1), with the top 5% ranging from 8.7% to 12.6%.

A preliminary analysis of the leaf HC yields from six locally cultivated cotton plants found a HC yield of 7.94% in one plant. In comparison, HC yields from our locally cultivated commercial sunflowers ranged from 2.75 to 3.85%, as we expected, in a crop that has been extensively selected for seed production that leads to an inadvertent selection against the production of protective phytochemicals in the leaves.

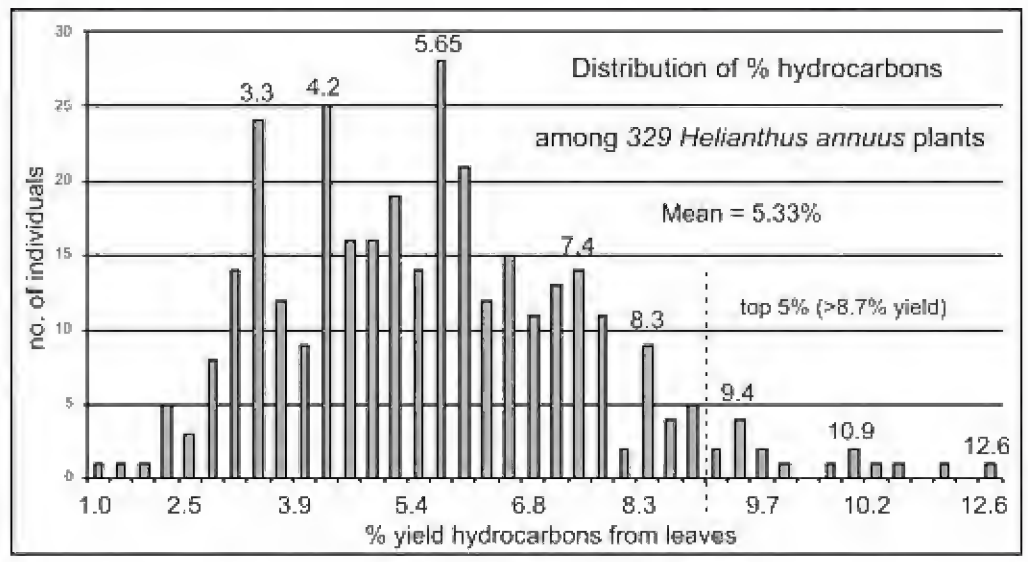

Figure 1. Frequency distribution of HC yields for 329 *H. annuus* plants (from Adams et al., 2017).

A comparison between sunflowers and cotton characteristics shows considerable differences:

characteristics	sunflowers (commercial)	cotton (commercial)
annual/ perennial	annual	perennial (but grown as an annual)
habit	herbaceous	woody
flowering	natural, 1 flower head/plant (natural: many heads/plant)	induced by growth regulators or drought, many flowers/plant (depending on photoperiod)
leaf life	lower leaves yellow and die	generally defoliate for harvest
natural habitat	temperate, North America	dry tropics (or dry sub-tropics)
origin	from *H. annuus*, North America	complex genetics from taxa from around the world (Wendel and Grover, 2015).

Annual sunflowers, herbaceous plants, live only to reproduce (by seed), whereas cotton, a woody, perennial, having evolved with a dry season to induce flowering and seed formation, has adapted to a long lifetime, in which annual seed production is not critical for short-term survival. However, maintaining plant defensive chemicals and storing energy metabolites for cotton to survive the dry season are important.

The evolution of modern cotton (*Gossypium* sp.) encompasses an improbable series of events that involved transoceanic, long-distance dispersal with hybridization involving two diploids, one from the Old World and one from the New World, forming the modern cultivated allo-tetraploid, *G. hirsutum* (the reader is urged to read the informative account by Wendel and Grover, 2015).

Although there are several papers on the conversion of cotton field stubble to liquid fuels (see Putun, 2010; Putuan et al., 2006; Akhtar and Amin, 2011 and references therein), there appear to be no surveys of the yields of non-polar HC extractables in cotton.

As a part of our research on the investigation of contemporary crops for alternative, renewable sources of petrochemical feedstocks and fuels, the present paper reports on the yields of HC from cotton (*Gossypium* sp.) cultivars and accessions.

MATERIALS AND METHODS

Plant Materials:
Ontogenetic variation in HC yields study:
Commercial, cultivated cotton - FiberMax 1320, dryland, dark, loam soil, JP TeBeest Farm, 36° 25' 0.6" N, 101° 32' 17.3" W, 3258 ft., Oslo, TX, avg. annual rainfall, 19.3". The eight (8) lowest growing, non-yellowed mature leaves were collected at random from each of 10 cotton plants, at square bud, 1st open flower, 1st boll, and 1st boll completely opened stages. The leaves were air dried in paper bags at 49° C in a plant dryer for 24 hr or until 7% moisture was attained.
HC yields of 30 cotton accessions representing photoperiodic and non-photoperiodic forms of two species:
Cultivated at the USDA-ARS Southern Plains Agricultural Research Center, College Station, TX, 30 37' 5.00" N, 96 21' 50" W, 354 ft., subsurface drip irrigation, sandy soil, annual rainfall 40". The lowest growing, non-yellowed, mature leaf was collected at random, from each of 4-5cotton plants and bulked for an accession sample. Different accessions varied in growth stage from square bud, 1st flower, and 1st boll as the accessions were being grown for seed production. These accessions represent both photoperiodic and non-photoperiodic types as well as obsolete cultivars within the two commercial tetraploid cotton species, *G. hirsutum* and *G. barbadense*. These accessions were collected worldwide and are maintained by the USDA National Cotton Germplasm Collection.
HC yields 21 cotton accessions grown for drought testing:
Cultivated at the USDA-ARS Plant Stress and Germplasm Development Research Center, Lubbock, TX, 33 35' 36.3" N, 101 54' 4.2" W, 3243 ft., light, sandy soil, avg. annual rainfall 19.2". The lowest growing, non-yellowed, mature leaf was collected at random, from each of 10 cotton plants and bulked for an accession sample. Different accessions varied in growth stage from square bud to 1st flower. Some supplemental water was applied during the growing season to attain germination and limited growth to reflect plant stress responses, similar to dryland production, otherwise the plants were watered only by natural rainfall. These accessions represent a diverse pool of *G. hirsutum* germplasm with different genetic backgrounds from the USDA National Cotton Germplasm Collection.

Leaves were ground in a coffee mill (1mm). 3 g of air dried material (7% moisture) was placed in a 125 ml, screw cap jar with 20 ml pentane, the jar sealed, then placed on an orbital shaker for 18 hr. The pentane soluble extract was decanted through a Whatman paper filter into a pre-weighed aluminum pan and the pentane evaporated on a hot plate (50°C) in a hood. The pan with hydrocarbon extract was weighed and tared.

The shaker-pentane extracted HC yields are not as efficient as soxhlet extraction, but much faster to accomplish. To correct the pentane yields to soxhlet yields, one sample was extracted in triplicate by soxhlet with pentane for 8 hrs. All shaker extraction yields were corrected to oven dry wt. (ODW) multiplication of 1.085. For the cultivated TeBeest cotton, the shaker yields were corrected by the increased soxhlet extraction efficiency (CF = x1.56). For the arid land accessions, the soxhlet CF was x1.31 and for the accessions grown at College Station, the soxhlet CF was x1.69.

RESULTS

Ontogenetic variation in HC yields in FiberMax 1320, grown dryland, are given in Table 1. Notice (Fig. 2) that the DW of 8 leaves (lvs) (from each plant) reach a maximum at the 1st flower stage, and then declined. However, % HC yield continued to increase throughout the growing season (due to the decline of leaf DW). It seems likely that as the bolls mature and seed are filled, carbohydrates from the leaves are metabolized into sugars that are transported to the bolls for utilization. Non-polar hydrocarbons such as waxes, terpene hydrocarbons, alkanes, alkenes, etc. are thought to be largely inert

and not subject to catabolism. Notice that non-polar hydrocarbons (HC, as g DW/ 8 leaves) increased from square bud stage to 1st flower, remained constant until 1st boll-set, then declined at 1st boll-opened stage (Fig. 2). This seems to imply that most (~80% 0.355 g/0.440 g, Table 1) of the HC are not catabolized and converted to sugars or other metabolites that might be utilized for during the maturation of the bolls and seeds. Approximately ~80% of the non-polar hydrocarbons remain in the leaves (at least through the boll-opening stage (additional research is in planned to further examine the fate of non-polar HC).

Table 1. Ontogenetic variation in pentane soluble hydrocarbon (HC) yields in FiberMax 1320, grown dryland using eight leaves (lvs) per plants and dry weight (DW) of leaves.

collection growth stage	DW for 8 lvs/plant, std err. mean	% HC yield, std err. mean	Range of yields(%)	HC g/ 8 lvs DW, std err. mean
14949 Cotton, cult Oslo, square bud stage	5.49 g, 0.32	4.05%, 0.15	(3.31 - 4.56)	0.222 g, 0.016
14949 Cotton, cult Oslo, 1st flower stage	7.46 g, 0.34	6.05%, 0.35	(4.78 - 7.84)	0.451 g, 0.053
14949 Cotton, cult Oslo, 1st boll set	6.29 g, 0.36	6.99%, 0.31	(4.95 - 8.28)	0.440 g, 0.034
14949 Cotton, cult Oslo, 1st boll open, seeds maturing	4.43 g, 0.286	8.02%, 0.25	(6.65 - 8.90)	0.355 g, 0.027

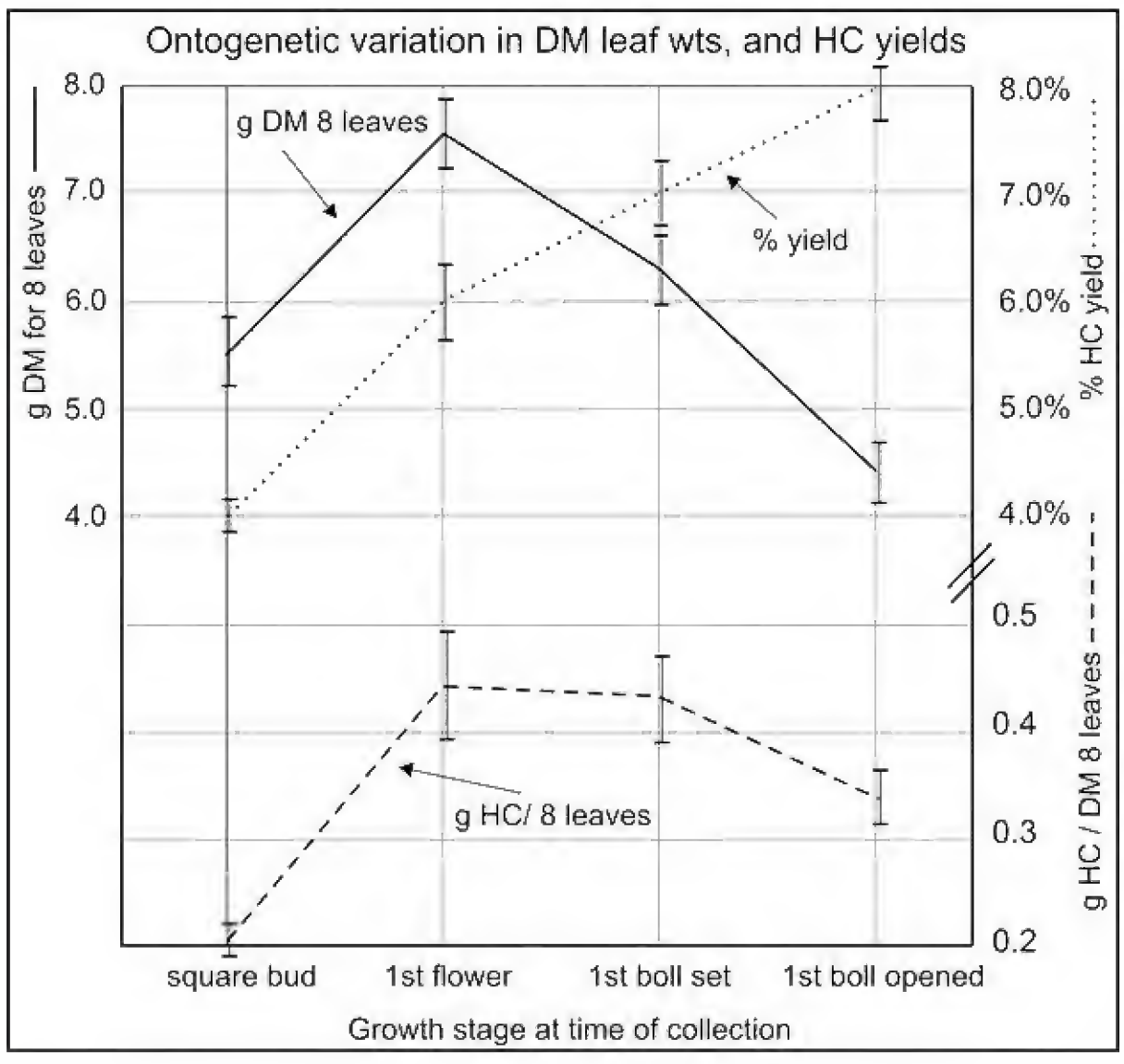

Figure 2. Ontogenetic variation in HC yields (as % HC yield and g HC/g dry leaves) in FiberMax 1320.

The survey of arid land cotton accessions growing dryland at Lubbock, TX revealed (Table 2) that % HC yield ranged from a low of 2.88% (14972, 16TXLWSA057) to highs of 5.78% (14961,

16TXLWSA039) and 5.54% (14964, 16TXLWSA043). Yields based on g HC/ g leaf DW ranged from 0.017 (14971, 16TXLWSA056) to 0.043 (14961, 16TXLWSA039 and 14965, 16TXLWSA047).

The correlation between % HC yield and avg. leaf DW was non-significant (r = -0.103). Thus, one might be able to breed for increases (up to some point) in both % HC yield and leaf DW in the same genotype.

Table 2. Cotton screening for leaf HC of arid land accessions at USDA, Lubbock, TX.

Lab # , Plot No.	USDA identifier	g avg leaf DW	% yield HC*	g HC yield/ g leaf DW
14954 L1, 16TXLWSA002	DP1212	0.579	4.87	0.028
14955 L2 , 16TXLWSA012	SA-0464	0.542	5.30	0.029
14956 L3 , 16TXLWSA015	SA-0476	0.733	4.54	0.033
14957 L4 , 16TXLWSA016	SA-1049	0.600	3.83	0.023
14958 L5 , 16TXLWSA021	SA-1598	0.530	4.83	0.026
14959 L6 , 16TXLWSA029	STV5458	0.570	4.35	0.025
14960 L7 , 16TXLWSA036	SA-0473	0.493	3.69	0.018
14961 L8 , 16TXLWSA039	SA-1484	0.737	5.78 Hi 1	0.043 Hi
14962 L9 , 16TXLWSA041	SA-1269	0.627	4.08	0.027
14963 L10 , 16TXLWSA042	SA-1555	0.635	3.55	0.023
14964 L11 , 16TXLWSA043	SA-3128	0.632	5.54 Hi 2	0.035
14965 L12 , 16TXLWSA047	SA-2289	0.852	5.06	0.043 Hi
14966 L13 , 16TXLWSA049	FM2011	0.781	3.31 Lo	0.026
14967 L14 , 16TXLWSA050	PHY72	0.627	4.20	0.026
14968 L15 , 16TXLWSA052	SA-1762	0.836	3.49	0.029
14969 L16 , 16TXLWSA053	SA-1759	0.647	3.73	0.024
14970 L17 , 16TXLWSA055	SA-0429	0.682	3.88	0.026
14971 L18 , 16TXLWSA056	STV474	0.471	3.55	0.017 Lo
14972 L19 , 16TXLWSA057	PHY375	0.917	2.88 Lo	0.026
14973 L20 , 16TXLWSA059	SA-2169	0.773	4.59	0.035
14974 L21 , 16TXLWSA062	SA-1599	0.893	4.34	0.039
14975 L22 , Pima,	SJ-FR05	1.018	2.78	0.028
r (leaf wt, % yield) = -0.103 ns				

The survey of USDA germplasm cotton accessions grown with supplemental irrigation at College Station, TX, found % HC yields were very high, with four accessions yielding 11.34, 12.32, 13.23 and 13.73% (Table 3). These HC yields are in the top 2% reported by Adams et al. (1986) and top 1% for sunflowers (Fig. 1, Adams et al. 2017).

Per plant HC yields (g HC/ g leaf DW) varied from 0.023 g to 0.172 g, a 7-fold range (Table 3). Hopi (14992) had a high % HC yield (10.03%), but it was the lowest per plant HC yield (0.023 g/ plant). In contrast, China 86-1 (14997) with the second highest % HC yield (13.23%), had the highest per plant HC yield (0.172, Table 3). The correlation between % HC yield and avg. leaf DW was non-significant (r = 0.092 ns). Thus, as seen in the arid land accessions, it appears that one might breed (up to some maximum point) for both % HC yield and leaf DW in cotton. This seems counter intuitive, but it may be that cotton, being a perennial, and closely related to wild plants, may use the leaf hydrocarbons for plant defensive chemicals. If so, there may be an evolutionary advantage to fully protect plants with large leaves as well as those with small leaves. At this survey stage, we have not examined the amount of gossypol (a known defense chemical).

Table 3. Cotton screening for leaf HC at USDA germplasm center, College Station, TX.
For % yield HC: + = 10.01 - 11.00%; ++ = 11.01 - 13.73%.
For g HC yield/leaf DW: + = 0.110 - 0.137g (top 13%); ++ = 0138 - 0.172g (top 3%).

Lab acc Source	USDA identifier	g avg leaf DW (# plants)	% yield HC	g HC yield/ g leaf DW
14983, U1, Tanguisw LMW 12-40	GB-0085	1.335 (4)	5.97	0.080
14984, U2, Mono 57	GB-0204	1.360 (4)	7.37	0.100
14985, U3, Nevis 81	GB-0227	0.728 (4)	10.36 +	0.041
14986, U4, Ashmouni Giza 32	GB-0230	1.128 (4)	7.37	0.083
14987, U5, Ashabad 1615	GB-0790	0.866 (4)	7.01	0.061
14988, U6, Tadla 2	GB-1439	1.106 (4)	9.70	0.107
14989, U7, 3-79	na	0.720 (4)	7.06	0.051
14990, U8, Pima S-5	SA-1497	0.995 (4)	7.92	0.079
14991, U9, TAM 87N-5	SA-1710	0.764 (4)	6.64	0.051
14992, U10, Hopi	SA-0033	0.266 (4)	10.03 +	0.023 Low
14993, U11, Mexican #68	SA-0815	0.994 (4)	7.92	0.079
14994, U12, Christidis 53D7	SA-1166	0.706 (4)	13.73 ++Hi	0.097
14995, U13, Acala SJ-1	SA-1181	0.962 (4)	12.32 ++	0.119 +
14996, U14, 3010	SA-1403	1.463 (4)	9.08	0.133 +
14997, U15, China 86-1	SA-1419	1.300 (4)	13.23 ++	0.172 ++Hi
14998, U16, TM 1	SA-2269	1.244 (4)	11.09 ++	0.138 +
14999, U17, KL 85/335	SA-2589	0.812 (4)	10.25 +	0.083
15000, U18, KLM-2026	SA-2597	0.802 (4)	9.02	0.072
15001, U19,TAM 91C-34	SA-2910	1.006 (4)	10.85 +	0.109
15002,U20,Vir-7080Col.Macias17	SA-3348	0.896 (4)	11.34 ++	0.102
15003, U21,Palmeri, wild	TX-0005	0.398 (5)	7.92	0.032
15004, U22,Latifolium, wild	TX-0100	0.894 (5)	10.72 +	0.096
15005, U23,Latifolium, wild	TX-0104	0.967 (5)	9.25	0.089
15006, U24,Punctatum, wild	TX-0114	0.815 (5)	6.33	0.052
15007, U25,Morrili, wild	TX-0130	0.830 (5)	8.67	0.072
15008, U26,Marie-galante, wild	TX-0367	1.289 (5)	7.37	0.095
15009, U27,Richmondi, wild	TX-0462	0.973 (5)	9.93	0.097
15010, U28,Marie-galante, wild	TX-0866	0.511 (5)	8.05	0.041
15011, U29,Marie-galante, wild	TX-0878	0.692 (5)	4.50	0.031
15012, U30,Yucantanense, wild	TX-1046	0.728 (5)	3.29 Low	0.024 Low
r (leaf wt, % yield) = 0.092 ns				

Principal Coordinate Analysis (PCoA), utilizing 597 SSR bands, of the 30 accessions revealed the accessions are divided into *G. barbadense* and *G. hirsutum* (Fig. 3, left and right) (see Hinze et al., 2016 for further details on molecular marker analysis). The *G. barbadense* samples (8) are all improved accessions. The samples of *G. hirsutum* contain both wild and improved accessions forming a very loose group, but the wild accessions are mostly found in the upper-right quadrant of the ordination (Fig. 3).

Utilizing the g HC/ g leaf DW data, the above average HC yielding accessions are clearly clustered in a tightly grouped set of improved accessions (Fig. 3, dashed oval). Plotting the high and highest yielding samples revealed that all three of the high yielding samples (SA-1181, SA-1403, SA-2269, top 13%) and the highest yielding individual (SA-1419, top 3%) are found in that group (Fig. 3,

dashed oval). The discovery of the highest yielding individuals in a group of improved accessions is surprising, in view of the selection for increased cotton seed and fiber yields.

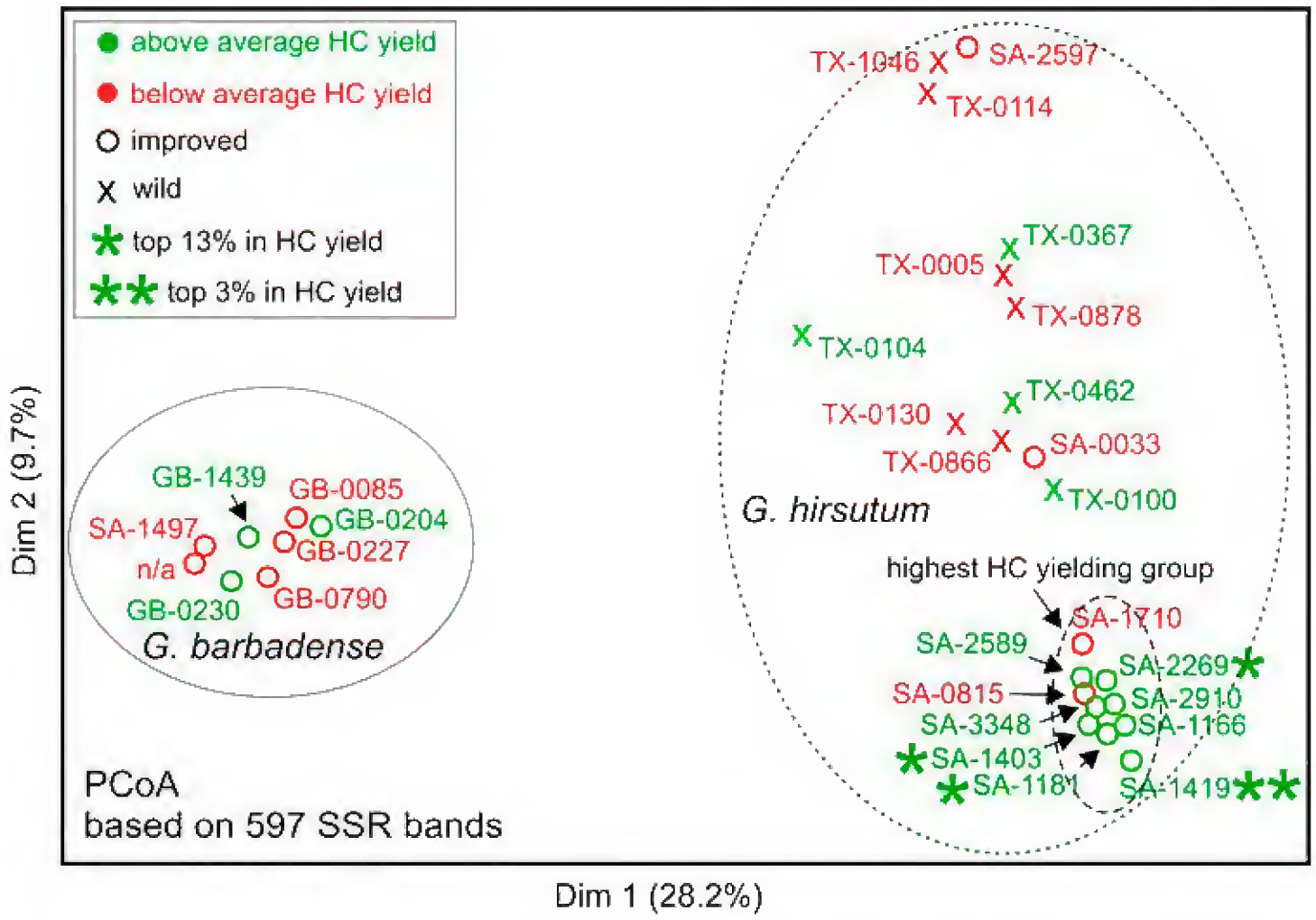

Figure 3. Principal Coordinate Analysis (PCoA) based on 597 SSR bands. The percent of variance accounted for among accessions is given on Dim 1 and Dim 2. See text for discussion.

It is also surprising that none of the wild accessions had high yields, although TX-0100 had a high % yield (10.72%), but having smaller leaves resulted in a moderate total g HC/ g leaf DW yield (Table 3). It is interesting that genetically (by SSR data), TX-0100 is ordinated nearest of any other wild accessions to the high HC yielding group (Fig. 3). It may be that back-crossing TX-0100 with SA-1419 might produce some useful progeny in the future.

CONCLUSION

By the very definition of 'survey', this report is preliminary. Nevertheless, it seems remarkable that a commercial crop, that has been bred and selected for seed (and lint) production, would sequester such high amounts of hydrocarbons in leaves, as found in many cotton accessions. These results raise many evolutionary questions, as well as numerous practical questions such as: Are the HC yields heritable? Are they environmentally induced? Can breeding increase these HC levels without detrimental effects on growth and hardiness? Clearly, much more research is needed (in progress).

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DNA sequencing and taxonomy of unusual serrate *Juniperus* from Mexico: Chloroplast capture and incomplete lineage sorting in *J. coahuilensis* and allied taxa

Robert P. Adams
Biology Department, Baylor University, Box 97388, Waco, TX 76798, USA, Robert_Adams@baylor.edu

M. Socorro Gonzalez-Elizondo, Martha Gonzalez-Elizondo, David Ramirez Noya
CIIDIR Unidad Durango, Instituto Politecnico Nacional,
Sigma 119, Durango, Dgo., 34234 Mexico
and

Andrea E. Schwarzbach
Department of Health and Biomedical Sciences, University of Texas - Rio Grande Valley,
Brownsville, TX 78520, USA.

ABSTRACT

Analysis of nrDNA, petN-psbM, trnS-trnG, trmD-trnT, and trnF-trnL of *Juniperus coahuilensis* and allied taxa of Mexico found typical *J. coahuilensis*, as well as individuals with: *coahuilensis* cp and hybrid ITS; *coahuilensis* cp and novel ITS sequence (La Parrilla type); novel Blue Fruited cp (blue fruited taxon) and *coahuilensis* ITS; plus Blue Fruited cp and La Parrila ITS. nrDNA data was examined and found to detect hybridization, chloroplast capture and incomplete lineage sorting. In addition, a new taxon was found with Blue Fruited (Blue Fruited) cp and *J. martinezii* ITS, suggestive of chloroplast capture. New records of *J. saltillensis* were confirmed from Zacatecas. A new record of *J. martinezii* from Durango was also confirmed. Several plants affiliated with either *J. martinezii*, or *J. flaccida* were in distinct clades showing the need for additional research on their volatile leaf oils, morphology and ecology to address their taxonomic status. And lastly, a very unusual population of junipers large, single stemmed trees with aff. *J. poblana* was found in Nayarit, with long and pendulous foliage. Analysis of the leaf volatile oils, ecology and morphology of this taxon is necessary (in progress) to ascertain its taxonomic rank. Published on-line www.phytologia.org *Phytologia 99(1): 62-73 (Jan 19, 2017).* ISSN 030319430.

KEY WORDS: *Juniperus coahuilensis, J. flaccida, J. martinezii, J. poblana*, Cupressaceae, hybridization, introgression, incomplete lineage sorting, nrDNA polymorphisms, petN-psbM DNA.

As a part of on-going research on *Juniperus*, recently, Adams (2016) found (by petN-psbM sequencing) that *Juniperus arizonica*, previously known only from Arizona and New Mexico, occurs in northern Sonora and Chihuahua, trans-Pecos Texas in the Franklin Mtns., Hueco Mtns., Hueco Tanks State Park, Quitman Mtns., Eagle Mtns. and Sierra Vieja Mtns., primarily on igneous material. These trans-Pecos juniper populations have previously been identified as *J. coahuilensis*.

Additional examination of populations of *J. coahuilensis* in the Trans-Pecos, Texas region (Adams 2017) revealed that situation was more complex with a relatively sharp demarcation between *J. arizonica* and *J. coahuilensis* (Fig. 1). The zone of contact and likely hybridization is in Hueco Tanks State Park, Quitman Mtns., and Anima Mtns. and this appears to be a region of introgression northward from *J. coahuilensis* (Fig. 1).

Although it appeared that the *J. coahuilensis* at La Zarca, MX was a pure population (Adams 2017), new specimens of aff. *J. coahuilensis* with violet, reddish and blue colored fruits have been discovered in north central Mexico that do not fit the current *Juniperus* keys (Adams 2014). The present distribution map of *J. coahuilensis* is shown in Figure 2.

The purpose of this paper is to report on the results of DNA sequencing for these new, morphologically variable samples in an effort to better understand the variation in the serrate junipers of Mexico, with particular emphasis on *J. coahuilensis* and its allies.

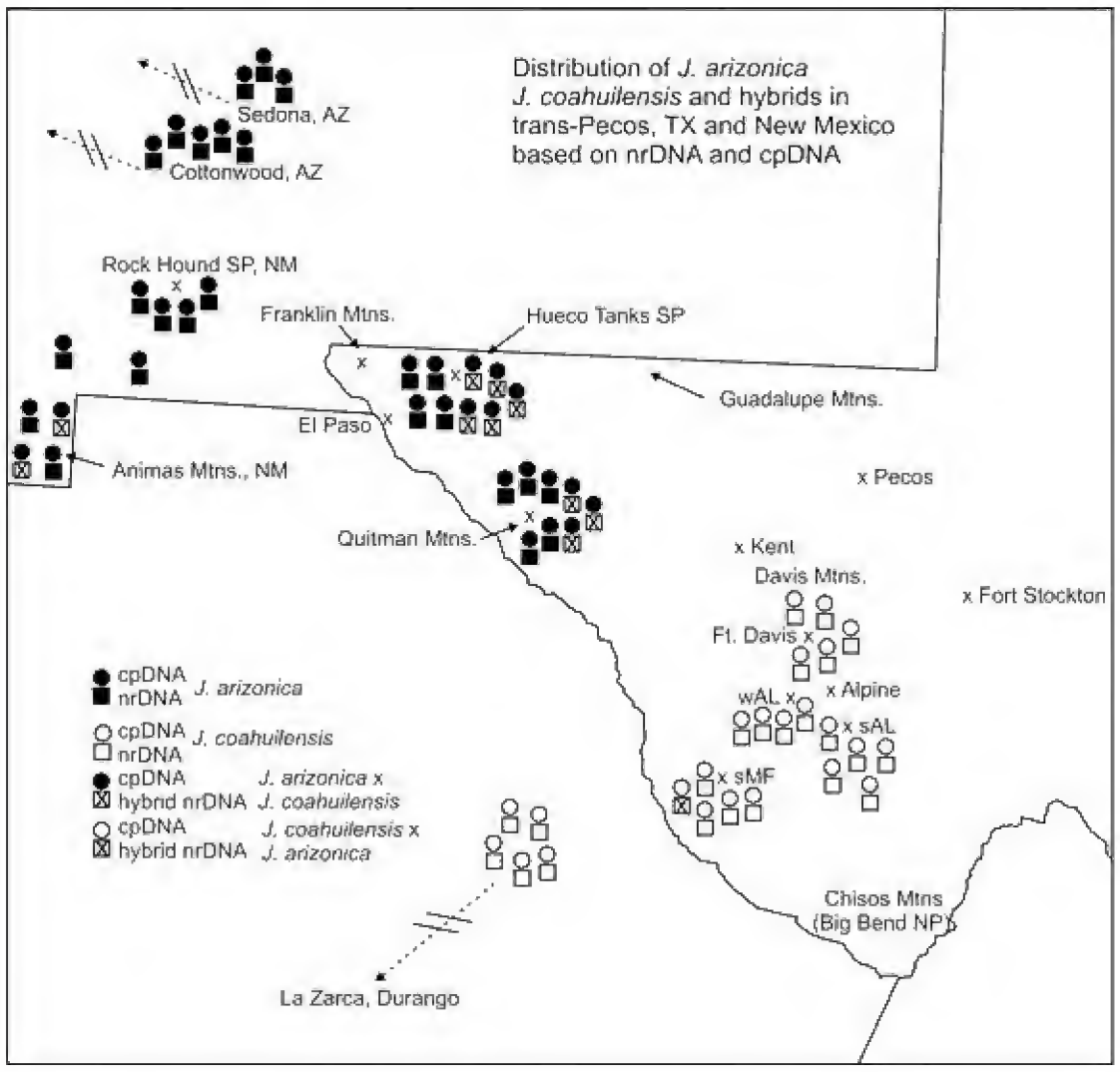

Figure 1. Plant distribution map showing their classification as *J. arizonica, J. coahuilensis*, or hybrids based on the results from both nrDNA and cpDNA analysis. From Adams (2017).

MATERIALS AND METHODS

Plant material and populations studied:

J. coahuilensis, large population with thousands of trees. Mexico, Durango, 85 km n. of La Zarca on Mex. 45, 26° 21' N, 105° 16.66' W,1740m, 10 Dec 1991, *Robert P. Adams 6829-6831*,

J. coahuilensis, large population in *Bouteloua* grassland, multi-stemmed tree, 4 m tall, female, female cones glaucous, blue-pinkish when mature. Mexico, Durango, at km 18 on Mex. 45, north of Durango, pollen shed in fall. bark exfoliating in narrow strips. 24° 09.067'N, 104° 42.462' W, 1938 m, 7 May 2004, Coll. *R. P. Adams 10241, 10242*.

J. aff. *coahuilensis,* shrub or tree 3-6 m, seed cones globose, fleshy, bright rose to salmon colored, sweet, 1(2)-seeded, on limestone, Mexico, Durango, Mpio. Nombre de Dios, San Jose de La Parrilla, 23° 44' 20" N, 104° 07' 20" W, 2120 m, 27 Aug 2004, Coll. *Socorro Gonzalez 6988,* Lab Acc. *Robert P. Adams 10454,*

J. aff. *coahuilensis,* Plant on limestone, with unusual seed cones: fibrous, bluish appearance because of the dense glaucous cover on a green surface, one seed [Not fleshy, nor rose or salmon, nor sweetish as in *J. coahuilensis*]; bark thin, fibrous, gray-brown, Mexico, Durango, Mpio. Nombre de Dios, San Jose de La Parrilla; on limestone, 23° 44' 20" N, 104° 7' 20" W, 2120 m, 27 Aug 2004, Coll. *Socorro Gonzalez 6989,* Lab Acc. *Robert P. Adams 10455,*

J. aff. *coahuilensis* hybrid?, Plant with unusual seed cones: fleshy as found in *J. coahuilensis* (present in the same site), but differs having dull purple to dull rose color, glaucous, seed cones in dense groups; branches firm, ascendant; bark thin, fibrous, gray-brown, Mexico, Durango, Mpio. Nombre de Dios, San Jose de La Parrilla; on limestone. 23° 44' 20" N, 104° 7' 20" W, 2120 m, 27 Aug 2004, Coll. *Socorro Gonzalez 6990;* Lab Acc. *Robert P. Adams 10456,*

J. aff. *coahuilensis,* Abundant shrubs, 2-3 m, seed cones rose-pale cherry, without glaucous cover, Mexico, Durango, Mpio. Guanaceví; SE of Guanaceví, on road to Durango, 25° 53' 14" N, 105° 50' 59"

W, 1990 m, 27 Aug 2004, Coll. *Socorro Gonzalez and M. Gonzalez-Elizondo7005;* Lab Acc. *Robert P. Adams 10459,*

J. aff. *coahuilensis,* Abundant, trees on limestone, to 3 m, seed cones fleshy, red-orange, sweet, Mexico, Durango, Mpio. Nombre de Dios, S of El Porvenir and NE of San José de La Parrilla, 23° 46' 30" N, 104° 09' 30" W, 1980 m, 4 Nov 2004, Coll. *Socorro Gonzalez 7016-1, 7016-2,* Lab Acc. *Robert P. Adams 10503, 10504,*

J. aff. *coahuilensis,* shrub-trees, on limestone, seed cones violet colored, somewhat fibrous and resinous, Mexico, Durango, Mpio. Nombre de Dios, NE of San José de La Parrilla, 23° 46' N, 104° 9' W, 1980 m, 4 Nov2004, Coll. *Socorro Gonzalez* 7017a, Lab Acc. *Robert P. Adams 10505,*

J. aff. *coahuilensis,* shrub-trees, on limestone, seed cones: densely grouped, fleshy, sweet, reddish-orange, 1(2) seeds; thin, fibrous bark; on branches pale gray to whitish, Mexico, Durango, Mpio. Nombre de Dios, 0.4 km SW of San Jose de La Parrilla; on limestone, 23° 44' 20" N, 104° 7' 20" W, 2120 m, 4 Nov2004, Coll. *Socorro Gonzalez* 7019-1, 7019-2, Lab Acc. *Robert P. Adams 10511, 10512,*

J. cf. flaccida, Short trees, 1.5-3 m tall; bark on branches papery and exfoliating, inner bark smooth, reddish; no seed cones, similar to *J. flaccida,* but in a very dry habitat in the Chihuahuan desert region, Mexico, Durango, Mpio. Lerdo, Sierra del Rosario, nearly atop the mountain, with Yucca and oak scrub; on limestone, 25° 38' 44" N, 103° 54' 40" W, 2700 m, 8 Apr 2008, Coll. *M. S. Gonzalez-Elizondo et al. 7375 a,b;* Lab Acc. *Robert P. Adams 14616, 14617.*

J. aff. *martinezii/ durangensis,* Shrub, seed cones orangish color and fibrous, with pinyon pine and oaks. Mexico, Durango, Mpio. Panuco, Sierra de Gamón, NW slopes, 24° 35' N, 104° 16' W, 2500 m, 4 June 2008, Coll. *M. S. Gonzalez-Elizondo et al. 7391 a,b;* Lab Acc. *Robert P. Adams 14618, 14619,*

J. aff. *saltillensis,* Abundant shrub 1-1.8 m, dark blue seed cones, somewhat glaucous, Mexico, Zacatecas, Sierra de Mazapil, Mpio. Concepción del Oro, 24° 37' 21" N, 101° 28' 05" W, 2850-2900 m, 16 Oct 2009, Coll. *M. S. Gonzalez-Elizondo and M. Gonzalez-Elizondo 7567,7568;* Lab Acc. *Robert P. Adams 14620, 14621*

J. aff. *poblana,* uncommon young trees (saplings) 2 m, in oak woodland dominated by *Quercus resinosa,* Mexico, Nayarit, Mpio. El Nayar, SW of Mesa del Nayar on road to Ruiz, Km 86.8; S of bridge of arroyo del Fraile, E of El Maguey, 22° 10' 08" N, 104° 43' 51" W, 1150 m, 19 Jan. 2016, Coll. *M. S. Gonzalez-Elizondo and M. Gonzalez-Elizondo 8381 with L. López, A. Torres Soto;* Lab Acc. *Robert P. Adams 14896*

J. aff. *poblana,* large, single stemmed trees, foliage long and pendulous, abundant trees, up to 25 m high, on strongly rocky slope, forest of *Juniperus-Clusia* with elements of mesophytic forest (Magnolia) and tropical forest (*Bursera, Opuntia, Pilosocereus purpusii*) as well as *Agave attenuata* and *Yucca jaliscensis,* Mexico, Nayarit, Mpio. El Nayar, SW of Mesa del Nayar on road to Ruiz; NE of El Maguey, 22° 07'40" N, 104° 47' 47" W, 1430 m, 19 Jan. 2016, Coll. *M. S. Gonzalez-Elizondo and M. Gonzalez-Elizondo 8379a,b,c,d, with L. López, A. Torres Soto;* Lab Acc. *Robert P. Adams14897-14900,*

J. martinezii, new record for Durango, Abundant tree with drooping branchlets, pale grayish-green foliage with white resin marks, Mexico, Durango, Mpio. Vicente Guerrero, Sierra de Organos, near the border of state of Zacatecas, northernmost known population of *J. martinezii.* The closest population is about 220 km to the SE [Aguascalientes, San José de Gracia (acc. Pérez de la Rosa 1985) 23° 47' 28" N, 103° 49' 44" W, 2225 m, 21 Jan 2016, Coll. *M. S. Gonzalez-Elizondo and M. Gonzalez-Elizondo) 8384;* Lab Acc. *Robert P. Adams 14901,*

J. aff. *coahuilensis,* Shrub, blue seed cones, Mexico, Durango, Mpio. Nombre de Dios, 4 km w of San José de La Parrilla, 23° 43' N, 104° 08' W, 2150 m, 25 Oct 1983, Coll. *M. S. Gonzalez-Elizondo et al. 2776;* Lab Acc. *Robert P. Adams 14902,*

J. aff. *coahuilensis,* Shrub, blue seed cones, Mexico, Durango, Mpio. Tepehuanes, SE edge of town, 25° 20' N, 105° 43' W, 1800 m, 10 Sep 1989, *O. Bravo 288*; Lab Acc. *Robert P. Adams 14903,*

J. aff. *coahuilensis,* Shrub, blue seed cones, Mexico, Durango, Mpio. Santiago Papasquiaro, 9 km por el camino a Los Altares, 25° 06' N, 105° 27' W, 1940 m, 30 July 1990, Coll. *A. Benitez P. 1646;* Lab Acc. *Robert P. Adams 14904,*

Voucher specimens for new collections 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° 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. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010). The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.) or Sequencher v. 5 (genecodes.com). Sequence datasets were analyzed using Geneious v. R7 (Biomatters. Available from http://www.geneious.com/), the MAFFT alignment program. Further analyses utilized the Bayesian analysis software Mr. Bayes v.3.1 (Ronquist and Huelsenbeck 2003). For phylogenetic analyses, appropriate nucleotide substitution models were selected using Modeltest v3.7 (Posada and Crandall 1998) and Akaike's information criterion.

RESULTS AND DISCUSSION

Sequencing nrDNA, petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF resulted in 4,351 bp of concatenated sequence data. A Bayesian tree shows the placement of most of the samples collected as *J.* aff. *coahuilensis* (*10241, 10242, 10503, 10504, 10505*) are in the clade with typical *J. coahuilensis* (shaded box, Fig. 2). However, an adjacent clade (cross-hatched box, Fig. 2) contains two sub-clades: blue seed cones plants (*14902, 14903, 14904*) and La Parrilla plants, with very variable seed cone colors from violet to bluish to orange (*14055, 10454, 10456, 10459, 10511*).

Plants *14620, 14621*, *J.* aff. *saltillensis* from Zacatecas, Sierra de Mazapil, Mpio. Concepción del Oro, are nested, loosely in a clade with *J. saltillensis* (Fig. 2). Additional research on the leaf volatile oils, ecology and morphology (in progress) may prove these to be a new variety of *J. saltillensis*.

Sample *14901*, collected as *J. martinezii* from Durango, Mpio. Vicente Guerrero, Sierra de Órganos, near the border of state of Zacatecas, is in a clade with *J. martinezii* (Fig. 2). This is the first report of *J. martinezii* from Durango and is the northernmost known population of *J. martinezii*. The closest population is about 220 km to the SE (Aguascalientes, San José de Gracia, Perez de la Rosa, 1985).

Two other collections (*14618, 14619*, shrubs, seed cones orangish color and fibrous, with pinyon pine and oaks. Mexico, Durango, Mpio. Panuco, Sierra de Gamón) with affinities to both *J. martinezii* and *J. durangensis*, were placed in a clade with *J. martinezii* and *J. durangensis* (Fig. 2). There is some support for it being in a distinct clade (51%, Fig. 2), but additional research is needed on the leaf volatile oils, ecology and morphology (in progress) to determine if this taxon is a new variety of *J. martinezii* or perhaps a new species.

Plants *14616, 14617*, collected as *J.* aff. *flaccida*, were short trees, 1.5-3 m tall with the bark on branches papery and exfoliating, and inner bark smooth, reddish. These samples were in a well supported

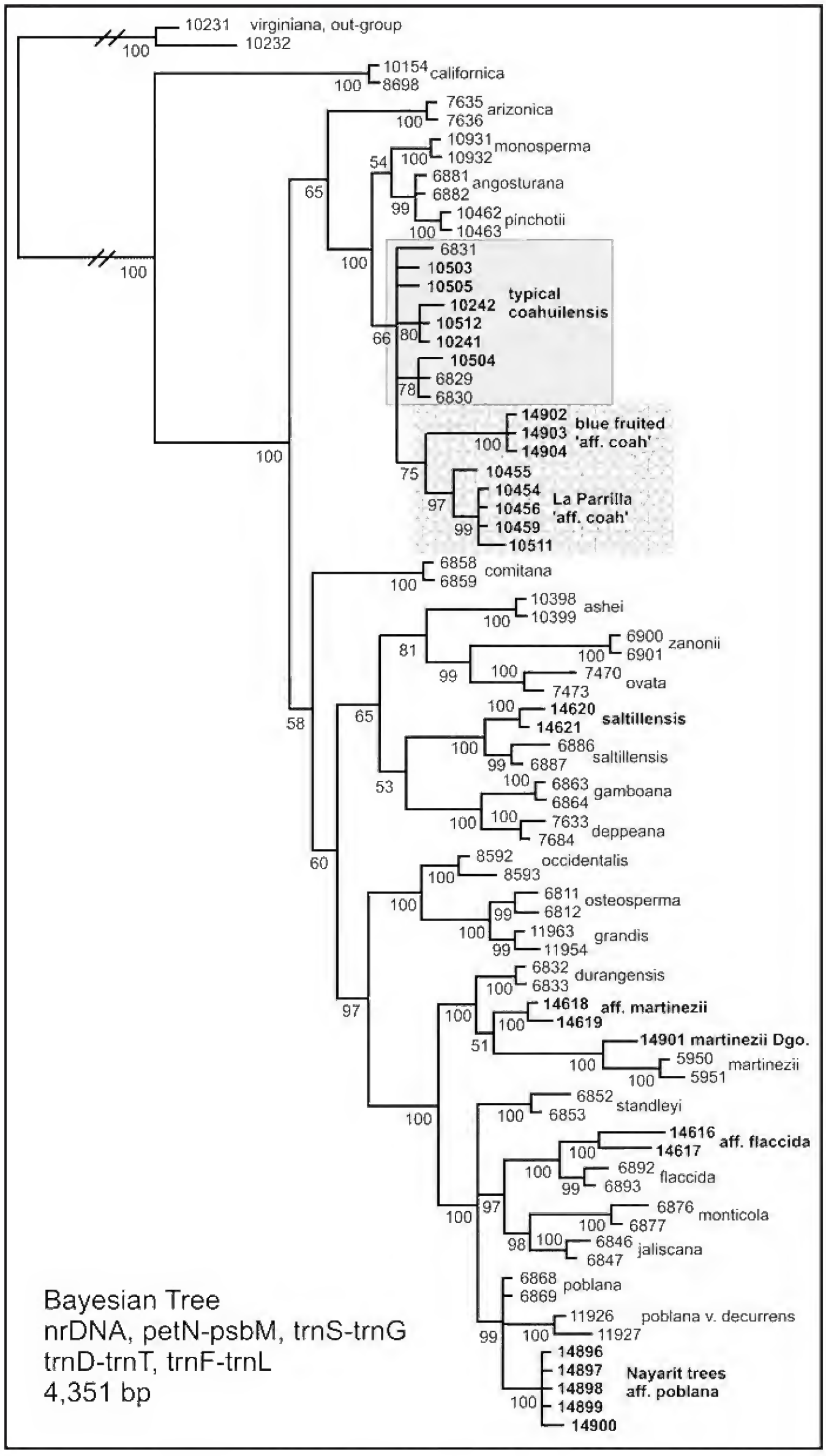

Figure 2. Bayesian tree of serrate leaved *Juniperus* of North America. Numbers next to branch points are posterior probabilities as percents. Note the typical *J. coahuilensis* (shaded box) and the adjacent clade (cross-hatched box). See text for discussion. Samples in boldface print are new collections. Samples in regular font are the reference set of serrate junipers.

clade with *J. flaccida*, but yet, quite distinct (Fig. 2). The site is in a very dry habitat in the Chihuahuan desert region, Mexico, Durango, Mpio. Lerdo, Sierra del Rosario. No seed cones were found (April, 2008), so new collections with seed cones are needed. Clearly, additional research is needed on the leaf

volatile oils, ecology and morphology (in progress) to determine if this taxon might be a new variety of *J. flaccida*.

And lastly, a very unusual population with aff. *poblana*, was found with large, single stemmed trees, and foliage long and pendulous in Nayarit. Analysis of their DNA did place them (*14986, 14897, 14898, 14899, 14900*) in a large clade with *J. poblana* and *J. p.* var. *decurrens* (Fig. 2). However, they are quite distinct and well supported as a separate clade. Analysis of the leaf volatile oils, ecology and morphology (in progress) should be sufficient to determine if this taxon is a new species, or perhaps another (new) variety of *J. poblana*.

A detailed examination of variable nrDNA (ITS) sites of *J. coahuilensis* aff. samples, as well as *J. coahuilensis* from the Trans-Pecos, Texas region is shown in Table 1. Overall, *J. coahuilensis* and the aff. samples from Mexico do not have as many variable sites as found in the Trans-Pecos region (see also Adams, 2017).

Mapping the classification of individuals based on ITS and cp (petN) data shows (Fig. 3) only four samples in Durango that have both ITS and cpDNA of *J. coahuilensis* (as found in the Trans-Pecos, Texas area).

The cpDNA of the blue fruited taxon (black filled circles, Fig. 3) was found in combination with various types of ITS DNA in central and southern (La Parrilla area) Durango. The cpDNA of typical *J. coahuilensis* was found in both northern and southern Durango (Fig. 3). Two of the blue fruited samples (black filled circle, open diamond, Fig. 3) were found in central Durango, and the third sample was found in the La Parrilla area. Two samples with La Parrilla type ITS (LaPar, Table 1; black square, Fig. 3) were found in the La Parrilla area and are in northwestern Durango.

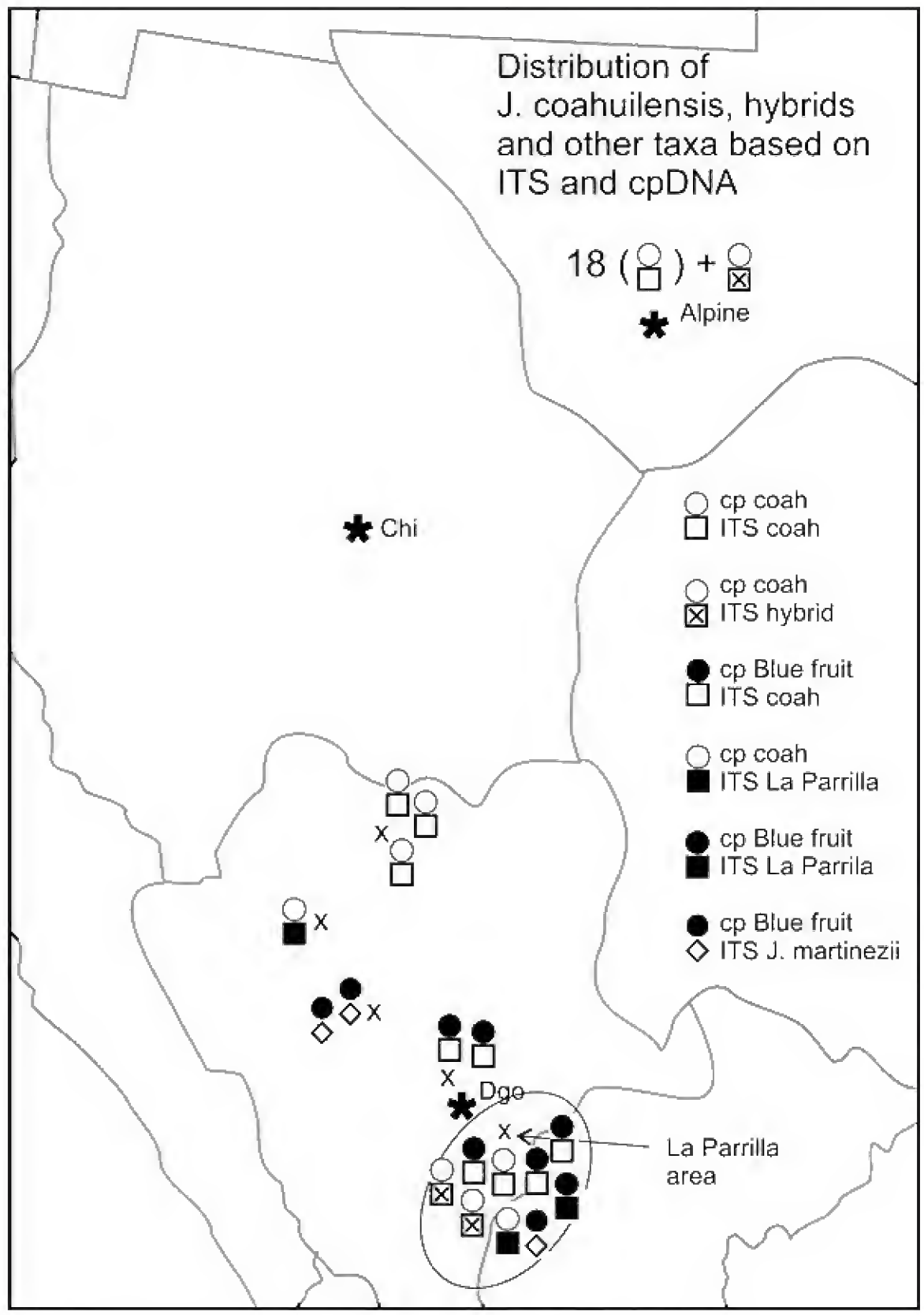

Figure 3. Map of *J. coahuilensis* and aff. samples by their cpDNA (petN) (circles) and ITS DNA (squares). Data in the Trans-Pecos, Texas area from Adams (2017.

Two samples, putatively hybrids based on their ITS, were found in the La Parrilla area (crossed squares, Fig. 3). All six of the cpDNA/ ITS types were found in the La Parrilla area (Fig. 3). It may be that other areas are equally as diverse, but additional sampling is needed to address this question.

Several of the nrDNA (ITS) sites display interesting geographic patterns. ITS site 191 (A,G, A/G) has considerable variation in the Trans-Pecos, Texas region (Fig. 4) and continues into northern Durango. However, no other A/G sites were found in central and southern (La Parrilla) Durango. This may be the result of hybridization/ introgression from some juniper in the Alpine area.

One individual with site 191 (A) was found south of Alpine and another found in the La Parrilla area of southern Durango.

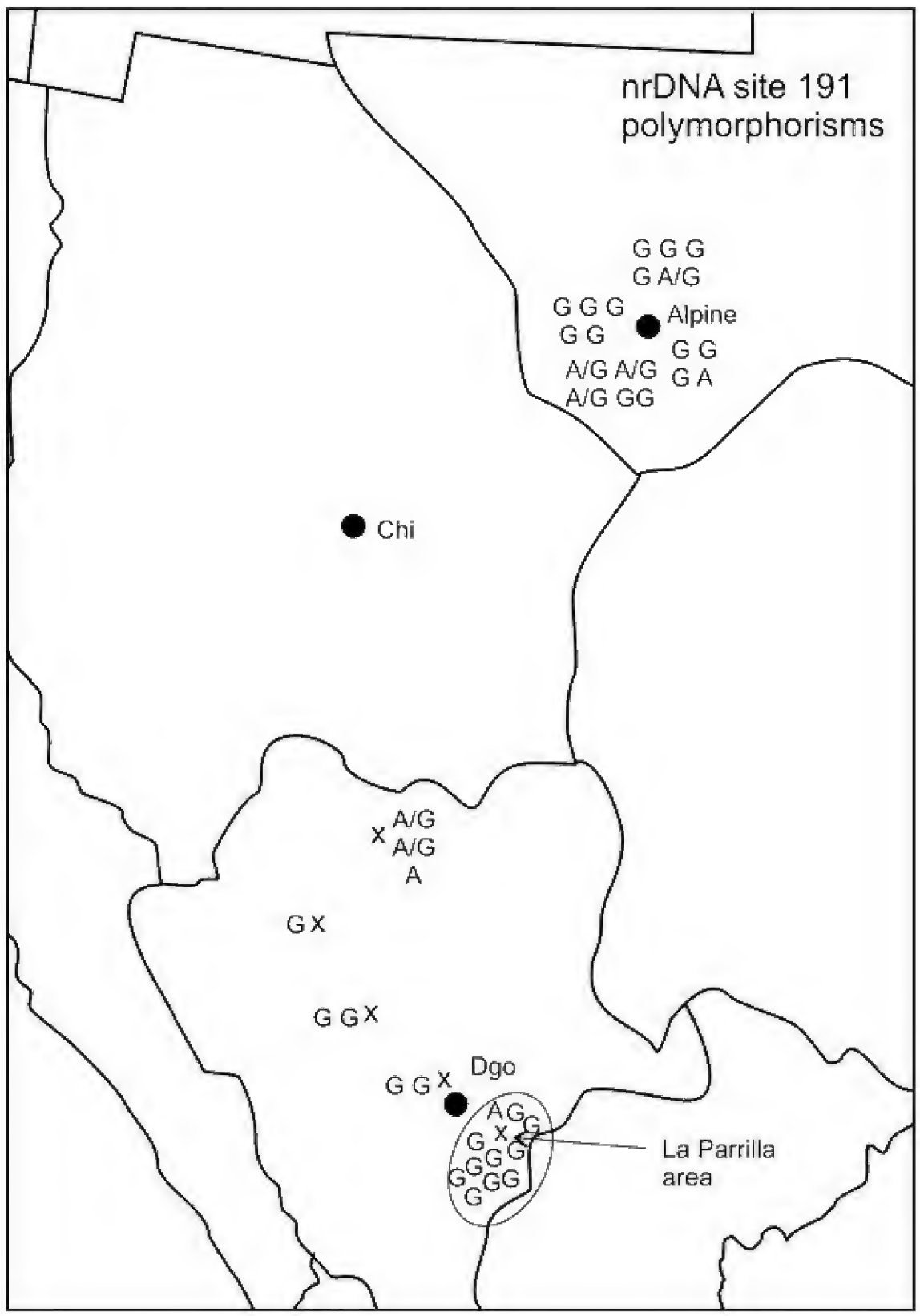

Figure 4. Geographic variation in ITS site 191. See text for discussion.

ITS site 196 featured the deletion of T in many samples ranging from the Alpine to central and southern Durango (Fig. 5). All three BF (blue fruited) and the 'LaPar' ITS type samples had the 196 deletion (Table 1). Plants *10454, 10456*, and *14903* appear to be hybrids. The deletion caused slippage during sequencing, so all the sites downstream from 196 were polymorphic. To remedy this problem, a new internal reverse primer was synthesized and used to reverse-sequence the immediate 700 bp past site 196 to obtain clean sequences from some plants. It is not known if this deletion is of contemporary or ancient origin.

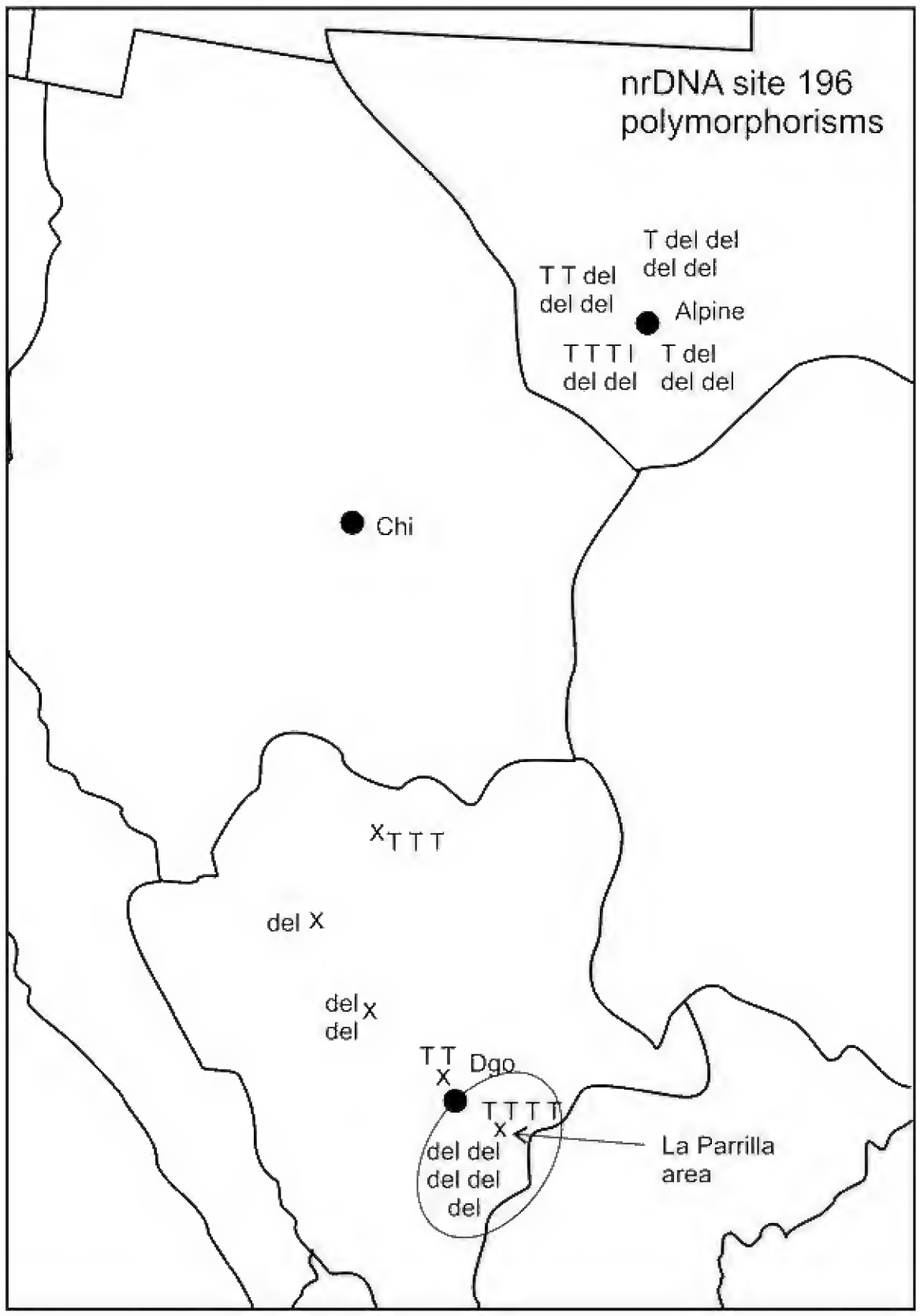

Figure 5. Geographic variation in ITS site 196. See text for discussion.

Mapping ITS site 303 provided a novel pattern not seen in other ITS sites. The presence of C/T polymorphisms for site 303 in the Trans Pecos area (Fig. 6) was not found in Mexico (nor in AZ, NM, see Adams, 2017). This seems to imply that the event was modern and due to hybridization with some unknown extant or extinct juniper in the Trans-Pecos area. Of interest to this study was the finding many plants with either C or T, but no plants with C/T in Durango.

In addition, the three BF (blue fruited) plants each contained G at site 303 (Table 1) and are shown (Fig. 6) with two in central Durango and one in the La Parrilla area. In addition, G (site 303) is also found in *J. martinezii* (Table 1). This site, no doubt, supported the placing of the BF junipers in a clade with *J. martinezii* in a NJ tree based on ITS sequences (data not shown), suggesting the BF taxon has a nuclear affinity to *J. martinezii*. However, sequences from the four cp gene regions was concatenated to nrDNA data in the construction of the Bayesian tree (Fig. 2), and this led to the positioning of the BF taxon loosely in the *J. coahuilensis* clade (Fig. 2).

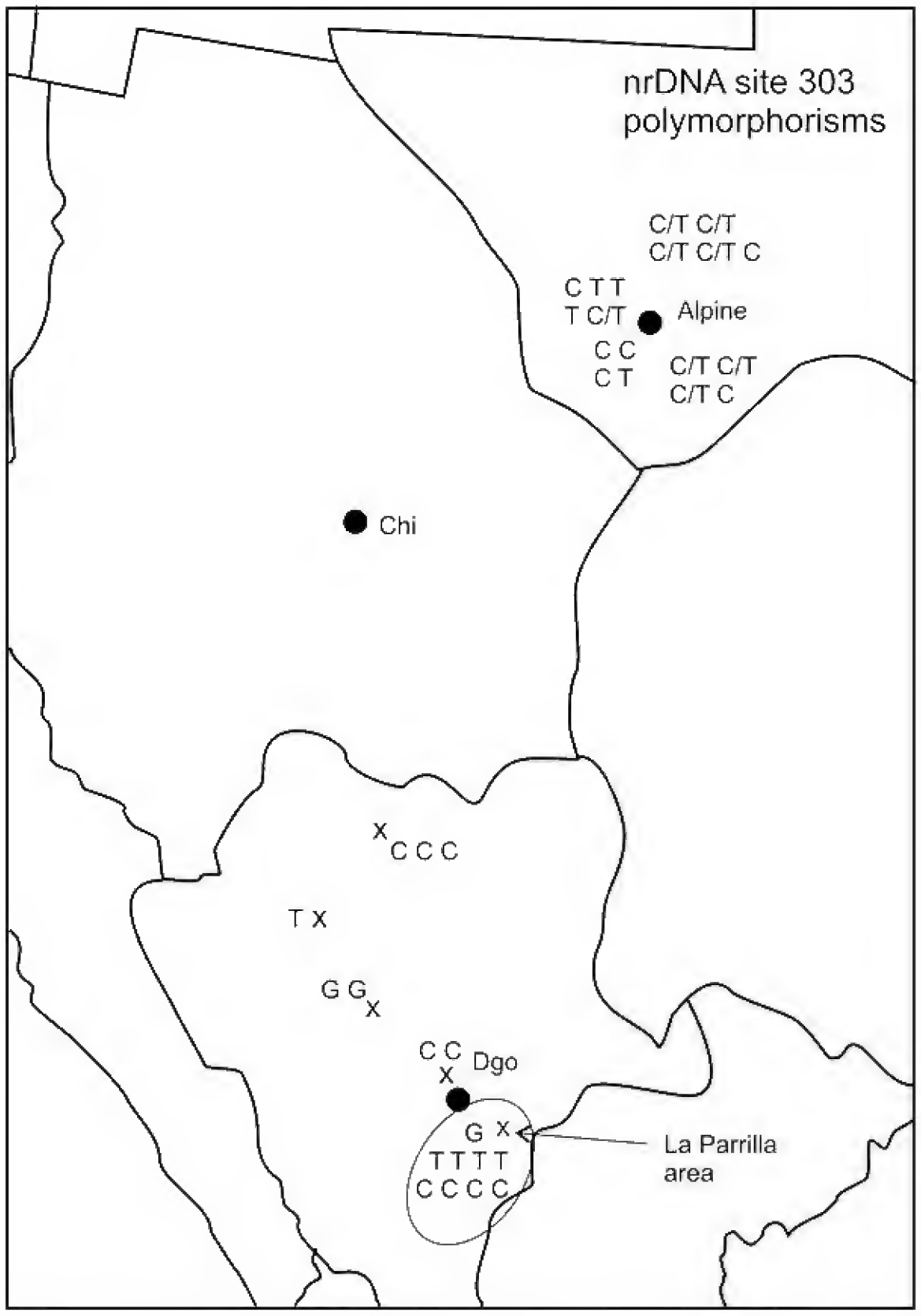

Figure 6. Geographic variation in ITS site 303. See text for discussion.

Finally, examination of ITS site 1116 presents an interesting situation in that every case with C/T at site 1116 (Table 1) has a deletion (del) at 196 (Table 1). Re-examination of the nrDNA sequence for 14814 revealed that the site 196 contains mostly T, there is a small (ca. 20% C peak). From 196 onward, small peaks (ca. 20% high) are present in the sequence. The del at 196, the slippage of the sequence for ca. 20% of the DNA strains perfectly explains the minor bases from 196 onward. This suggests that the plant is of backcross origin and that incomplete lineage sorting has not yet removed the minority copies that contain a del in 196). It should be noted that several samples (Table 1) have a del at 196 but have either a clean C or T at 1116.

The pattern seen for site 1116 (Fig. 7) suggests (as seen in Fig. 5) hybridization throughout the range of *J. coahuilensis* from Alpine to southern Durango, with the presence of numerous plants with C or T at site 1116.

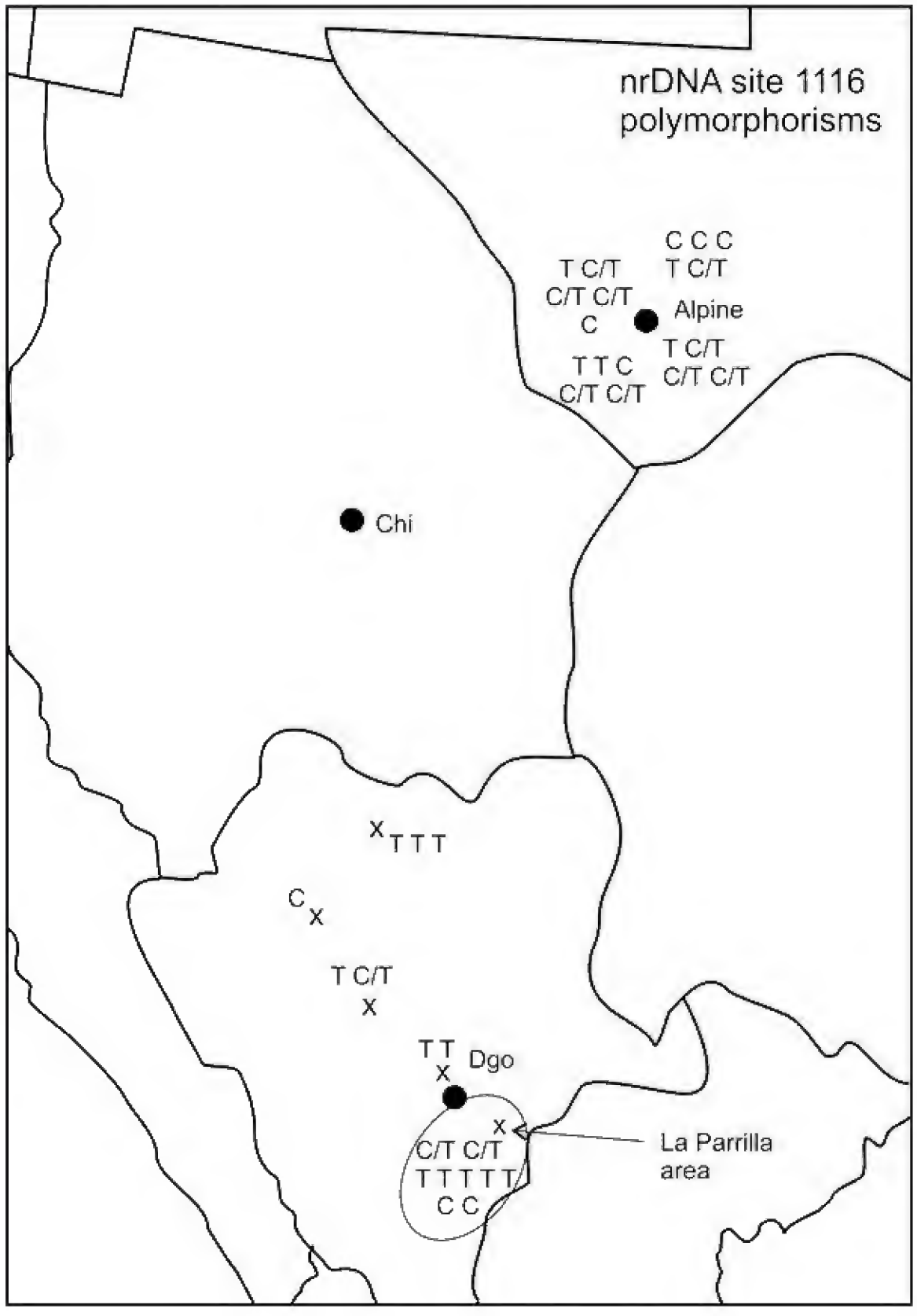

Figure 7. Geographic variation in ITS site 1116. See text for discussion.

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Table 1. Variable sites in nrDNA for *J. arizonica* (ariz), *J. coahuilensis* (coah) and 'blue, violet, bluish' fruited (BF), and La Parrilla type nrDNA (LaPar). del = deletion, mart = *J. martinezii* nrDNA.

sample	petN	ITS	191	196	302	**303**	304	318	**533**	543	1116	1148	#poly
az10634Sedona 181A/C	ariz	ariz	G	T	A	C	T	T	A	**C/G**	T	C	2
az10635Sedona 681A/C	ariz	ariz	G	T	A	C	T	T	A	**C/G**	T	C	2
az10636Sedona	ariz	ariz	G	T	A	C	T	T	A	**C/G**	T	C	1
az14908Cottonwood	ariz	ariz	G	T	A	C	T	T	A	G	T	C	0
az14909Cottonwood	ariz	ariz	G	T	A	C	T	T	A	**C/G**	T	C	1
az14910Cottonwood	ariz	ariz	G	T	A	C	T	T	A	C	T	C	0
az14912Cottonwood	ariz	ariz	G	T	A	C	T	T	A	C	T	C	0
az14913Cottonwood 121C/T	ariz	ariz	G	T	A	C	T	T	A	**C/G**	T	C	2
az7635RockHoundSP	ariz	ariz	G	T	A	C	T	T	A	C	T	C	0
az7636RockHoundSP	ariz	ariz	G	T	A	C	T	T	A	C	T	C	0
az7637RockHoundSP	ariz	ariz	G	T	A	C	T	T	A	**C/G**	T	C	1
az10630RockHSP	ariz	ariz	G	T	A	C	T	T	A	C	T	C	0
coa14807sofAlpine	coah	coah	G	**del**	**A/G**	**C/T**	**C/T**	**C/T**	T	C	**C/T**	**C/T**	6
coa14808sofAlpine	coah	coah	G	T	A	C	T	**C/T**	T	C	T	**C/T**	2
coa14810sofAlpine	coah	coah	G	**del**	A	**C/T**	**C/T**	T	T	C	**C/T**	C	4
coa14811sofAlpine	coah	coah	A	**del**	**A/G**	**C/T**	T	T	T	C	**C/T**	C	3
coa14812wofAlpine	coah	coah	G	**del**	**A/G**	**C/T**	T	**C/T**	T	C	C	C	4
coa14813wofAlpine 313A/G	coah	coah	G	T	A	**C/T**	T	**C/T**	T	C	T	**C/T**	4
coa14814wofAlpine	coah	coah	G	**del**[6]	A	C	T	T	T	C	**C/T**	C	1
coa14815wofAlpine	coah	coah	G	**del**	**A/G**	**C/T**	T	**C/T**	T	C	**C/T**	C	5
coa14816wofAlpine	coah	coah	G	**del**	A	**C/T**	T	**C/T**	T	C	**C/T**	**C/T**	5
coa14817FtDavis	coah	coah	G	T	A	C	T	**C/T**	T	C	T	**C/T**	2
coa14818FtDavis	coah	coah	G	**del**	A	T	T	T	T	C	C	C	1
coa14819FtDavis	coah	coah	G	**del**	G	T	T	T	T	C	C	C	1
coa14820FtDavis 689G/T	coah	coah	**A/G**	**del**	**A/G**	**C/T**	T	T	T	C	**C/T**	C	6
coa14821FtDavis	coah	coah	G	**del**	**A/G**	T	T	T	T	C	C	C	2
coa14822sofMarfa	coah	coah	**A/G**	T	A	C	T	T	T	C	T	C	1
coa14823sofMarfa	coah	coah	G	T	A	C	T	T	T	C	**C/T**	C	1
coa14824sofMarfa	coah	coah	G	**del**	**A/G**	T	T	T	T	C	C	C	2
coa14825sofMarfa	coah	**AxC**	**A/G**	T	A	C	T	T	**A/T**	C	T	C	2
coa14826sofMarfa	coah	coah	**A/G**	del	na	na	na	na	T	C	**C/T**	C	3
coa6829km85, nLaZarca, rose	coah	coah	**A/G**	T	A	**C**	T	T	T	C	T	C	1
coa6830km85, nLaZarca, rose	coah	coah	A	T	A	**C**	T	T	T	C	T	C	0
coa6831km85, nLaZarca, rose [1]	coah	coah	**A/G**	T	A	**C**	T	T	T	C	T	C	2
coa10241k18 nDgo blue-pink [2]	BF	coah	G	T	A	**C**	T	T	T	**C/T**	T	C	2
coa10242k18 nDgo blue-pink [3]	BF	coah	G	T	A	**C**	T	T	T	**C/T**	T	C	2
coa10503LaParr red,sweet Fr	BF	coah	G	T	A	**C**	T	**C/T**	T	C	T	**C/T**	2
coa10504LaParr red,sweet	coah	coah	A	T	A	**C**	T	T	T	C	T	C	0
coa10505LaParr violet Fr	BF	coah	G	T	A	**C**	T	T	T	C	T	C	0
coa10512LaParr red-orange Fr	BF	coah	G	T	A	**C**	T	T	T	C	T	C	0
coa10454LaParr, rose Fr	coah	hyb?	G	**del**	A	**T**	T	T	T	C	**C/T**	C	2
coa10455LaParr bluish Fr [5]	BF	LaPar	G	**del**	**A/G**	**T**	T	T	T	C	C	C	4
coa10456LaParr rose-purple [4]	coah	hyb?	G	**del**	A	**T**	T	T	T	C	**C/T**	C	3
coa10459Guan rose-red,no blo	coah	LaPar	G	**del**	A	**T**	T	T	T	C	C	C	1
coa10511LaParr red-orange Fr	coah	LaPar	G	**del**	A	**T**	T	T	T	C	C	C	1
coaBF14902LaParr blueFr	BF	mart	G	**del**	A	**G**	T	T	T	C	T	C	1
coaBF14903Tepeh blueFr	BF	mart	G	**del**	A	**G**	T	T	T	C	**C/T**	C	2
coaBF14904SPapa blueFr	BF	mart	G	**del**	A	**G**	T	T	T	C	T	C	1
mart5950 J. martinezii	mart	mart	G	T	A	**G**	T	T	T	C	T	C	0
mart5950 J. martinezii	mart	mart	G	T	A	**G**	T	T	T	C	T	C	0

[1] 240A/G; [2]603A/G; [3]503C/T; [4]731A/G; [5]308A/G, 665C/T; [6]T with ca. 20% C at site 196

The taxa of *Dictyomorpha* (Chytridiomycota, *in praesens tempus*)

Will H. Blackwell, Peter M. Letcher and **Martha J. Powell**
Biological Sciences, The University of Alabama, Tuscaloosa, AL 35487

ABSTRACT

Dictyomorpha (initially known, among Chytridiomycetes, as *Pringsheimiella*), an endoparasite of types of 'water molds' (e.g. *Achlya*), is relatively unusual in being a heterothallic chytrid. As traditionally recognized, *Dictyomorpha* belongs to Family Olpidiaceae, Order Chytridiales. The genus was long considered monotypic, *D. dioica* the only taxon known. However, an additional variety (*D. dioica* var. *pythiensis*) was eventually described, seemingly based exclusively on occurrence in a different host (*Pythium*). Without explanation, this variety was subsequently elevated (different author) to species. We reviewed the two, putative taxa of *Dictyomorpha* in an attempt to determine whether varietal or specific status is preferable. Based on apparent morphological distinctions evident in existing literature and illustrations, the rank of species is supported, viz. *Dictyomorpha dioica* and *D. pythiensis*. We also consider whether *Dictyomorpha* should remain in Phylum Chytridiomycota, or, rather, if this genus is perhaps more appropriately placed in Phylum Cryptomycota ("Superphylum" Opisthosporidia). Published on-line www.phytologia.org *Phytologia 99(1): 74-82 (Jan 19, 2017).* ISSN 030319430.

KEY WORDS: *Achlya*, aquatic fungi, chytrid, *Dictyomorpha*, endoparasite, *Nucleophaga*, Olpidiaceae, Oomycetes, *Plasmophagus*, *Pringsheimiella*, *Pythium*, resting spores, *Rozella*, sporangia, zoospores.

Dictyomorpha (originally *Pringsheimiella*)—an endobiotic, single-celled genus producing small, posteriorly uniflagellate zoospores—has been considered a member of the Olpidiaceae; this family contains holocarpic forms (simple thallus converting, asexually, entirely to a sporangium), lacking rhizoids (i.e., lacking "vegetative" structures). Although the Olpidiaceae has traditionally been placed in Order Chytridiales (Class Chytridiomycetes), recent placements of some members have indicated other relationships (as will be discussed). The name *Dictyomorpha* ('net-form') would seem [incorrectly] to imply a 'network' or 'multicellularity;' mutual compression of zoosporangia in [sorus-like] clusters in the host (cf. Mullins, 1961; Karling, 1977)—resultant from multiple zoospore infections (cf. Couch, 1939; Karling, 1977)—imparts this illusion. *Dictyomorpha* should not be confused with *Dictyuchus* (name = 'net-holder'), an unrelated genus [of Oomycetes] in which a single sporangium may contain a network of (its own) zoospore-cysts (cf. Blackwell and Powell, 1999). Karling (1977)—and previously Couch (1939), ref. *Pringsheimiella*—noted that, superficially, *Dictyomorpha* may resemble [perhaps be mistaken for] *Dictyuchus* (until one realizes that the "*Dictyuchus*-like" appearance of *Dictyomorpha* is the result of a combination of the morphology of *Dictyomorpha* and its host, e.g., *Achlya*—and not simply the consequence of morphological development of a single organism).

Dictyomorpha (for many years thought to contain only *D. dioica*) was known as a parasite of *Achlya* (*A.* "*flagellata*;" cf. Couch, 1939; Mullins, 1961); *D. dioica* is relatively distinct among Chytridiomycota in being heterothallic (apparently existing as morphologically similar, male and female strains). A new variety (*D. dioica* var. *pythiensis*) was later discovered in a species of *Pythium* (Sarkar and Dayal, 1988). *Dictyomorpha dioica* was thought to be morphologically uniform, in spite of recognition of this additional variety (see Sarkar & Dayal, 1988); however, this variety was eventually recognized as a species by Dick (2001) who provided no supporting evidence for his elevation of taxonomic level. The zoospores of *Dictyomorpha*—and its resting spores (these formed as the result of sexual reproduction, by motile gametes seemingly identical to zoospores)—bear resemblance to those of *Rozella*, cf. Mullins (1961). *Rozella* had been considered a genus of Chytridiomycota, but some species

are now classified elsewhere (discussed herein)—raising questions as to correct phylum placement of *Dictyomorpha*. Our study questions the uniformity of *Dictyomorpha*, examines potential taxa in the genus (their 'rank'), and reconsiders relationships of this genus among Fungi and related organisms.

TAXONOMIC HISTORY OF *DICTYOMORPHA* (Figures 1 - 20)

Dictyomorpha was described as a genus of Chytridiales (Mullins, 1961). Illustration (Fig. 1) of [what turned out to be] sporangia of this organism [in its host, *Achlya*] is, however, traceable to Pringsheim (1860, specimens from Germany). Pringsheim, though, provided no legitimate name for this organism, incorrectly interpreting the motile cells he observed (his plate 22, fig. 5 and plate 23, fig. 3) as a stage (antherozoids) in the life-cycle of *Achlya* [unrelated genus of Oomycetes]. *Achlya* and other Saprolegniaceae do not possess flagellated gametes. Motile cells [actually zoospores] figured by Pringsheim are uniflagellate (flagellum at or toward one pole), Figs. 1,3. Zoospores of *Achlya* and other Saprolegniaceae are biflagellate (*Achlya* is laterally biflagellate). Motile cells do not seem to have been illustrated by Cornu (1872); however, the organism seen by him (similar to that illustrated by Pringsheim) was placed in Cornu's new genus, *Woronina* (non-chytridiomycetous organism—classified in the Plasmodiophoromycetes, e.g., Alexopoulos, 1962). Sparrow's (1943, fig. 44A) illustration of the organism seen by Cornu (1872) matches generally with that illustrated by Pringsheim (1860). Couch (1939)—noted in Sparrow (1943)—described 'Pringsheim's organism,' not as a Plasmodiophoromycete, but more correctly as a chytridialean genus—under his proposed name, *Pringsheimiella* (acknowledging Pringsheim's illustration). Couch (1939), based on collections in North and South Carolina, accurately described zoospores of *Pringsheimiella* as posteriorly uniflagellate. Couch, realizing that *Pringsheimiella* had been known just in its asexual phase, determined *P. dioica* (then the only taxon) to be heterothallic—among the first members of the Chytridiales shown to be so—male and female strains necessary for sexual reproduction (and production of resting spores, Figs. 11-12). Couch noted potential physiological (not morphological) differences between certain strains. Sparrow (1960) recognized *Pringsheimiella* Couch (1939). Mullins (1961) was uncertain that the organisms seen by Pringsheim (1860) and Couch (1939) were the same; however, Karling's (1977) illustration of this organism compares well with those of Couch and Pringsheim. There is little doubt that Pringsheim's fig. 1, plate 23, is of sporangia (in *Achlya*) of what would be described as *Pringsheimiella* (Couch, 1939) and *Dictyomorpha* (Mullins, 1961). Mullins was concerned that Pringsheim didn't observe [the zoospore as having] the lipoid body of chytrid zoospores; however, certain of Pringsheim's illustrations (plate 23, fig. 3) suggest this feature.

Mullins (1961) reviewed the taxonomy/nomenclature of *Pringsheimiella* Couch (1939), concluding the generic name was preoccupied; Mullins indicated that "*Pringsheimiella*" was employed by Höhnel, in "1919" in vol. "17" of Ann. Mycol., as the name of an alga. Nielsen and Pedersen (1977) noted that Höhnel's use of this algal name was actually in 1920 (vol. 18). Regardless, because of Höhnel's prior usage, *Pringsheimiella* Couch (1939) is a later homonym (illegitimate). Mullins (1961) supplied a legitimate, substitute name *Dictyomorpha* [*nomen novum*] for *Pringsheimiella* Couch. Mullins re-collected *Dictyomorpha* (Highlands, NC area) and restudied the life cycle—providing additional description and illustrations (including zoospore variation, see Fig. 9), and depositing slide material (additional to that of Couch, re: *Pringsheimiella*) in the UNC herbarium. Still, only one species was recognized in *Dictyomorpha*; this species, named *D.* "*dioica*" by Mullins (1961), would *seem* to have been transferred from *Pringsheimiella* (*P.* "*dioica*;" Couch, 1939). One might assume this species name would be cited "*Dictyomorpha dioica* (Couch) Mullins"—and it is so cited by Karling (1977) and Dick (2001). However, *Index Fungorum* currently (correctly we believe) lists the citation as "*Dictyomorpha dioica* Couch ex Mullins"—doubtless because Couch (1939) provided no Latin diagnosis when he described genus *Pringsheimiella* and species *P. dioica* (relegating Couch to having 'proposed' the epithet "*dioica*" rather than legitimately publishing it). Mullins (1961) provided a combined, Latin genus/species

description for *Dictyomorpha/D. dioica*, validating both. Authorship of the name *D. dioica* could, in fact, be cited as either "Couch ex Mullins" or "Mullins" (cf. *International Code...*, Article 46.5).

Pursuant to Mullins (1961), *Dictomorpha* was still thought to contain only *D. dioica*, without subspecific taxa, until Sarkar and Dayal (1988), based on material from India, described *D. dioica* var. *pythiensis* (see our Figs. 15-20)—occurring in *Pythium aphanidermatum*—automatically creating *Dictyomorpha dioica* var. *dioica* [not mentioned by Sarkar and Dayal]. While attempts by Sarkar and Dayal to infect hosts (including *Achlya*) other than *Pythium aphanidermatum* with *D. dioica* var. *pythiensis* were unsuccessful—var. *pythiensis* being apparently host-specific—they indicated a "close morphological similarity" between their variety and typical *D. dioica*, noting no consistent morphological differences; they felt, therefore, that var. *pythiensis* could not be justified as a new species. Since host specificity was nonetheless considered important in deciphering entities within the Olpidiaceae (Sparrow, 1960; Mullins and Barksdale, 1965), Sarkar and Dayal (1988) deemed varietal recognition appropriate. As noted by Sarkar and Dayal, Mullins and Barksdale (1965) demonstrated an increased host range for *Dictyomorpha dioica* [i.e., var. *dioica*]; successful infections included a total of eight identified (and two unidentified) species of *Achlya* (primarily in Subgenus *Achlya*)—including the original host (*A. flagellata*)—and also, *Thraustotheca clavata*; *Pythium* was not included in their investigation. Questionable evidence from early literature (Pringsheim, 1860) suggested that *D. dioica* may have occurred in *Saprolegnia* (cf. Mullins, 1961); however, *Saprolegnia* isolates tested (Mullins and Barksdale, 1965) were immune to such infection. The "*Saprolegnia*" identified by Pringsheim (1860, his plate 22) was apparently a mixture of *Achlya* and *Dictyuchus* (the latter not involving *Dictyomorpha*).

In a nomenclatural summary, Dick (2001)—placing *Dictyomorpha* in Family Rozellopsidaceae, Order Rozellopsidales (Order "*insertae sedis*")—recognized two species, "*Dictyomorpha dioica* (J. N. Couch) J. T. Mullins" and "*Dictyomorpha pythiensis* (N. Sarkar & R. Dayal) M. W. Dick, stat. nov." Proper author citation of *D. dioica* [i.e., Couch ex Mullins] has already been discussed. Of concern is Dick's (2001) recognition of var. *pythiensis* (Sarkar & Dayal, 1988) at species level, since Dick offered no justification for this status change (no distinguishing features of the taxa were noted). As we mentioned, Sarkar and Dayal had recognized "*pythiensis*" as a variety of *D. dioica* (not a separate species) because "*pythiensis*" was based, by them, on host specificity—occurring in *Pythium*, not *Achlya*—rather than on morphology. There was thus a need to determine if there are in fact morphological differences between the two alleged taxa within *Dictyomorpha*.

AT WHICH RANK SHOULD THE TAXA OF *DICTYOMORPHA* BE RECOGNIZED?

The question hence remains: Should the two 'entities' (var. *dioica* and var. *pythiensis*) within *Dictyomorpha dioica* be considered varieties (Sarkar and Dayal, 1988) or species (Dick, 2001)? Dick presented no evidence for his decision to recognize *Dictomorpha dioica* and *D. pythiensis* as distinct species. If there is no reliable difference between these 'taxa' other than host occupied (implied by Sarkar and Dayal, 1988), varietal status would be (at most) the appropriate taxonomic category. Even if this 'host difference' is accompanied by only one, minor, morphological difference, varietal status is perhaps still preferable. But if there is separation of taxa by host infected *and* by several morphological differences, species recognition should be considered. Reexamination of literature (including illustrations) was essential to this determination, since living material is not currently available; future collection of *Dictyomorpha* is obviously important to further understanding of the genus.

Comparison of illustrations of [what eventually came to be known as] *Dictyomorpha dioica* in Pringsheim (1860), Couch (1939), Mullins (1961), Karling (1977) and Sarkar and Dayal (1988)—reference our Figs. 1-20—suggests (in addition to occurrence in mutually exclusive hosts) that morphological differences do exist between "var. *dioica*" and "*var. pythiensis*." Eight (**8**) potential differences we noted in these illustrations—not always congruent with statements in text of the articles—

include: **1**. Shape of zoosporangium—"*dioica*," typically spherical (Figs. 1-2), except as altered by mutual compression; "*pythiensis*," generally oval (Figs. 17-18). **2**. Sporangial discharge "tube"—"*dioica*," merely a papilla (Figs. 6-7); "*pythiensis*," occurring as an actual (sometimes somewhat elongated) tube (Figs. 16-18). **3**. Number of sporangia in host cell—"*dioica*," often numerous (Fig. 1); "*pythiensis*," ranging from one to eight, illustrated (Sarkar and Dayal, 1988) as six or fewer (Figs. 17-18). **4**. Location of sporangia in host—"*dioica*," occurring in vegetative (often distal/apical) portions of host-hyphae (Figs. 1,2,6); "*pythiensis*," occurring at various points in vegetative hyphae and, notably, in oogonia (Figs. 17-18). **5**. Sporangial wall—"*dioica*," relatively thin and pliable (Fig. 7); "*pythiensis*," firmer and more definite in shape (Figs. 17-18). **6**. Zoospores—"*dioica*," illustrated (cf. Fig. 3) as typically somewhat elongated (Pringsheim, 1860) or irregular (spherical to elongate, e. g., Mullins, 1961; Karling, 1977); "*pythiensis*," illustrated (cf. Fig. 15) as essentially spherical (Sarkar and Dayal, 1988, though stated by them to be elongate). **7**. Resting-spore outer wall—"*dioica*," roughened, undulate, reticulate, or obscurely spiny (Figs. 11-14); "*pythiensis*," more distinctly spiny (Fig. 20), although the spines are typically small. We note that Karling (1977) illustrated (see, for example, fig. 36 of his plate 8) the outer resting-spore wall of 'typical' *D. dioica* as more obviously (though still minutely) spiny than did either Couch (1939) or Mullins (1961). **8**. 'Extra' structure ('compartment') surrounding the already double-walled resting spore(s)?—"*dioica*," one to several resting spores contained (often loosely) in a sometimes thick-walled, polygonal to square or rounded, extra 'cell' or 'compartment' (Figs. 11,12,14) produced by the host (illustrated: Couch, 1939; Mullins, 1961; Karling, 1977); "*pythiensis*," no extra 'host compartment' surrounds resting spores, though host-hyphae may form septa (Fig. 20) in response to infection (cf. Sarkar and Dayal, 1988).

Certainly, not all characters are distinguishable between "*dioica*" and "*pythiensis*." For example: Zoosporangial, and resting-spore, diameters of "*dioica*" were indicated (respectively) to be 15 to 20 µm, and 15 to 17 µm (Mullins, 1961); for *pythiensis*, these same parameters were (respectively) observed at 12 to 20 µm, and 14.95 to 18.95 µm (Sarkar and Dayal, 1988). Regardless of precise form, the small zoospores of the two taxa are also of similar dimensions (ca. 3 µm; cf. Couch, 1939; Mullins, 1960; Sarkar and Dayal, 1988). The resting spores (other than degree of 'spiny' appearance of the outer wall) are not only similar between the two taxa of *Dictyomorpha*, but reminiscent as well of the resting spores of *Rozella* (to which *Dictyomorpha* may be related; cf. Mullins, 1961, p. 386, last paragraph).

Characters (whether potentially distinguishing or not) perceived through study of literature are subject to further investigation should live material of *Dictyomorpha* become available. Regardless, sufficient morphological differences seem evident in various illustrations—in consort with delimitation by host infected—to support recognition of the varieties of *Dictyomorpha dioica*—*D. dioica* var. *dioica* and *D. dioica* var. *pythiensis* (Sarkar and Dayal, 1988)—as separate species (Dick, 2001, although Dick gave no explanation for this change in taxonomic status). We thus accept (duly noting here proper authorship) two species within *Dictyomorpha*: *D. dioica* J. N. Couch ex J. T. Mullins (1961) and *D. pythiensis* (N. Sarkar & R. Dayal) M. W. Dick (2001). We cannot, though, concur with Dick's inclusion of *Dictyomorpha* in the Rozellopsidaceae (Rozellopsidales); this category contains biflagellate forms, e.g., *Rozellopsis*. Zoospores of *Dictyomorpha* are definitely uniflagellate (Couch, 1939; Mullins, 1961; Karling, 1977; Sarkar and Dayal, 1988), cf. Figs. 3,8,9,15.

POSSIBLE SYSTEMATIC RELATIONSHIPS OF GENUS *DICTYOMORPHA*

Dictyomorpha—traditionally placed in the order Chytridiales [class Chytridiomycetes, phylum Chytridiomycota]—was considered a member of the family Olpidiaceae (simple, holocarpic forms lacking rhizoids). The Olpidiaceae included such seemingly similar genera as: *Olpidium*, *Olpidiomorpha*, *Rozella*, *Plasmophagus*, *Nucleophaga* and *Sphaerita* (cf. Sparrow, 1960; Karling, 1977). But molecular information has shed new light upon relationships of some Olpidiaceae. For example, certain species of *Olpidium* place within the clade of Zygomycetes (James et al., 2006); and, species of both *Rozella*

(Karpov et al., 2014) and *Nucleophaga* (Corsaro et al., 2014) have relationships within phylum Cryptomycota (superphylum Opisthosporidia). Bearing, as it does, morphological similarity (of zoospores and resting spores) to *Rozella* (cf. Mullins, 1961; Karling, 1977), *Dictyomorpha* could conceivably place in the Cryptomycota, rather than the Chytridiomycota; just how closely *Dictyomorpha* is related to *Rozella*, remains to be determined. We do note that in *Dictyomorpha*, in contrast to *Rozella*, the sporangial walls are readily distinguishable from the wall of the host (cf. Mullins, 1961, p. 386). However, only molecular/genetic analysis will answer ultimate questions of generic and phylum relationships. The puzzle of the systematic relationship of *Dictyomorpha* is, in fact, quite similar to that of *Plasmophagus* (Blackwell et al., 2016). Unfortunately, these obligately parasitic organisms are not generally available in culture collections—nor may they typically be cultured in the absence of their hosts (cf. Mullins, 1961; Mullins and Barksdale, 1965; re: *Dictyomorpha dioica*)—rendering molecular analysis elusive. Future collecting of such organisms—so that molecular analyses will have at least the possibility of being performed—is essential to ultimate resolution of systematic problems. There is continuing need for broad surveys of "hydromycoflora"—such as that of Czeczuga (1995) in north-east Poland—to "enrich our knowledge of biology of many aquatic fungi species." *Dictyomorpha dioica* was indeed found by Czeczuga, in one of 31 lakes sampled (the host for this organism, however, was not indicated).

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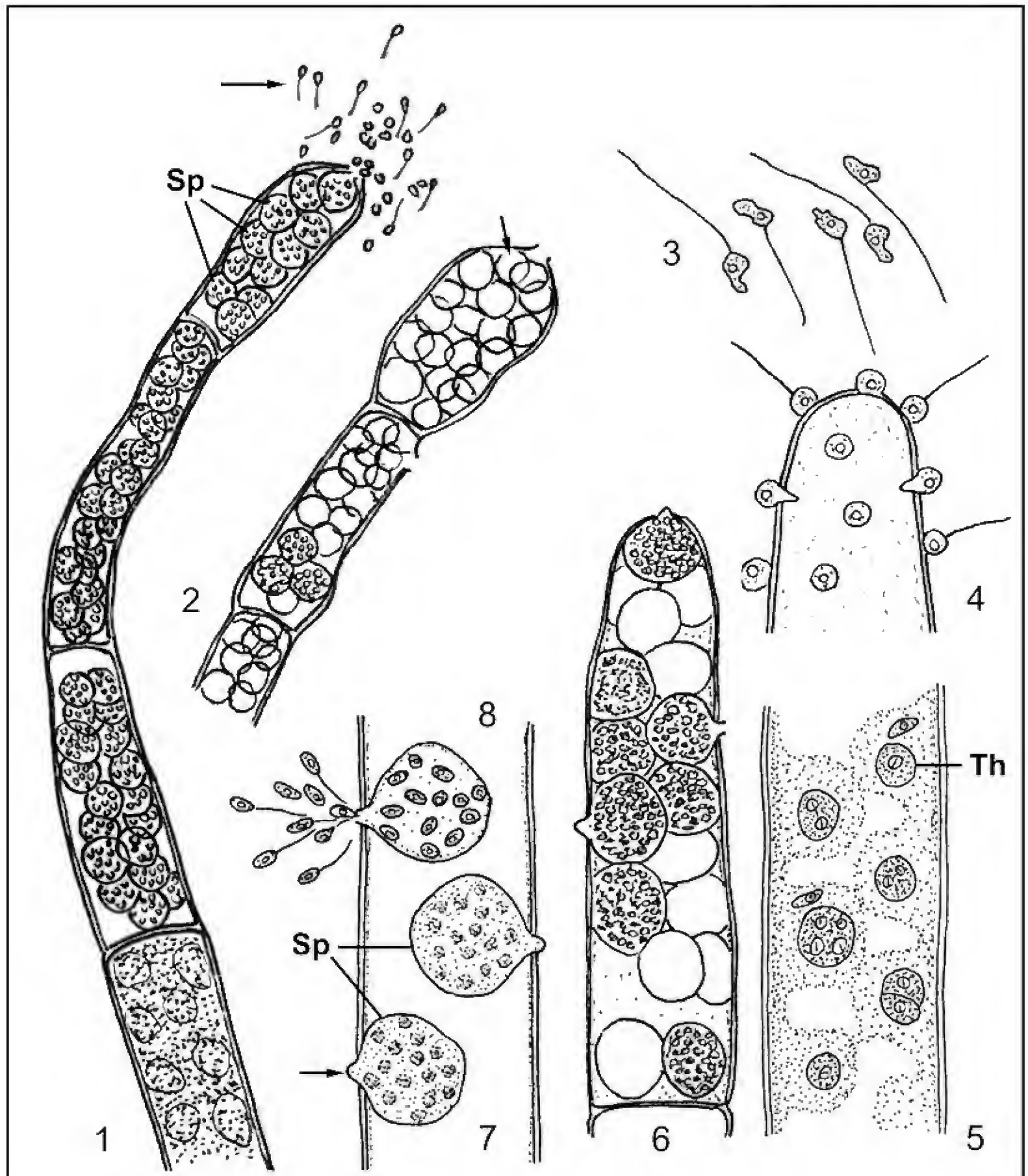
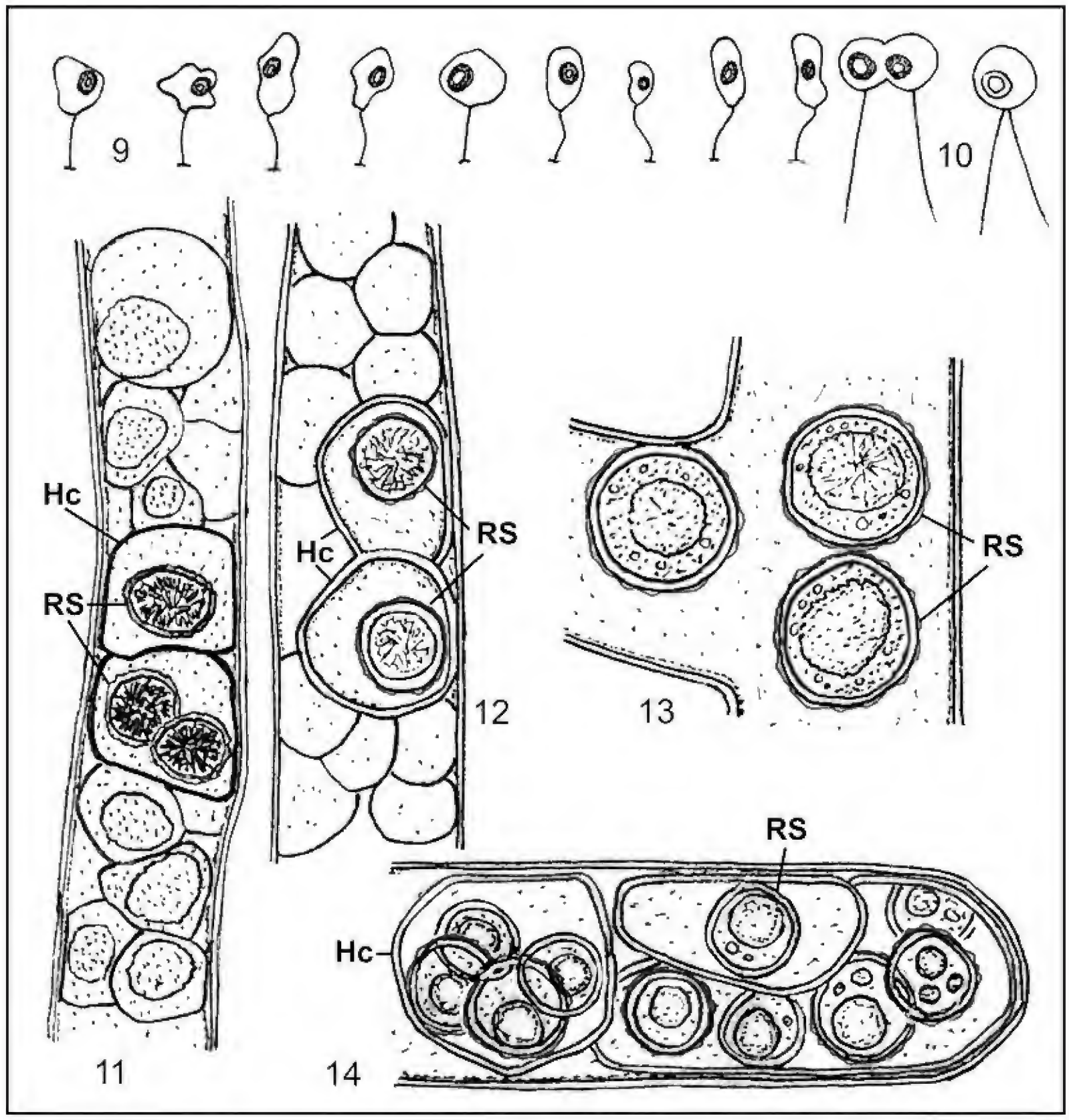
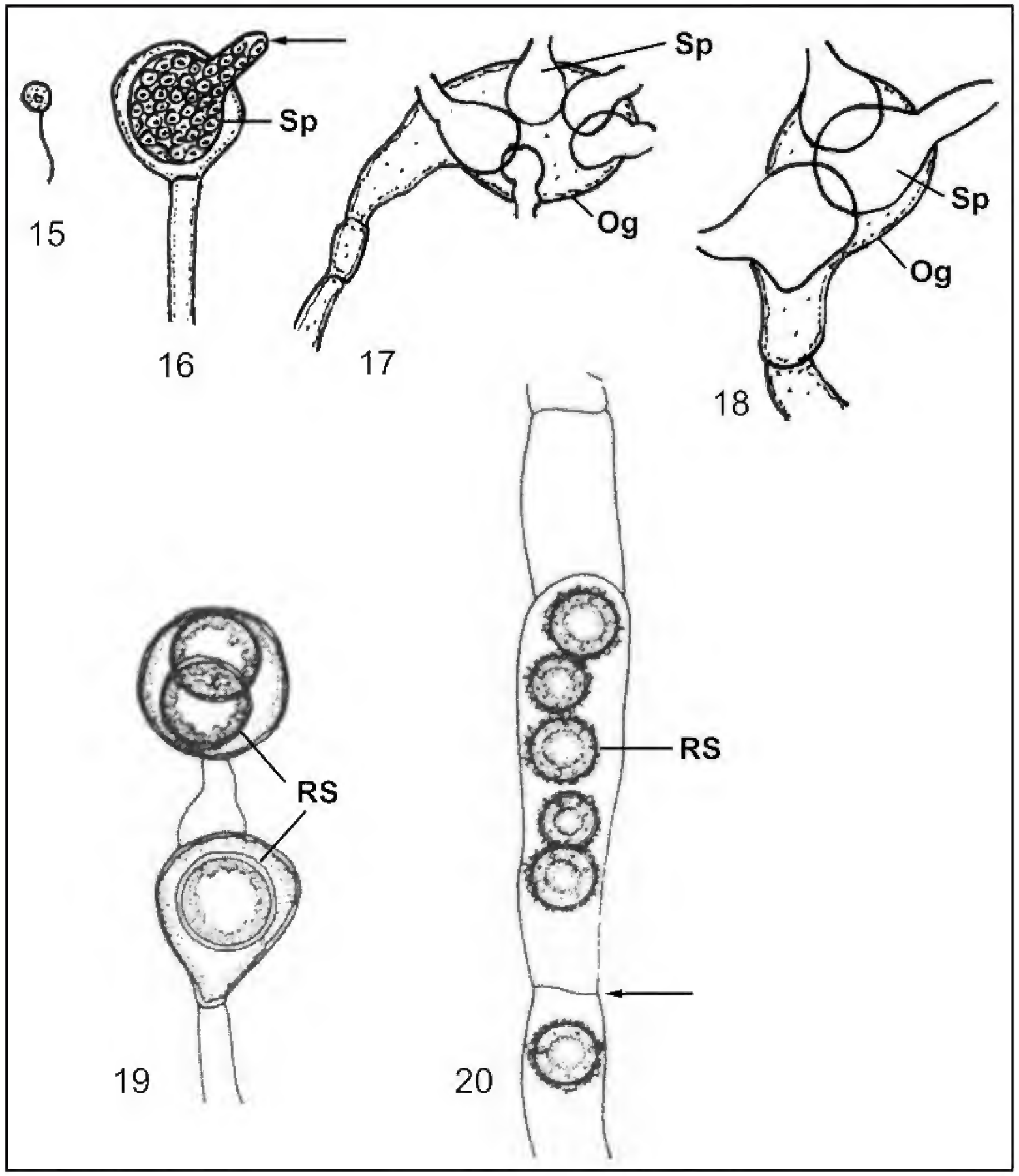


Figs. 1-8: ***Dictyomorpha dioica***. **1**: Sporangia (Sp), generally spherical in form, in host (*Achlya*); zoospores released at tip of host filament (arrow). **2**: Discharged sporangia (arrow). **3**: Variable (often elongate) shape of posteriorly uniflagellate zoospores. **4**: Zoospores infecting host (*Achlya*) by their apical ends. **5**: Young thalli (Th) developing in host. **6**: Maturing, and also empty, sporangia inside apical portion of host hypha. **7**: Maturing sporangia (Sp); note exit-papilla (arrow). **8**: Mature sporangium; zoospores released, through papilla, laterally from host filament. Figs. 1-3 after Pringsheim (1860), 4-5 after Mullins (1961), 6 after Couch (1939) and Mullins (1961), 7-8 after Mullins (1961).



Figs. 9-14: ***Dictyomorpha dioica***. **9**: Range of zoospore form. **10**: 'Zoospores' fusing, as gametes, to form zygote. **11-14**: Resting spores (RS) in various maturation stages (in hyphae of host, *Achlya*); note 'extra cells' ('host compartments' = Hc), each surrounding one to several resting spores (Figs. 11, 12, 14); wall of 'host compartments' sometimes thickened (12); outer resting-spore wall roughened, reticulate or 'undulate' (11-12), sometimes sub-spiny (13). Figs. 9-10 after Mullins (1961), 11-12 after Couch (1939), 13 after Mullins (1961), 14 based generally on Couch (1939) and Karling (1977), among others.



Figs. 15-20: ***Dictyomorpha pythiensis***. **15**: Zoospore. **16**: Sporangium (Sp) in host (*Pythium*), exit-tube forming (arrow). **17-18**: Emptied sporangia (Sp) in host oogonium (Og); sporangia generally oval, exit-tubes persistent. **19**: Resting spores (RS) in host oogonia. **20**: Resting spores (RS), inside host hypha, exhibiting minutely but distinctly spiny walls; special 'host compartments' (potentially enclosing resting spores) lacking, but extra hyphal septa may form (arrow). Figs. 15-20 after Sarkar and Dayal (1988).

Mandevilla torosa (Apocynaceae), treated as having two allopatric intergrading varieties in Mexico

Billie L. Turner
Plant Resources Center The University of Texas Austin, TX 78712
billie.turner@austin.utexas.edu

ABSTRACT

Mandevilla coulteri S. Wats. is treated as a variety within the widespread **M. torosa**, the former largely confined to north-central Mexico, but passing into var. **torosa** southwards.

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Mandevilla torosa (Jacq.) Woodson, the Type from Jamaica, with populations extending into southern Mexico, is treated as composed of two intergrading varieties, a more southern typical var. **torosa** and a more northern var. **coulteri**, the latter largely confined to Coahuila, Nuevo Leon and Tamaulipas but grading into var**. torosa** south-wards; this dichotomy was first proposed by Williams (1999) in his doctoral thesis but not published. Unfortunately he applied the varietal name "karwinskii" to the more northern elements, the latter typified by a Karwinski collection from southern Mexico (probably Oaxaca). He should have adopted the varietal name "coulteri," for the northern populations, which is typified by a Coulter collection from the state of Coahuila.

I have more or less adopted the key to the two varieties provided by Williams, but with the addition of leaf shapes:

Mandevilla torosa (Jacq.) Woodson, Ann. Missouri Bot. Gard. 19: 64 1932.

Key to varieties

1. Corolla tubes mostly 4-6 mm long; leaves mostly obovate, or rounded at their apices; plants typically vine-like.....var. **torosa**
1. Corolla tubes mostly 7-9 mm long; leaves mostly elliptic with acute apices; plants perennial herbs or subshrubs.....var. **coulteri**

var. **coulteri** (S. Wats) B.L. Turner, **var. nov.**
Based upon *Echites coulteri* S. Wats., Proc. Amer. Acad. Arts 18: 113. 1883.

The name is typified by *Coulter 957*, this collected in the state of Coahuila, S. of Saltillo, according to Williams (1999). There are some 60 specimens of the variety at LL- TEX, all remarkably alike and possessing the characters attributed to var. **coulteri** by the present author. Williams applied the name var. *karwinskii* to all of these sheets, largely because he had not examined the type concerned; Alvorado-Cadenas and Morales (2014) correctly note its synonymy under their concept of **Mandevilla torosa;** they also placed var. **coulteri** in synonymy under **M. torosa**, which belies the taxonomy proposed herein.

Distribution of the two taxa in Mexico, along with intermediates, is show in Fig. 1. Williams mapped, but did not annotate or name the intermediate sheets. Those sheets which I have accepted as intermediates (and mapped accordingly) follow:

TAMAULIPAS (two collections): Hidalgo, *Hinton et al. 24709*; 11 mi W of Victoria. *Graham & Johnston 4133.*

SAN LUIS POTOSI: *Barkley et al. 854; Irving 167* [both collections near Cd. de Maiz].

QUERETARO: (7 sheets, all intermediate) *Carranza 930*; *Carranza & Silva 5873a*; *Fernandez & Rzedowski 3425*; *Rubio 1866, 1250*; *Servin 1042*; *Zamudio & Carranza 6651.*

Alvorado-Cadenas and Morales (2014) noted two collections of **M. torosa** from Veracruz that I have not examined. I have mapped these as var. **torosa**, but these too might be intermediates. Indeed, with DNA analysis it is possible that typical **Mandevilla torosa** (in Mexico) will be found confined to the Yucatan Peninsula and that intermediates between these and var. **coulteri** are deserving of formal recognition.

It should be noted that Morales (1998) stated "*Mandevilla karwinskii* is closely related to *M. torosa* but can be recognized by its very narrowly elliptic (or almost linear) to spatulate leaf blades and mucroulate to rarely acute leaf apices, its usually sub-erect habit, and the usually continuous to obscurely moniliform follicles." He did not clearly delineate the two taxon, either morphologically or geographically, as perceived by Williams, or the present author.

LITERATURE CITED

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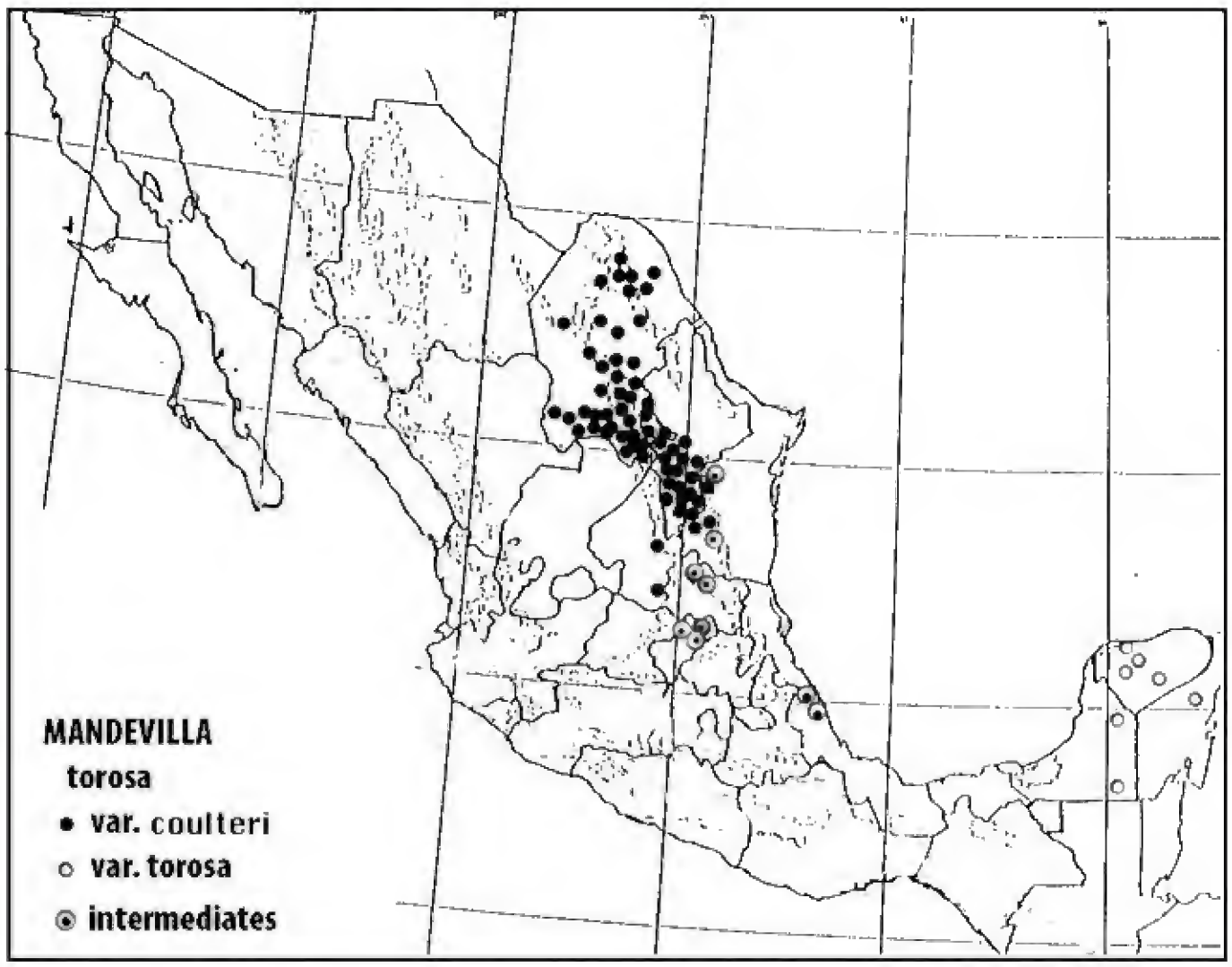

Fig. 1. Distribution of *Mandevilla torosa* in Mexico