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RRH: Morphometrics of North American creosote bush

Morphometric analysis of the North American creosote bush (*Larrea tridentata,* Zygophyllaceae) and the microspatial distribution of its chromosome races

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Abstract Polyploidy is a major mechanism of chromosome evolution and speciation in flowering plants. Delineation of polyploid populations as species or subspecies is complicated because of the uncertainties of distinguishing closely-related diploids and polyploids in field conditions. Here we evaluate the practical identification of polyploids—using geographic distributions and morphological features—in the North American creosote bush (*Larrea tridentata*, Zygophyllaceae). Regarded as a classical autopolyploid complex, *L. tridentata* comprises diploids, tetraploids, and hexaploids distributed throughout the Chihuahuan, Sonoran, and Mojave Deserts. Transect sampling on cytotype range boundaries revealed strong spatial structuring of the chromosome races, and infrequent sympatry, over small spatial scales (<50 km). Intercytotype hybrids were rare, with putative triploids and pentaploids comprising <1% of the sampled plants (N = 1,197). In morphometric analyses of stem, flower, and leaf characteristics (N = 14 traits), we found significant ploidy effects in several cytotype comparisons; reproductive structures and foliage characteristics were particularly discriminatory. Significant transect and ploidy × transect effects for most traits suggest, however, that environmental and genic factors influence plant architecture and morphology. Nonetheless, discriminant function analysis with a combined morphometric data set correctly assigned 68.9% of plants to ploidal level. Pollen diameters increased significantly with ploidal level, providing another potentially informative trait for comparisons of reproductive plants. Taken together, the spatial distribution and morphometric data presented here suggest that the majority of *L. tridentata* plants could, in principle, be assigned to cytotype in the field. However, because of potential misclassifications we suggest recognition of the *L. tridentata* cytotypes as subspecies.

Keywords Desert Plants; Geographic Variation; Polyploidy; Incipient Speciation; Cryptic Species

Introduction

Genome duplication poses many challenges to plant systematics. While polyploidy creates strong reproductive barriers—an important consideration for species delimitation—recently diverged diploid and polyploid populations may be difficult to distinguish on the basis of simple morphological and anatomical characteristics (Joly and Bruneau 2007; Mandáková and Münzbergová 2008; Španiel et al. 2008). Moreover, due to its very rapid pace, polyploidization is not usually associated with reciprocal monophyly or clear-cut molecular marker differences that are increasingly leveraged in taxonomic studies (Hardy and Vekemans 2001; Sang et al. 2004; Ramsey et al. 2008; Rebernig et al. 2010). Polyploidy thus remains an enigma to evolutionary biologists: it is widely recognized as a mechanism of plant speciation, but usually considered inadequate for the delimitation of species or subspecies (Lewis 1967; Mayr 1992; Thompson and Lumaret 1992; Soltis et al. 2007). Moreover, because allopolyploids sometimes exhibit a characteristic "intermediacy" of morphology and molecular marker profiles that is absent from autopolyploids, the mode of origin for a polyploid lineage may strongly influence its ultimate taxonomic treatment (Lewis 1980; Ramsey and Schemske 1998, 2002; Soltis et al. 2010; Ramsey and Ramsey 2014).

Although plant systematists have traditionally favored conservative treatments of polyploid species complexes (Stebbins 1950, 1974; Lewis 1967; Raven et al. 1968; Grant 1971; Cronquist 1978), taxonomic opinion may be changing in the 21st century. For example, there is increasing recognition of cryptic species in a wide range of animal and plant groups (McDaniel and Shaw 2003; Bickford et al. 2007; Dennis and Hellberg 2010;

Scheffers et al. 2012). Technological advancements related to morphometric measurements, DNA content estimation, and environmental data may streamline the identification of diploid vs. polyploid populations in the wild (Husband and Schemske 1998; Brysting and Elven 2000; Suda et al. 2007; Mandáková and Münzbergová 2008; Laport et al. 2012; Laport et al. 2013). Finally, conservation efforts (Severns and Liston 2005) or management of invasive species (Treier et al. 2009; Green et al. 2013) in some plant genera may be dependent upon recognition of polyploid populations. Soltis et al. (2007) argue that, in the best-studied autopolyploid systems, diploids and tetraploids warrant recognition at the species level and that, by extension, botanists may be greatly underestimating the number of plant species worldwide. Taxonomic treatment of polyploid complexes may also have significant impacts on perceived geographic patterns of plant species diversity, due to the frequent occurrence of polyploidy in herbaceous taxa of temperate and arctic regions (Löve 1964; Johnson et al. 1965; Löve and Löve 1967; Grant 1981; Ramsey and Ramsey 2014).

Here we consider two fundamental issues related to the taxonomic recognition of diploid, tetraploid, and hexaploid populations of an autopolyploid species complex, the North American creosote bush (*Larrea tridentata* (DC.) Coville; Zygophyllaceae). First, we assess the microspatial distributions of the cytotypes where they naturally come into contact, which speaks to classification of populations on the basis of ecological associations and geographic data. Secondly, we investigate the morphometric features of the cytotypes growing in natural contact zones at their respective range boundaries, which is critical for the practical field identification in the absence of chromosome counts or genetic analyses. Building upon recent studies of creosote bush phylogeography

(Laport et al. 2012), and geographic distributions (Laport et al. 2013), these analyses allow us to evaluate the taxonomic treatment of *L. tridentata* chromosome races in the context of morphological, phylogenetic, and biological species concepts.

Materials and Methods

Study system

Larrea tridentata is a widespread and dominant species of the North American warm deserts. The taxon comprises three cytotypes and a geographically restricted tetraploid variety (*Larrea tridentata* var. *arenaria* L. D. Benson) distributed across the Chihuahuan Desert (diploid; $2n=2x=26$), Sonoran Desert (principally tetraploid; $2n=4x=52$), and Mojave Desert (principally hexaploid; $2n=6x=78$) (Yang 1968; Yang and Lowe 1968; Yang 1970; Barbour 1969). While exhibiting complex spatial distributions, these chromosome races are seemingly allopatric and rarely co-occur within populations (Hunter et al. 2001; Laport et al. 2012). *Larrea tridentata* was derived by long-distance dispersal from South America in the Pleistocene or late Pliocene; five species of *Larrea* are recognized in Chile and Argentina, including the sister species to the North American creosote bush, *Larrea divaricata* Cav. (Raven 1963; 1972; Barbour 1969; Porter 1974; Hunter et al. 2001; Lia et al. 2001; Laport et al. 2012). Evidence from cytogenetic, isozyme, and DNA molecular studies suggest an autopolyploid origin of the *L. tridentata* chromosome races (Sternberg 1976; Wells and Hunziker 1976; Hunziker et al. 1972, 1977; Poggio et al. 1989; Cortes and Hunziker 1997).

Transect establishment and ploidy analysis

To identify parapatric and sympatric occurrences of cytotypes, we established six transects (30-50 km in length) across the range boundaries of diploid and tetraploid plants (southeastern Arizona) as well as tetraploid and hexaploid plants (southwestern Arizona and southeastern California), based on a prior investigation of *L. tridentata* cytogeography in the southwestern U.S. and northern Mexico (Laport et al. 2012; Fig. 1). Range boundaries with hexaploids were studied separately for the tetraploid sand dune endemic, *L. tridentata* var. *arenaria*, and the more widespread tetraploid *L. tridentata* (two transects each). Transects were established in 2008-2010 from January through April by random sampling of plants at localities selected at 3-7 km intervals to span the distance between previously identified occurrences of pure cytotype populations (Laport et al. 2012). All populations occurred in natural, relatively undisturbed desert habitats offset at least 25-50 m from paved roads and agricultural land use. Vegetation composition varied substantially across sites and transects, reflecting their occurrence in the Chihuahuan, Sonoran, and Mojave Deserts (Laport and Minckley 2013; Laport unpublished data); in all cases, *L. tridentata* was a dominant component of the plant community.

Flow cytometry was performed on desiccated leaf tissues to evaluate the cytotype composition of study sites and the occurrence of interploidy hybrids, using previously-described methods (Laport et al. 2012). At each site we sampled 20-75 plants. We permanently marked each plant with uniquely numbered metal tags and recorded their

GPS coordinates to enable relocation and plant measurements conducted in spring 2010. Vouchers for all specimens were deposited at the University of Arizona Herbarium (ARIZ 418859-419563). Morphometric analyses were performed on 12 randomly-selected plants at each single-cytotype site and 12 plants of each cytotype (24 total plants) at mixed-cytotype sites.

Stem traits

The heights of the three tallest stems of each plant (Fig. 2A) were measured in the field using a telescoping surveyor's rod (Crain Philly Metric Leveling/Measuring Rod, Model 92023); additionally, the nodes in the top 30 cm of each stem were counted. Because the dune endemic *L. tridentata* var. *arenaria* is reported to have distinctive pendulous stem tips (Turner et al. 1995, Felger 2000), we evaluated the "droopiness" of the three tallest stems (for all plants and cytotypes) by measuring the lateral and vertical projections of stems from a point 20 cm below the stem terminus. For evaluating this trait, the stem was manually held in a fully erect position to measure 20 cm from the terminus, and then allowed to "droop" naturally for measurement using a 30 cm ruler.

Flower structures

Through the natural flowering periods of each cytotype, ten haphazardly-selected flowers on each plant were measured in the field at maturity. For each flower, we used digital calipers (Control Company, Model 3415) to determine (1) length and (2) width of two haphazardly-selected petals; (3) length of two haphazardly-selected stamens; and (4) length of the pistil (Fig. 2B).

Leaf traits

On each sampled plant, three branches (~15 cm in length) were collected, pressed, and returned to the lab for measurements of leaf characteristics. Leaf traits were measured on five haphazardly-selected leaves from each of the three branches using digital calipers. Measured leaf traits included: (1) petiole length; (2) midrib length; (3) right leaflet width; (4) left leaflet width; (5) sinus width; (6) total leaf length; and (7) total leaf width (Fig. 2C). These measured traits include two characters (total leaf width and sinus width) that were previously measured by Yang et al. (1977) in comparisons of *L. tridentata* and the South American *L. divaricata*.

Pollen size

As an approach to evaluating anatomical and cell size differences between cytotypes, we measured the size of pollen grains collected in 2011 and 2012. In total, we sampled 35 plants from eight field sites (San Pedro 2, 3, 4; Kofa 2; Salton 4; Algodones N 4, Algodones S 3; Figs. 1, 3), including representatives of diploid, tetraploid, and hexaploid *L. tridentata* as well as *L. tridentata* var. *arenaria*. Pollen was collected from dehiscing anthers of multiple flowers on each plant using strips of felt glued to toothpicks; pooled pollen for each plant was stored in glass vials prior to staining on glass microscope slides with lactophenol aniline blue (Kearns and Inouye 1993). For each plant, we measured diameters of at least 25-50 stained pollen grains, as viewed under a compound light microscope (400× total magnification) equipped with an ocular micrometer (N = 1,125 pollen grains).

Data analysis

To evaluate spatial clustering of chromosome races in sites where they co-occurred, we used the *R* package FOSSIL to calculate intra- and inter-cytotype distances between all individuals using the latitude and longitude coordinates of each individual plant (Vavrek 2011). We then converted the distance matrix into a list with the *R* package RESHAPE (Wickham 2007) and generated a null distribution for cytotype pairwise-distances based on 1,000 random permutations of each point locality; differences between observed intra- and intercytotype distance values were compared to null distributions, with a significance threshold of P=0.05.

Statistical analyses of all morphometric measurements were conducted in JMP (v.9; SAS Institute Inc., Cary, NC, U.S.A.). The ratio of lateral stem tip projection to vertical stem tip projection was computed as a dimensionless value from untransformed measurements. All other measurements were natural log-transformed prior to analysis to improve the distribution of residuals.

Comparison of stem, flower, and leaf traits were made independently for the different transect types (i.e., separately for diploid-tetraploid, tetraploid-hexaploid, and tetraploid *L. tridentata* var. *arenaria*-hexaploid transects) using multivariate analysis of variance. MANOVA models included ploidy, transect, ploidy × transect, and population (nested under ploidy and transect) as effects. We then used univariate ANOVA to analyze stem, leaf, and flower traits individually, with correction by sequential Bonferroni (Rice 1989). To evaluate the utility of morphometric traits to distinguish *L. tridentata* chromosome races, we performed discriminant function analysis (DFA) across all six transects. DFA models were performed separately by trait type (stem, flower, leaf) and with a combined data set (14 total traits). Consistency of trait differences between cytotypes were also inspected visually using two-character scatterplots.

We compared pollen diameters (untransformed) between chromosome races using an ANOVA model that included ploidy and plant (nested under ploidy) as factors. A Tukey HSD test was used for post-hoc comparisons between cytotypes (Tukey 1953; Kramer 1956).

Results

Flow cytometry

Analysis of nuclei fluorescence via flow cytometry revealed the occurrence of diploid and tetraploid cytotypes (San Pedro and Gila transects) as well as tetraploid and hexaploid cytotypes (Salton, Kofa, Algodones N, and Algodones S transects), as anticipated from the location of transects on the range boundaries of the *L. tridentata* chromosome races (Figs. 1, 3). DNA content histograms exhibited coefficients of variation averaging 3.67% (range 1.65 – 8.41%), with approximately one-tenth of the samples exhibiting coefficient of variation values exceeding 5.00%. Inferred DNA contents for the combined data set (N = 1,197 plants) were trimodal and corresponded to the diploid, tetraploid, and hexaploid values previously identified from broad geographic sampling across the Chihuahuan, Sonoran, and Mojave Deserts (Fig. 3; Laport et al. 2012). Nonetheless, shifts in inferred DNA content were observed across transects, with the Salton transect in particular having relatively lower values (Fig. 3). For the purposes of this study, we recognize plants with 2C values of 0.85 – 1.30 pg as diploids, 1.80 – 2.30 pg as tetraploids, and 3.00 – 3.70 pg as hexaploids. Plants on the Salton transect with 2C values of 2.70 – 3.10 pg are also recognized as hexaploid, with the caveat that further cytogenetic analysis is warranted for these populations (see Discussion). Putative F1 hybrids were identified on the Gila and San Pedro transects (one triploid each) and the Kofa and Algodones S transects (two pentaploids each); these putative hybrids exhibited DNA content estimates that were clearly intermediate to the above-defined 2C categories on their respective transects.

Cytotype distributions

The Gila and San Pedro transects (boundary of Chihuahuan and Sonoran Deserts in southeastern Arizona) comprised four sites (N = 85 cytotyped plants) and six sites (N = 323 cytotyped plants), respectively (Fig. 1). Sites on the Gila transect were essentially “pure” diploid or tetraploid, with one location (Gila 3) having a single triploid plant amidst 24 sampled diploids (Fig. 3). Sites on the San Pedro transect displayed a similar pattern where most sites were “pure” diploid or tetraploid and one location (San Pedro 4) had a single triploid plant among 49 sampled diploids. However, one location along this transect (San Pedro 3) also had large numbers of both cytotypes (52 diploid plants and 23 tetraploid plants) (Figs. 1, 3). Permutation tests revealed significant spatial clustering of chromosome races at San Pedro 3, with average plant distances of 77.1 m (diploid-diploid), 85.4 m (diploid-tetraploid), and 44.7 m (tetraploid-tetraploid).

The Kofa transect (southwestern Arizona) and Salton transect (southeastern California) had three sites (N = 160 cytotyped plants) and seven sites (N = 243 cytotyped plants), respectively (Fig. 1). Sites on the Salton transect comprised tetraploid or hexaploid plants, with no mixture of plants within populations; on the Kofa transect, one location (Kofa 2) harbored tetraploids (12 plants), pentaploids (2 plants), and hexaploids (36 plants) (Figs. 1, 3). Cytotype distributions were significantly clustered at the Kofa 2 site, with average plant distances of 110.6 m (tetraploid-tetraploid), 146.3 m (tetraploid-hexaploid), and 116.5 m (hexaploid-hexaploid).

The Algodones N and S transects—located in southeastern California on the boundary between the tetraploid dune endemic *L. tridentata* var. *arenaria* and hexaploid *L. tridentata*—had six sites (N = 217 cytotyped plants) and five sites (N = 169 cytotyped plants), respectively (Fig. 1). Although sites on these transects predominately comprised tetraploid or hexaploid plants, there were three mixed sites on the Algodones N transect and one mixed site on the Algodones S transect (Figs. 1, 3). Algodones N 4 and Algodones S 3 in particular harbored co-existing cytotypes (11 tetraploids and 6 hexaploids; 31 tetraploids, 2 pentaploids, 17 hexaploids, respectively). Permutation tests indicated that tetraploid *L. tridentata* var. *arenaria* plants were not spatially clumped at Algodones N 4 or Algodones S 3; average plant distances were 99.3 and 118.6 m (tetraploid-tetraploid) vs. 97.4 and 109.0 m (tetraploid-hexaploid), respectively, for these two sites. Hexaploids were spatially clumped at Algodones S 3 (mean distances of 88.1 m for hexaploid-hexaploid comparisons vs. 109.0 m hexaploid-tetraploid comparisons) but not Algodones N 4 (mean distances of 69.4 m for hexaploid-hexaploid comparisons vs. 97.4 m hexaploid-tetraploid comparisons).

Stem characteristics

Cytotypes were moderately distinguished by stem traits, especially for comparisons of diploids vs. tetraploids and *L. tridentata* var. *arenaria* vs. hexaploids (Table 1; Fig. 4A). For the diploid-tetraploid transects, MANOVA (Wilks' $\Lambda = 0.017$, $F_{357,715} = 5.689$, $P<0.0001$) indicated significant effects of ploidy ($F_{3,238} = 181.661$, $P<0.0001$), transect ($F_{3,238} = 8.643$, $P<0.0001$), ploidy × transect ($F_{3,238} = 12.867$, $P<0.0001$), and population ($F_{18,674} = 10.327$, $P<0.0001$). Univariate ANOVA indicated significant ploidy effects for individual stem characters after correction by sequential Bonferroni (Table 1). In general, we found tetraploids to have taller stems with fewer terminal nodes and increased pendulance compared to diploid plants (Fig. 4A). Stem height and node density seem especially discriminatory, with the vast majority of diploids being ≤1.5 m in height and approaching (or exceeding) a node density of one per centimeter of terminal stem length (Table 1; Fig. 4A).

Comparisons of tetraploids and hexaploids also indicated differences for stem traits (Table 1). For the tetraploid-hexaploid transects of *L. tridentata*, MANOVA (Wilks' $\Lambda = 0.007$, $F_{321,642} = 8.280$, $P<0.0001$) revealed significant effects of ploidy ($F_{3,214}$

= 64.189, $P<0.0001$), transect ($F_{3,238} = 42.291$, $P<0.0001$), ploidy × transect ($F_{3,214} = 10.290$, $P<0.0001$), and population ($F_{15,591} = 24.181$, $P<0.0001$). In univariate ANOVA tests, however, only node density differed significantly between tetraploid and hexaploid cytotypes (Table 1). For transects comparing tetraploid *L. tridentata* var. *arenaria* and hexaploid *L. tridentata*, MANOVA (Wilks' $\Lambda = 0.010$, $F_{405,817} = 7.276$, $P<0.0001$) indicated significant effects of ploidy ($F_{3,272} = 267.900$, $P<0.0001$), transect ($F_{3,272} = 49.398$, $P<0.0001$), ploidy × transect ($F_{3,272} = 10.674$, $P<0.0001$), and population ($F_{24,789} = 22.673$, $P<0.0001$). Univariate tests revealed significant ploidy effects for stem height and pendulance but not node density (Table 1). In general, stem traits appear to have minimal utility in distinguishing tetraploid and hexaploid plants of *L. tridentata*, but are reasonably discriminating for tetraploid *L. tridentata* var. *arenaria* vs. hexaploid *L. tridentata*. The vast majority of surveyed *L. tridenata* var. *arenaria* plants were more than 1.25 m in height and had stem pendulance indices (horizontal:vertical ratios) greater than 0.30 (Table 1; Fig. 4A).

Flower characteristics

Floral characters were more consistently different between *L. tridentata* chromosome races than vegetative characters, and are of potential utility in discriminating diploid vs. tetraploid as well as tetraploid vs. hexaploid cytotypes. For the diploid-tetraploid transects, MANOVA (Wilks' $\Lambda = 0.023$, $F_{476,4301} = 14.096$, $P<0.0001$) indicated significant effects of ploidy ($F_{4,1075} = 328.608$, $P<0.0001$), transect ($F_{4,1075} = 27.562$, $P<0.0001$), ploidy × transect ($F_{4,1075} = 49.063$, $P<0.0001$), and population ($F_{24,3751} =$

16.518, $P<0.0001$). Univariate ANOVA indicated ploidy effects for all individual floral characters, including petal length and width, stamen length, and pistil length; in all cases, diploids had smaller structures than tetraploids (Table 2). Stamen and petal lengths were particularly discriminatory in diploid-tetraploid transects, as these reproductive structures were <9.0 mm in length for the vast majority of diploid plants (Table 2; Fig. 4B).

Floral characters also differed substantially between tetraploid and hexaploid plants. For the tetraploid-hexaploid transects of *L. tridentata*, MANOVA (Wilks' Λ = 0.028, $F_{404,3597}$ = 12.862, $P<0.0001$) revealed significant effects of ploidy ($F_{4,899}$ = 267.638, $P<0.0001$), transect ($F_{4,899}$ = 60.442, $P<0.0001$), ploidy × transect ($F_{4,899}$ = 12.601, $P<0.0001$), and population ($F_{20,2983}$ = 13.556, $P<0.0001$). For transects comparing tetraploid *L. tridentata* var. *arenaria* and hexaploid *L. tridentata*, MANOVA (Wilks' Λ = 0.023, $F_{512,4609}$ = 14.054, $P<0.0001$) identified significant effects of ploidy ($F_{4,1152}$ = 238.994, $P<0.0001$), transect ($F_{4,1152}$ = 17.089, $P<0.0001$), ploidy × transect ($F_{4,1152}$ = 8.730, $P<0.0001$), and population ($F_{32,4250}$ = 13.786, $P<0.0001$). As observed for diploid-tetraploid transects, all four individual floral traits differed significantly between cytotypes in the tetraploid-hexaploid transects (Table 2). Petal length and stamen length distinguished the vast majority of tetraploid and hexaploid plants in their respective transects, with hexaploids having the larger reproductive structures (Table 2; Fig. 4B). However, the absolute size range for stamen and petals (approximately 7.0 to 12.0 mm) was relatively constant across transect type; and thus tetraploids occupied the upper end of the absolute size distribution (9.0 to 12.0 mm) in diploid-tetraploid transects and the lower end of the absolute size distribution (7.0 to 9.0 mm) in tetraploid-hexaploid transects (Table 2; Fig. 4B).

Leaf characteristics

Cytotypes were moderately distinguished by leaf characters, which unlike stem traits may be particularly useful for comparisons of tetraploid and hexaploid plants. For the diploid-tetraploid transects, MANOVA (Wilks' $\Lambda = 0.022$, $F_{833,11840} = 10.341$, $P<0.0001$) indicated significant effects of ploidy ($F_{7,1692} = 215.119$, $P<0.0001$), transect ($F_{7,1692} = 36.002$, $P<0.0001$), ploidy × transect ($F_{7,1692} = 24.489$, $P<0.0001$), and population ($F_{42,7940} = 41.752$, $P<0.0001$). Univariate ANOVA indicated ploidy effects for many, but not all, individual leaf characters after correction by sequential Bonferroni: midrib length, leaf and leaflet blade widths, and sinus width comparisons were statistically significant but petiole length and leaf length were not (Table 3). In general, we found diploids to have broader leaves and shorter midribs than tetraploids. The distinguishing power of measured leaf traits may be limited, however, by population and transect-level variability (Table 3; Fig. 4C). For example, there was a linear relationship between leaf length and leaf width on the San Pedro transect, with diploids tending to have broader leaves for a given leaf length; on the Gila transect, however, diploids generally had both broader and longer leaves than tetraploids (Fig. 4C).

Leaf characteristics may be of greater utility in comparisons of tetraploid and hexaploid cytotypes than either stem or floral characters. For the tetraploid-hexaploid transects of *L. tridentata*, MANOVA (Wilks' $\Lambda = 0.030$, $F_{749,11668} = 10.184$, $P<0.0001$) revealed significant effects of ploidy ($F_{7,1668} = 165.543$, $P<0.0001$), transect ($F_{7,1668} = 59.104$, $P<0.0001$), ploidy × transect ($F_{7,1668} = 71.951$, $P<0.0001$), and population

($F_{693,11664} = 7.375$, $P<0.0001$). With the exception of petiole length, all individual leaf traits differed significantly between tetraploids and hexaploids in ANOVA tests (Table 3). For transects comparing tetraploid *L. tridentata* var. *arenaria* and hexaploid *L. tridentata*, MANOVA (Wilks' $\Lambda = 0.022$, $F_{1001,14132} = 10.261$, $P<0.0001$) indicated significant effects of ploidy ($F_{7,2019} = 92.987$, $P<0.0001$), transect ($F_{7,2019} = 13.423$, $P<0.0001$), ploidy × transect ($F_{7,2019} = 27.889$, $P<0.0001$), and population ($F_{56,10878} = 32.044$, $P<0.0001$). For univariate ANOVA tests, blade size and sinus traits differed significantly between cytotypes while petiole and midrib lengths did not (Table 3). For both *L. tridentata* and *L. tridentata* var. *arenaria*, we found tetraploids to have shorter and narrower leaves than hexaploids (Fig. 4C). For comparisons of tetraploid and hexaploid *L. tridentata* in particular, the vast majority of tetraploids had leaves less than 7.0 mm in length and width (Table 3; Fig. 4C).

Combined morphometric analyses

For discriminant function analyses performed across all six transects, we found significant cytotype effects for stem traits (Wilks' $\Lambda = 0.663$, $F_{9,2648} = 54.079$, $P<0.0001$; 48.0% of plants correctly classified to cytotype), flower traits (Wilks' $\Lambda = 0.790$, $F_{12,9205} = 71.366$, $P<0.0001$; 39.4% of plants correctly classified to cytotype), and leaf traits (Wilks' $\Lambda = 0.458$, $F_{21,16503} = 245.439$, $P<0.0001$; 46.6% of plants correctly classified to cytotype). Discriminant function analysis based on the complete morphology data set was likewise significant (Wilks' $\Lambda = 0.288$, $F_{42,3104} = 38.560$, $P<0.0001$) with 68.9% of plants assigned correctly to ploidal level.

Pollen size

Pollen grain diameter differed statistically among the sampled *L. tridentata* (ANOVA, $F_{34,1090} = 10.122$, $P<0.0001$), with significant effects of ploidy ($F_{3,1090} = 50.056$, $P<0.0001$) and plant specimen ($F_{31,1090} = 6.556$, $P<0.0001$; Fig. 5). In post-hoc tests, tetraploid *L. tridentata* did not differ significantly from tetraploid *L. tridentata* var. *arenaria*, but all other comparisons (diploid-tetraploid, tetraploid-hexaploid, diploid-hexaploid) were statistically significant. On average, pollen from tetraploid plants was approximately 1.2 μm larger than pollen from diploid plants, and pollen from hexaploid plants was approximately 1.2 μm larger than pollen from tetraploid plants. Nonetheless, there was overlap in pollen grain sizes sampled from diploid, tetraploid, and hexaploid plants (Fig. 5).

Characteristics of putative hybrids

Putative F1 hybrids had phenotypic characteristics that were in most regards intermediate to those of nearby diploids and tetraploids (for triploids) or tetraploids and hexaploids (for pentaploids) (Fig. 4). For example, triploid plants at San Pedro 4 and Gila 3 exhibited stamen and petal lengths of approximately 9.0 to 10.0 mm, while reproductive structures of diploids and tetraploids were in general smaller and larger, respectively (Fig. 4B). Stem heights of triploids were similarly intermediate to those of nearby diploids and tetraploids, but stem node density differed markedly between the two discovered triploid plants (Fig. 4A).

Pentaploids discovered at study site Kofa 2 were intermediate in height and stem node density to co-occurring tetraploid and hexaploid plants (Fig. 4A, C). However, one pentaploid at Kofa 2 had relatively large reproductive structures, as typically observed in hexaploids, while the other had smaller reproductive structures, as typically observed in tetraploids (Fig. 4B). The two pentaploids identified at the *L. tridentata* var. *arenaria*-hexaploid *L. tridentata* site Algodones S 3 exhibited variable morphological and architectural features (Fig. 4).

Discussion

Early students of chromosome evolution were sympathetic to the taxonomic recognition of allo- and autopolyploids at the species or subspecies level (Blakeslee 1921; Müntzing 1936, 1937; Clausen et al. 1940, 1945; Löve 1964). Since the mid-1900s, however, plant systematists have struggled with the classification of polyploid complexes, with two principle arguments against the delineation of taxa on the basis of ploidal level (Mosquin 1966; Lewis 1980; Soltis et al. 2007). The first and more common argument is that chromosome number cannot reliably be diagnosed in the field or herbarium, and that it is thus impractical to recognize polyploids (particularly autopolyploids) in the framework of Linnean classification (Cronquist 1978; Mayr 1992). The second argument, sometimes voiced by botanists studying polyploid groups, is that closely-related diploids and polyploids may have non-trivial gene exchange via semi-fertile F1 hybrids (i.e. triploids, pentaploids, etc.) or *de novo* polyploid formation from unreduced gametes—and may fail the criterion of reproductive isolation required under the biological species concept (Lewis 1967; Thompson and Lumaret 1992). A final complication to the systematics of polyploids was the widespread belief, advocated by Stebbins (1950, 1974), that allopolyploids vastly outnumber autopolyploids in nature. While a handful of plant biosystematists pushed for greater recognition of autopolyploidy (Mosquin 1966, 1967; H. Lewis 1967; W. Lewis 1967; Estes 1969), polyploids lacking cytogenetic or morphological evidence of hybridity were for the most part viewed as an evolutionary curiosity through the latter half of the twentieth century (Soltis et al. 2007; Ramsey and Ramsey 2014).

As a practical solution to the issue of sibling species, Grant (1981) suggested that the morphological and biological species concepts have fundamentally different goals, and that evolutionary biologists may study polyploidy as a mechanism of speciation (via reproductive isolation) even while considering them conspecific with their diploid progenitors. However, this approach is not entirely satisfactory (Severns and Liston 2005; Soltis et al. 2007; Judd et al. 2007), as species are widely viewed as fundamental biological and ecological units that are not expected to circumscribe multiple reproductively isolated units adapted to different environmental conditions (Scheffers et al. 2012). In the context of rare plant conservation, for example, it is potentially problematic that diploid and polyploid populations are usually considered a single taxon.

The goal of this study was to evaluate the potential for taxonomic delineation of autopolyploid chromosome races using integrative geographical and morphological data in a manner that is practical for field conditions and in herbaria. These issues have been considered in a number of polyploid groups studied over the past 70 years (Smith 1946; Skalińska 1947; Solbrig 1964, 1971; Mosquin 1967; MacDonald and Chinappa 1988; Hardy et al. 2000; Nakagawa 2006). For example, Smith (1946) compared several morphological and architectural traits for diploid, tetraploid, and hexaploid cytotypes of *Sedum pulchellum* in the greenhouse, while Solbrig (1971) performed morphometric analyses of diploid and tetraploid populations of *Gutierrezia sarothrae* sampled in the field (including what is perhaps the first quantitative attempt to resolve phylogenetic relationships within a polyploid species complex). For its part, prior work on *L. tridentata* sends mixed signals on morphological divergence of the chromosome races. By growing diploid, tetraploid, and hexaploid seedlings under controlled conditions, Barbour (1969) showed that the cytotypes differed in some morphological features even when small. Yang (1970) argued that creosote bush cytotypes could be recognized by geographically-structured phenotypic variation, but these studies included few sympatric or parapatric comparisons of the chromosome races that could disentangle environmental vs. genetic influences on morphology. Thus, Whitford et al. (1996) and De Soyza et al. (1997) interpreted canopy architecture differences among plants growing in New Mexico (probable diploids) and California (probable hexaploids) on distinctive soil substrates as reflecting differences in water availability rather than ploidy. While inspired by prior work in *L. tridentata* and other autopolyploid complexes, this project increases the number of plant specimens and traits considered, expands the scale of sampling and geographic replication used, emphasizes attributes of plants growing in the wild, and uses multivariate statistics. The North American creosote bush is now among the best-studied autopolyploid complexes in the context of geographic distribution and morphometric trait variation—what conclusions can be reached about the practical identification of its chromosome races?

While dependent on prior sampling efforts, geography provides very good inference of ploidal level in creosote bush, especially at medium to large spatial scales (Laport et al. 2012; Laport and Minckley 2013; Laport et al. 2013). Diploids in particular occur allopatrically to polyploids across vast stretches of the North American desert landscape and apparently share an abrupt range boundary with tetraploids that coincides with the margins of the Chihuahuan and Sonoran Deserts. Range boundaries between tetraploid and hexaploid *L. tridentata* are more convoluted and spatially localized, but are nonetheless well-documented and predictable to some degree by ecological niche modeling (Laport et al. 2013). Moreover, transect sampling on the diploid-tetraploid and tetraploid-hexaploid range boundaries reveals micro-spatially structured cytotype distributions, which correlates with ecological differences, and infrequent sympatry (Fig. 1). In sympatry, cytotypes are spatially clustered, and F1 hybrids (e.g., triploids and pentaploids) are rare, comprising ~0.5% of the plants sampled in this study (Figs. 1, 3). While more complex than envisioned by classical studies (Yang 1967, 1970; Yang and Lowe 1968; Barbour 1969; Hunziker et al. 1977), the ecological and spatial distributions of *L. tridentata* chromosome races are adequately distinct for inclusion within taxonomic descriptions of the group.

In general, flow cytometry provides very good inference of ploidal level in the genus *Larrea* (Laport et al. 2012), but in this study we observed lower-than-expected DNA contents on the Salton transect as well as bimodal DNA content distribution among putative tetraploids on the Salton and Kofa transects (Fig. 3). Aneuploidy and intraspecific genome size variation are well-known from flowering plants, and further cytogenetic analyses (including chromosome counts) are warranted for populations on the aforementioned transects. On the other hand, the variation observed here for *L. tridentata* may simply reflect methodological issues (e.g., use of desiccated vs. fresh tissue, staining times, cytometer performance) or local environmental factors that affect the quality of plant materials (Kron et al. 2007; Bainard et al. 2011). For example, we used trout erythrocyte nuclei (TENs; BioSure, Grass Valley, CA) as an internal control for some of the samples, but most of the samples were run with a plant control material (*L. tridentata* tissue previously determined to be diploid or hexaploid; Laport et al. 2012). As *L. tridentata* tissue has high concentrations of phenolic compounds (Hunziker et al. 1977), the introduction of additional phenolic compounds into flow cytometry samples from *L. tridentata* internal control material relative to the TENs may influence binding of propidium iodide (PI) to DNA (Loureiro et al. 2006). Alternatively, the variation in DNA content estimates may relate to climatic variation and the season of collection (Knight and Ackerly 2002) or the age of sampled tissue, all of which may influence chromosome integrity, cytosolic compound concentrations, and the availability of DNA to PI binding (Loureiro et al. 2006; Bainard et al. 2011). In flow cytometry analyses of a subset of specimens on the Salton and Kofa transects that originally yielded unusually high or low DNA content estimates (N=30 samples), we recovered more typical 2C values (data not shown). Thus, we anticipate that apparent variation in DNA content is more likely to represent sampling or methodological factors than aneuploidy.

Ultimately, taxonomic delineation, of *L. tridentata* chromosome races depends on diagnostic morphological characteristics. Prior studies suggested that creosote bush cytotypes have different phenotypic tendencies, while acknowledging potential overlap of trait values between ploidal levels and the difficulty of collapsing complex traits, like growth form or canopy architecture, into simple measurements (Yang 1967, 1970; Barbour 1969; Whitford et al. 1996). For example, tetraploids are sometimes described as being tall and having relatively open canopy architecture, while hexaploids tend to be shorter and have conical or spherical canopies (Turner et al. 1995; Whitford et al. 1996). The dune creosote bush (*L. tridentata* var. *arenaria*) is reported to be tall-statured with distinctive pendulous stems (Benson and Darrow 1981). Stem trait measurements performed in this study revealed significant differences between the *L. tridentata* chromosome races (Table 1; Fig. 4A). Notably, tetraploids were found to be taller than diploids on the San Pedro and Gila transects, while the dune creosote had taller and more pendulous stems than nearby hexaploid specimens of *L. tridentata*. The principle difficulty with stem traits appears to be geographic variability; absolute stem trait values of tetraploids in particular varied greatly across Arizona and California (Fig. 4A), probably reflecting local soil attributes and water availability. Because of its narrow geographic distribution, specimens of *L. tridentata* var. *arenaria* may nonetheless be reliably diagnosed by stem attributes (Table 1).

Flower characteristics exhibit increased size with increased ploidal level: tetraploids have larger petals and longer stamens than diploids in eastern Arizona, while hexaploids have larger petals and longer stamens than tetraploids in western Arizona and eastern California (Table 2). Despite these intuitive differences in the ranking of flower sizes, in absolute terms the distribution of floral trait values was surprisingly consistent across our study transects (7 – 12 mm for petal lengths, 6 – 10 mm for stamen length, etc.; Fig. 4B). Thus, the flowers of tetraploids tended to have petals >9 mm in length when approaching the range of diploids (eastern Arizona; San Pedro and Gila transects) but <9 mm in length when approaching the range of hexaploids (western Arizona and California; Kofa, Salton, and Algodones transects) (Table 2; Fig. 4B). Reliable taxonomic use of flower characters would require testing of trait values across a broader range of the Chihuahuan, Sonoran, and Mojave Deserts than was sampled for this study. In the northwest quadrant of its range (north of the Mexican border and west of central Arizona), however, tetraploid and hexaploid *L. tridentata* seem to be identifiable on the basis of flower traits.

Unlike flowers, foliage size characters of *L. tridentata* do not increase in proportion to ploidal level, and there is more trait value overlap between cytotypes for foliage characters than floral characters (Tables 1, 2; Fig. 4). However, the consistently small leaf sizes (blade width, blade length, etc.) of tetraploids compared to diploids and hexaploids suggest that foliage characters may prove to be the more discriminatory trait type for diagnosing cytotype in the absence of ecological or geographic data (Table 3). Throughout the areas evaluated in this study (with the exception of a few sites on the San Pedro transect) tetraploids had leaf lengths and widths of 6 mm or less, while nearby diploids and hexaploids tended to have longer and broader leaves (Fig. 4C).

While qualitative character states and complex phenotypic traits may be informative for delineating the chromosome races of *L. tridentata*, we have emphasized simple and linear measurements because these are highly repeatable and testable using statistical approaches. Preliminary analyses of trait ratios and allometries derived from the aforementioned simple measurements were no better at discriminating chromosome races, and we are uncertain what other morphological trait types may be candidates for exploration. Beyond potential taxonomic uses, morphometric trait variation in *L. tridentata* warrants further investigation in the context of ecological adaptation. For example, cytotype differences in flower sizes may reflect intercytotype competition and reinforcement due to potential impacts of floral traits and display size on pollinator behavior; similarly, variation in leaf size may reflect different ecophysiological strategies involving transpiration and evaporative cooling.

Nomenclature for the North American creosote bush has a long and complicated history involving many authors, multiple species epithets, and at least two genera names (Cavanilles 1800; De Candolle 1824; Moricand 1833; Frémont 1845; Wislizenus 1848; Coville 1893; Vail 1899; Hunziker 1977). Investigation of this history—including extant voucher and type specimens—should be performed in conjunction with measurements of leaf and floral traits across a broader range of environments in the Chihuahuan, Sonoran, and Mojave Deserts. In this manner, taxonomic delineation of the *L. tridentata* chromosome races may proceed in confidence and with proper naming based on code-compliant rules. Nonetheless, we may speculate here on possible taxonomic schemes that could reasonably be applied to the North American creosote bush.

As reported above, the cytotypes of *L. tridentata* exhibit many distinctive features in their spatial distributions and habitat associations. Cytotypes rarely co-occur within populations—even at range boundaries between ploidal levels—and F1 hybrids comprise a very small fraction of plants sampled to date (Figs. 1, 3). There is also a discrete chloroplast DNA sequence difference between diploid and polyploid *L. tridentata* (Laport et al. 2012), suggesting that the cytotypes are, to some degree, genetically distinct. Despite statistical differences in their morphometric features, however, the cytotypes of *L. tridentata* cannot be unambiguously identified in the absence of chromosome counts or flow cytometry (Tables 1-3; Fig. 4). Therefore, recognition of the *L. tridentata* ploidal levels as subspecies appears justified. This designation is commonly applied in diploid animal and plant systems for populations with degrees of morphological, ecological, and genetic divergence comparable to those we have found (Rosenblum and Harmon 2010; Ng and Glor 2011). While highlighting the biological distinctions between ploidal levels and their apparent trajectory towards incipient speciation, subspecies ranking would not presume the degree of evolutionary independence carried by species ranking.

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Table 1. Mean trait values and statistical analyses (ANOVAs) for measured *L. tridentata* stem characteristics.

Trait	Comparison	Result	Effect test	*F*-statistic	*P*
Height (m)	2*x* (mean=1.33, SE=0.026) 4*x* (mean=1.71, SE=0.038)	ANOVA, $F_{119,240} = 9.039$ $P < 0.0001$	ploidy	$F_{1,110} = 27.619$	<0.0001
			transect	$F_{1,110} = 3.308$	0.0717
			transect × ploidy	$F_{1,110} = 3.034$	0.0843
			population [transect, ploidy]	$F_{6,110} = 2.241$	0.0445
Height (m)	4*x* (mean=1.15, SE=0.028) 6*x* (mean=1.08, SE=0.036)	ANOVA, $F_{107,216} = 21.246$ $P < 0.0001$	ploidy	$F_{1,99} = 1.783$	0.1849
			transect	$F_{1,99} = 0.098$	0.7577
			transect × ploidy	$F_{1,99} = 0.733$	0.3939
			population [transect, ploidy]	$F_{5,99} = 4.794$	0.0006
Height (m)	var. *arenaria* 4*x* (mean=1.79, SE=0.038) 6*x* (mean=1.17, SE=0.028)	ANOVA, $F_{135,275} = 23.671$ $P < 0.0001$	ploidy	$F_{1,124} = 56.936$	<0.0001
			transect	$F_{1,124} = 6.033$	0.0154
			transect × ploidy	$F_{1,124} = 2.324$	0.1299
			population [transect, ploidy]	$F_{8,124} = 5.263$	<0.0001
Number of Nodes	2*x* (mean=31.86, SE=0.609) 4*x* (mean=25.64, SE=0.389)	ANOVA, $F_{119,240} = 9.238$ $P < 0.0001$	ploidy	$F_{1,110} = 34.517$	<0.0001
			transect	$F_{1,110} = 0.058$	0.8099
			transect × ploidy	$F_{1,110} = 2.082$	0.1519
			population [transect, ploidy]	$F_{6,110} = 2.242$	0.0444

Number of Nodes	4*x* (mean=38.81, SE=0.667) 6*x* (mean=31.90, SE=0.515)	ANOVA, $F_{107,216} = 9.546$ $P < 0.0001$	ploidy	$F_{1,99} = 24.699$	<0.0001
			transect	$F_{1,99} = 16.967$	<0.0001
			transect × ploidy	$F_{1,99} = 2.671$	0.1054
			population [transect, ploidy]	$F_{5,99} = 1.739$	0.1326
Number of Nodes	var. *arenaria* 4*x* (mean=38.19, SE=0.382) 6*x* (mean=37.11, SE=0.545)	ANOVA, $F_{135,275} = 4.888$ $P < 0.0001$	ploidy	$F_{1,124} = 1.941$	0.1661
			transect	$F_{1,124} = 15.896$	0.0001
			transect × ploidy	$F_{1,124} = 0.044$	0.8345
			population [transect, ploidy]	$F_{8,124} = 1.939$	0.0599
Stem pendulance	2*x* (mean=0.03, SE=0.005) 4*x* (mean=0.11, SE=0.013)	ANOVA, $F_{119,240} = 1.746$ $P = 0.0001$	ploidy	$F_{1,110} = 22.595$	<0.0001
			transect	$F_{1,110} = 0.435$	0.5108
			transect × ploidy	$F_{1,110} = 0.020$	0.8884
			population [transect, ploidy]	$F_{6,110} = 0.425$	0.8611
Stem pendulance	4*x* (mean=0.26, SE=0.018) 6*x* (mean=0.23, SE=0.030)	ANOVA, $F_{107,216} = 2.241$ $P < 0.0001$	ploidy	$F_{1,99} = 0.209$	0.6482
			transect	$F_{1,99} = 11.838$	0.0009
			transect × ploidy	$F_{1,99} = 0.117$	0.7331
			population [transect, ploidy]	$F_{5,99} = 1.993$	0.0862
Stem pendulance	var. *arenaria* 4*x* (mean=0.41, SE=0.027) 6*x* (mean=0.20, SE=0.019)	ANOVA, $F_{135,275} = 3.148$ $P < 0.0001$	ploidy	$F_{1,124} = 21.755$	<0.0001
			transect	$F_{1,124} = 5.751$	0.0180
			transect × ploidy	$F_{1,124} = 0.1491$	0.7001
			population [transect, ploidy]	$F_{8,124} = 4.224$	0.0002

Table 2. Mean trait values and statistical analyses (ANOVA) for measured *L. tridentata* flower characteristics.

Trait	Comparison	Result	Effect test	*F*-statistic	*P*
Petal length (mm)	2*x* (mean=8.95, SE=0.045) 4*x* (mean=9.72, SE=0.044)	ANOVA, $F_{119,1078} = 18.206$ $P < 0.0001$	ploidy	$F_{1,110} = 23.501$	<0.0001
			transect	$F_{1,110} = 1.054$	0.3068
			transect × ploidy	$F_{1,110} = 0.005$	0.9465
			population [transect, ploidy]	$F_{6,110} = 0.882$	0.5110
Petal length (mm)	4*x* (mean=8.88, SE=0.048) 6*x* (mean=9.45, SE=0.058)	ANOVA, $F_{101,902} = 15.905$ $P < 0.0001$	ploidy	$F_{1,93} = 8.114$	0.0054
			transect	$F_{1,93} = 0.166$	0.6849
			transect × ploidy	$F_{1,93} = 1.700$	0.1954
			population [transect, ploidy]	$F_{5,93} = 1.213$	0.3095
Petal length (mm)	var. *arenaria* 4*x* (mean=8.70, SE=0.038) 6*x* (mean=9.45, SE=0.049)	ANOVA, $F_{128,1156} = 17.928$ $P < 0.0001$	ploidy	$F_{1,118} = 18.607$	<0.0001
			transect	$F_{1,118} = 0.000$	0.9843
			transect × ploidy	$F_{1,118} = 0.089$	0.7661
			population [transect, ploidy]	$F_{8,117} = 2.019$	0.0499
Petal width (mm)	2*x* (mean=4.40, SE=0.031) 4*x* (mean=5.26, SE=0.033)	ANOVA, $F_{119,1078} = 31.263$ $P < 0.0001$	ploidy	$F_{1,110} = 56.626$	<0.0001
			transect	$F_{1,110} = 0.234$	0.6299
			transect × ploidy	$F_{1,110} = 4.024$	0.0473
			population [transect, ploidy]	$F_{6,110} = 0.775$	0.5910
Petal width (mm)	4*x* (mean=4.24, SE=0.027) 6*x* (mean=5.20, SE=0.043)	ANOVA, $F_{101,902} = 27.163$ $P < 0.0001$	ploidy	$F_{1,93} = 55.280$	<0.0001
			transect	$F_{1,93} = 0.227$	0.6351
			transect × ploidy	$F_{1,93} = 2.475$	0.1191
			population [transect, ploidy]	$F_{5,93} = 0.655$	0.6583

Petal width (mm)	var. *arenaria* 4x (mean=4.29, SE=0.029) 6x (mean=4.76, SE=0.035)	ANOVA, $F_{128,1156} = 26.002$ $P < 0.0001$	ploidy	$F_{1,118} = 15.399$	0.0001
			transect	$F_{1,118} = 1.059$	0.3056
			transect × ploidy	$F_{1,118} = 0.918$	0.3400
			population [transect, ploidy]	$F_{8,117} = 0.636$	0.7457
Stamen length (mm)	2x (mean=8.07, SE=0.040) 4x (mean=8.60, SE=0.042)	ANOVA, $F_{119,1078} = 9.866$ $P < 0.0001$	ploidy	$F_{1,110} = 20.235$	<0.0001
			transect	$F_{1,110} = 0.056$	0.8130
			transect × ploidy	$F_{1,110} = 3.446$	0.0661
			population [transect, ploidy]	$F_{6,110} = 1.771$	0.1116
Stamen length (mm)	4x (mean=7.38, SE=0.047) 6x (mean=8.32, SE=0.059)	ANOVA, $F_{101,902} = 15.616$ $P < 0.0001$	ploidy	$F_{1,93} = 24.027$	<0.0001
			transect	$F_{1,93} = 18.084$	<0.0001
			transect × ploidy	$F_{1,93} = 1.356$	0.2472
			population [transect, ploidy]	$F_{5,93} = 1.469$	0.2075
Stamen length (mm)	var. *arenaria* 4x (mean=7.12, SE=0.035) 6x (mean=8.41, SE=0.048)	ANOVA, $F_{128,1156} = 15.861$ $P < 0.0001$	ploidy	$F_{1,118} = 86.107$	<0.0001
			transect	$F_{1,118} = 1.657$	0.2006
			transect × ploidy	$F_{1,118} = 0.546$	0.4615
			population [transect, ploidy]	$F_{8,117} = 1.135$	0.3451
Pistil length (mm)	2x (mean=8.73, SE=0.057) 4x (mean=9.57, SE=0.047)	ANOVA, $F_{119,1078} = 12.654$ $P < 0.0001$	ploidy	$F_{1,110} = 32.110$	<0.0001
			transect	$F_{1,110} = 4.594$	0.0343
			transect × ploidy	$F_{1,110} = 9.107$	0.0032
			population [transect, ploidy]	$F_{6,110} = 1.056$	0.3937

Pistil length (mm)	4*x* (mean=8.51, SE=0.048) 6*x* (mean=9.76, SE=0.066)	ANOVA, $F_{101,902} = 10.489$ $P < 0.0001$	ploidy	$F_{1,93} = 45.690$	<0.0001
			transect	$F_{1,93} = 1.898$	0.1716
			transect × ploidy	$F_{1,93} = 1.160$	0.2842
			population [transect, ploidy]	$F_{5,93} = 2.249$	0.0557
Pistil length (mm)	var. *arenaria* 4*x* (mean=8.46, SE=0.043) 6*x* (mean=9.56, SE=0.050)	ANOVA, $F_{128,1156} = 13.949$ $P < 0.0001$	ploidy	$F_{1,118} = 44.038$	<0.0001
			transect	$F_{1,118} = 2.164$	0.1440
			transect × ploidy	$F_{1,118} = 0.003$	0.9597
			population [transect, ploidy]	$F_{8,117} = 0.618$	0.7616

Table 3. Mean trait values and statistical analyses (ANOVAs) for measured *L. tridentata* leaf characteristics.

Trait	Comparison	Result	Effect test	*F*-statistic	*P*
Petiole length (mm)	2*x* (mean=1.02, SE=0.012) 4*x* (mean=0.95, SE=0.010)	ANOVA, $F_{119,1698} = 6.516$ $P < 0.0001$	ploidy	$F_{1,110} = 3.157$	0.0784
			transect	$F_{1,110} = 11.878$	0.0008
			transect × ploidy	$F_{1,110} = 1.164$	0.2831
			population [transect, ploidy]	$F_{6,110} = 4.193$	0.0008
Petiole length (mm)	4*x* (mean=0.74, SE=0.008) 6*x* (mean=0.78, SE=0.010)	ANOVA, $F_{107,1674} = 7.705$ $P < 0.0001$	ploidy	$F_{1,99} = 1.685$	0.1973
			transect	$F_{1,99} = 0.864$	0.3548
			transect × ploidy	$F_{1,99} = 12.238$	0.0007
			population [transect, ploidy]	$F_{5,99} = 5.483$	0.0002
Petiole length (mm)	var. *arenaria* 4*x* (mean=0.61, SE=0.006) 6*x* (mean=0.61, SE=0.007)	ANOVA, $F_{143,2025} = 6.578$ $P < 0.0001$	ploidy	$F_{1,132} = 0.099$	0.7538
			transect	$F_{1,132} = 0.069$	0.7836
			transect × ploidy	$F_{1,132} = 1.399$	0.2390
			population [transect, ploidy]	$F_{8,132} = 3.044$	0.0036
Midrib length (mm)	2*x* (mean=1.47, SE=0.013) 4*x* (mean=1.69, SE=0.013)	ANOVA, $F_{119,1698} = 10.897$ $P < 0.0001$	ploidy	$F_{1,110} = 25.666$	<0.0001
			transect	$F_{1,110} = 5.1094$	0.0258
			transect × ploidy	$F_{1,110} = 8.421$	0.0045
			population [transect, ploidy]	$F_{6,110} = 1.440$	0.2058

Midrib length (mm)	4*x* (mean=1.48, SE=0.013) 6*x* (mean=1.67, SE=0.016)	ANOVA, $F_{107,1674} = 13.335$ $P < 0.0001$	ploidy	$F_{1,99} = 12.432$	0.0006
			transect	$F_{1,99} = 22.420$	<0.0001
			transect × ploidy	$F_{1,99} = 0.072$	0.7888
			population [transect, ploidy]	$F_{5,99} = 3.851$	0.0031
Midrib length (mm)	var. *arenaria* 4*x* (mean=1.22, SE=0.010) 6*x* (mean=1.27, SE=0.013)	ANOVA, $F_{143,2025} = 17.443$ $P < 0.0001$	ploidy	$F_{1,132} = 0.720$	0.3977
			transect	$F_{1,132} = 0.021$	0.8863
			transect × ploidy	$F_{1,132} = 0.396$	0.5301
			population [transect, ploidy]	$F_{8,132} = 2.032$	0.0473
Right leaflet width (mm)	2*x* (mean=2.95, SE=0.023) 4*x* (mean=2.68, SE=0.020)	ANOVA, $F_{119,1698} = 17.568$ $P < 0.0001$	ploidy	$F_{1,110} = 12.528$	0.0006
			transect	$F_{1,110} = 1.553$	0.2154
			transect × ploidy	$F_{1,110} = 4.103$	0.0452
			population [transect, ploidy]	$F_{6,110} = 4.636$	0.0003
Right leaflet width (mm)	4*x* (mean=2.57, SE=0.020) 6*x* (mean=3.29, SE=0.028)	ANOVA, $F_{107,1674} = 19.973$ $P < 0.0001$	ploidy	$F_{1,99} = 90.188$	<0.0001
			transect	$F_{1,99} = 1.079$	0.3015
			transect × ploidy	$F_{1,99} = 21.040$	<0.0001
			population [transect, ploidy]	$F_{5,99} = 6.580$	<0.0001
Right leaflet width (mm)	var. *arenaria* 4*x* (mean=2.19, SE=0.016) 6*x* (mean=2.54, SE=0.021)	ANOVA, $F_{143,2025} = 17.625$ $P < 0.0001$	ploidy	$F_{1,132} = 25.963$	<0.0001
			transect	$F_{1,132} = 3.707$	0.0563
			transect × ploidy	$F_{1,132} = 0.000$	0.9870
			population [transect, ploidy]	$F_{8,132} = 3.402$	0.0014

Left leaflet width (mm)	2*x* (mean=2.99, SE=0.025) 4*x* (mean=2.70, SE=0.021)	ANOVA, $F_{119,1698} = 18.793$ $P < 0.0001$	ploidy	$F_{1,110} = 12.327$	0.0006
			transect	$F_{1,110} = 2.022$	0.1579
			transect × ploidy	$F_{1,110} = 4.479$	0.0366
			population [transect, ploidy]	$F_{6,110} = 4.573$	0.0004
Left leaflet width (mm)	4*x* (mean=2.63, SE=0.022) 6*x* (mean=3.47, SE=0.033)	ANOVA, $F_{107,1674} = 22.786$ $P < 0.0001$	ploidy	$F_{1,99} = 103.081$	<0.0001
			transect	$F_{1,99} = 0.0029$	0.9569
			transect × ploidy	$F_{1,99} = 31.334$	<0.0001
			population [transect, ploidy]	$F_{5,99} = 7.436$	<0.0001
Left leaflet width (mm)	var. *arenaria* 4*x* (mean=2.21, SE=0.016) 6*x* (mean=2.63, SE=0.023)	ANOVA, $F_{143,2025} = 19.441$ $P < 0.0001$	ploidy	$F_{1,132} = 32.969$	<0.0001
			transect	$F_{1,132} = 1.872$	0.1736
			transect × ploidy	$F_{1,132} = 0.023$	0.8809
			population [transect, ploidy]	$F_{8,132} = 3.734$	0.0006
Sinus width (mm)	2*x* (mean=4.06, SE=0.076) 4*x* (mean=4.50, SE=0.071)	ANOVA, $F_{119,1698} = 3.988$ $P < 0.0001$	ploidy	$F_{1,110} = 7.764$	0.0063
			transect	$F_{1,110} = 0.940$	0.3345
			transect × ploidy	$F_{1,110} = 2.433$	0.1217
			population [transect, ploidy]	$F_{6,110} = 0.821$	0.5558
Sinus width (mm)	4*x* (mean=4.33, SE=0.060) 6*x* (mean=5.65, SE=0.076)	ANOVA, $F_{107,1674} = 10.021$ $P < 0.0001$	ploidy	$F_{1,99} = 22.558$	<0.0001
			transect	$F_{1,99} = 6.289$	0.0138
			transect × ploidy	$F_{1,99} = 0.166$	0.6843
			population [transect, ploidy]	$F_{5,99} = 2.099$	0.0717

Sinus width (mm)	var. *arenaria* 4*x* (mean=3.91, SE=0.041) 6*x* (mean=4.60, SE=0.058)	ANOVA, $F_{143,2025} = 6.985$ $P < 0.0001$	ploidy	$F_{1,132} = 11.758$	0.0008
			transect	$F_{1,132} = 0.435$	0.5106
			transect × ploidy	$F_{1,132} = 2.137$	0.1461
			population [transect, ploidy]	$F_{8,132} = 1.012$	0.4303
Leaf length (mm)	2*x* (mean=6.79, SE=0.037) 4*x* (mean=7.02, SE=0.040)	ANOVA, $F_{119,1698} = 11.725$ $P < 0.0001$	ploidy	$F_{1,110} = 0.770$	0.3822
			transect	$F_{1,110} = 2.235$	0.1378
			transect × ploidy	$F_{1,110} = 19.685$	<0.0001
			population [transect, ploidy]	$F_{6,110} = 5.298$	<0.0001
Leaf length (mm)	4*x* (mean=6.02, SE=0.037) 6*x* (mean=6.84, SE=0.041)	ANOVA, $F_{107,1674} = 16.301$ $P < 0.0001$	ploidy	$F_{1,99} = 34.546$	<0.0001
			transect	$F_{1,99} = 6.048$	0.0157
			transect × ploidy	$F_{1,99} = 5.500$	0.0210
			population [transect, ploidy]	$F_{5,99} = 2.175$	0.0629
Leaf length (mm)	var. *arenaria* 4*x* (mean=4.82, SE=0.030) 6*x* (mean=5.40, SE=0.039)	ANOVA, $F_{143,2025} = 18.784$ $P < 0.0001$	ploidy	$F_{1,132} = 13.934$	0.0003
			transect	$F_{1,132} = 0.740$	0.3912
			transect × ploidy	$F_{1,132} = 7.982$	0.0055
			population [transect, ploidy]	$F_{8,132} = 4.966$	<0.0001
Leaf width (mm)	2*x* (mean=7.88, SE=0.055) 4*x* (mean=7.22, SE=0.053)	ANOVA, $F_{119,1698} = 7.877$ $P < 0.0001$	ploidy	$F_{1,110} = 22.056$	<0.0001
			transect	$F_{1,110} = 1.470$	0.2280
			transect × ploidy	$F_{1,110} = 9.655$	0.0024
			population [transect, ploidy]	$F_{6,110} = 3.036$	0.0087

Leaf width (mm)	4*x* (mean=6.16, SE=0.043) 6*x* (mean=7.53, SE=0.054)	ANOVA, $F_{107,1674} = 16.698$ $P < 0.0001$	ploidy	$F_{1,99} = 75.930$	<0.0001
			transect	$F_{1,99} = 6.866$	0.0102
			transect × ploidy	$F_{1,99} = 8.997$	0.0034
			population [transect, ploidy]	$F_{5,99} = 2.406$	0.0419
Leaf width (mm)	var. *arenaria* 4*x* (mean=5.26, SE=0.031) 6*x* (mean=5.94, SE=0.042)	ANOVA, $F_{143,2025} = 11.432$ $P < 0.0001$	ploidy	$F_{1,132} = 26.691$	<0.0001
			transect	$F_{1,132} = 1.823$	0.1792
			transect × ploidy	$F_{1,132} = 2.839$	0.0943
			population [transect, ploidy]	$F_{8,132} = 2.193$	0.0318

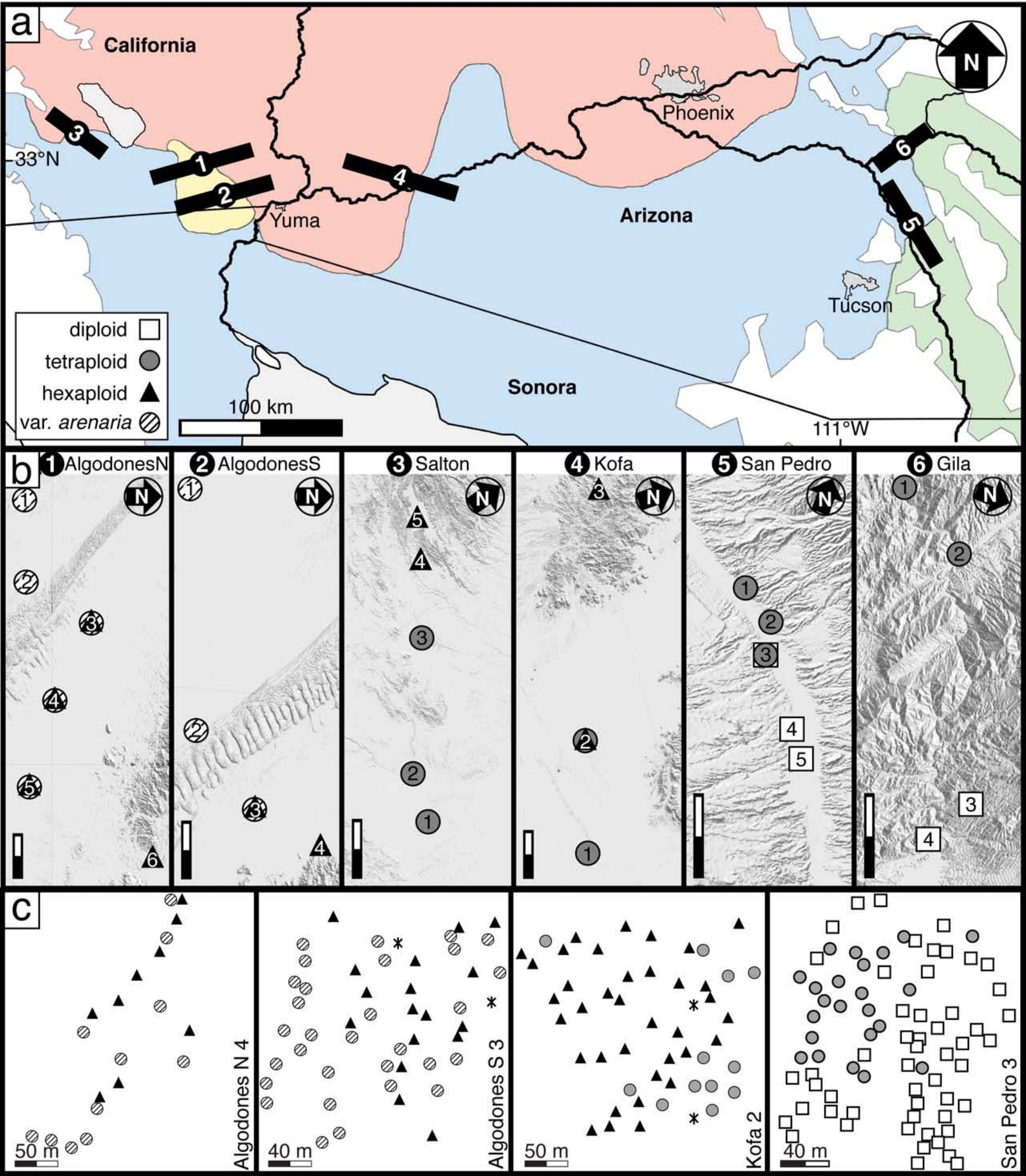
Fig. 1 Distribution of diploid, tetraploid, and hexaploid *L. tridentata* within cytotype contact zones of Arizona and California. **a.** Location of transects investigated in this study. The approximate geographic ranges of diploids, tetraploids, and hexaploids are shown by green, blue, and red shading, respectively. Yellow shading indicates the range of the tetraploid *L. tridentata* var. *arenaria*. Heavy black lines indicate major rivers. **b.** Spatial distributions of cytotype populations within studied transects on exaggerated topographic reliefs. Scale bar in each pane represents approximately 5 km. **c.** Micro-scale distributions of individuals in four sites found to harbor more than one cytotype. Stars at Algodones S 3 and Kofa 2 indicate pentaploids.

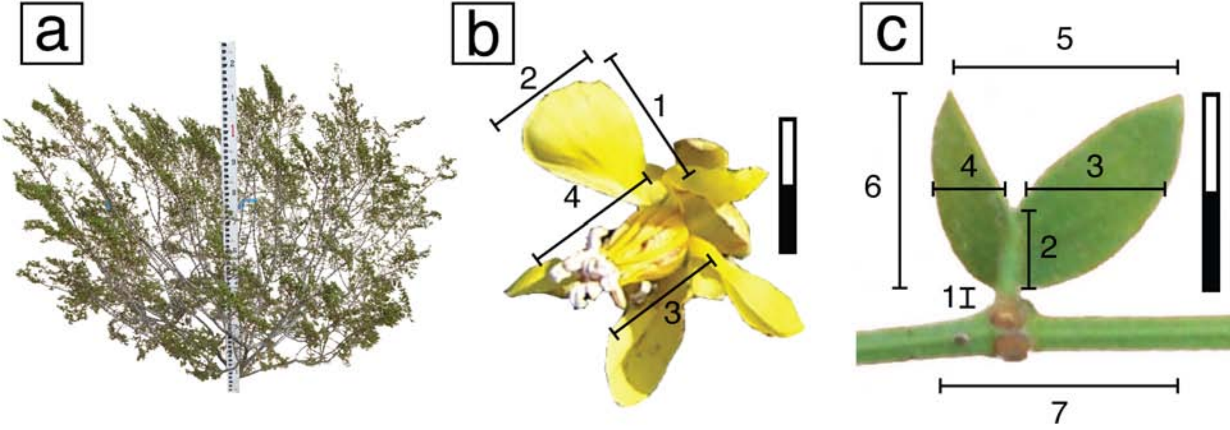
Fig. 2 Diagrams illustrating plant characteristics included in morphometric analyses (Tables 1-3). **a.** Stem traits, including height (for three tallest stems), tip droop, and number of nodes (in top 30 cm of three tallest stems). Surveyor rod in image indicates approximately 1.2m height. **b.** Floral traits, including petal length (1) and width (2), stamen length (3), and pistil length (4). Scale bar represents approximately 6 mm. **c.** Leaf traits, including petiole (1) and midrib lengths (2), widths of right (3) and left (4) leaflets, sinus width (5), leaf length (6), and leaf width (7). Scale bar represents approximately 6 mm.

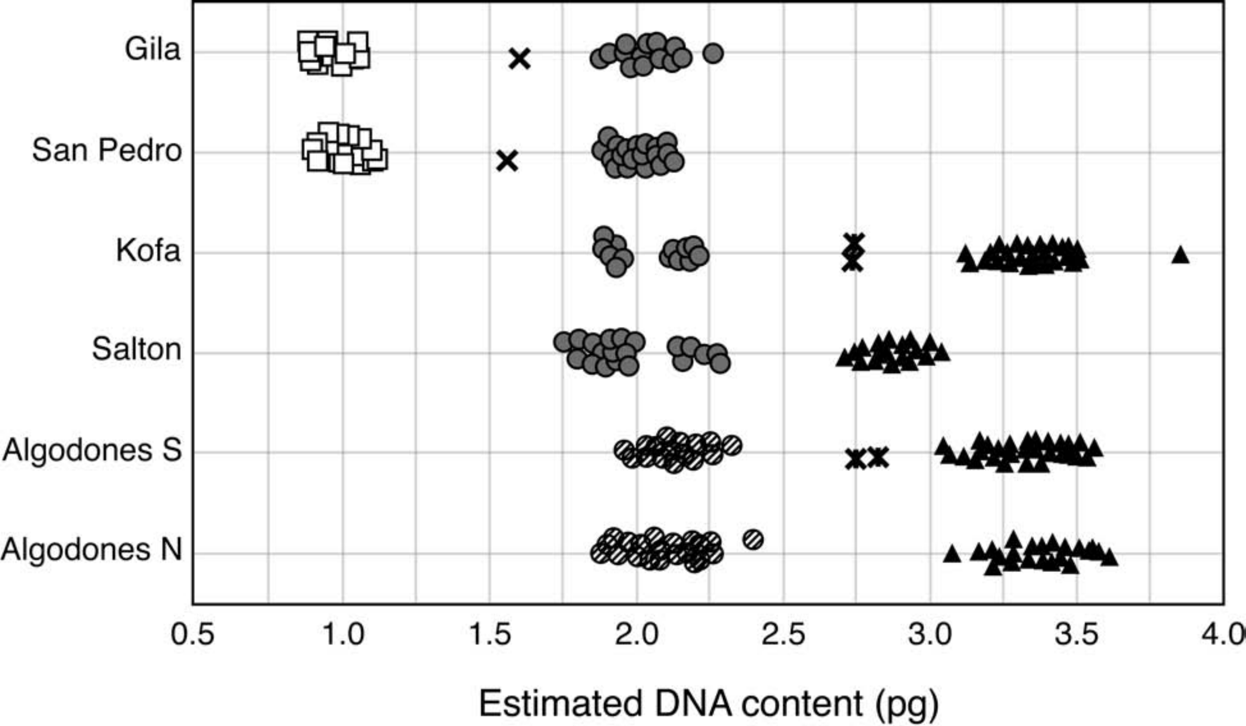
Fig. 3 Inferred plant DNA content values recovered from diploid-tetraploid, tetraploid-hexaploid, and *L. tridentata* var. *arenaria*-hexaploid contact zones. Icon shape indicates our classification of plants as diploids (squares), tetraploids (circles), and hexaploids (triangles). Odd-ploidy F1 hybrid triploids and pentaploids are represented by crosses and stars, respectively.

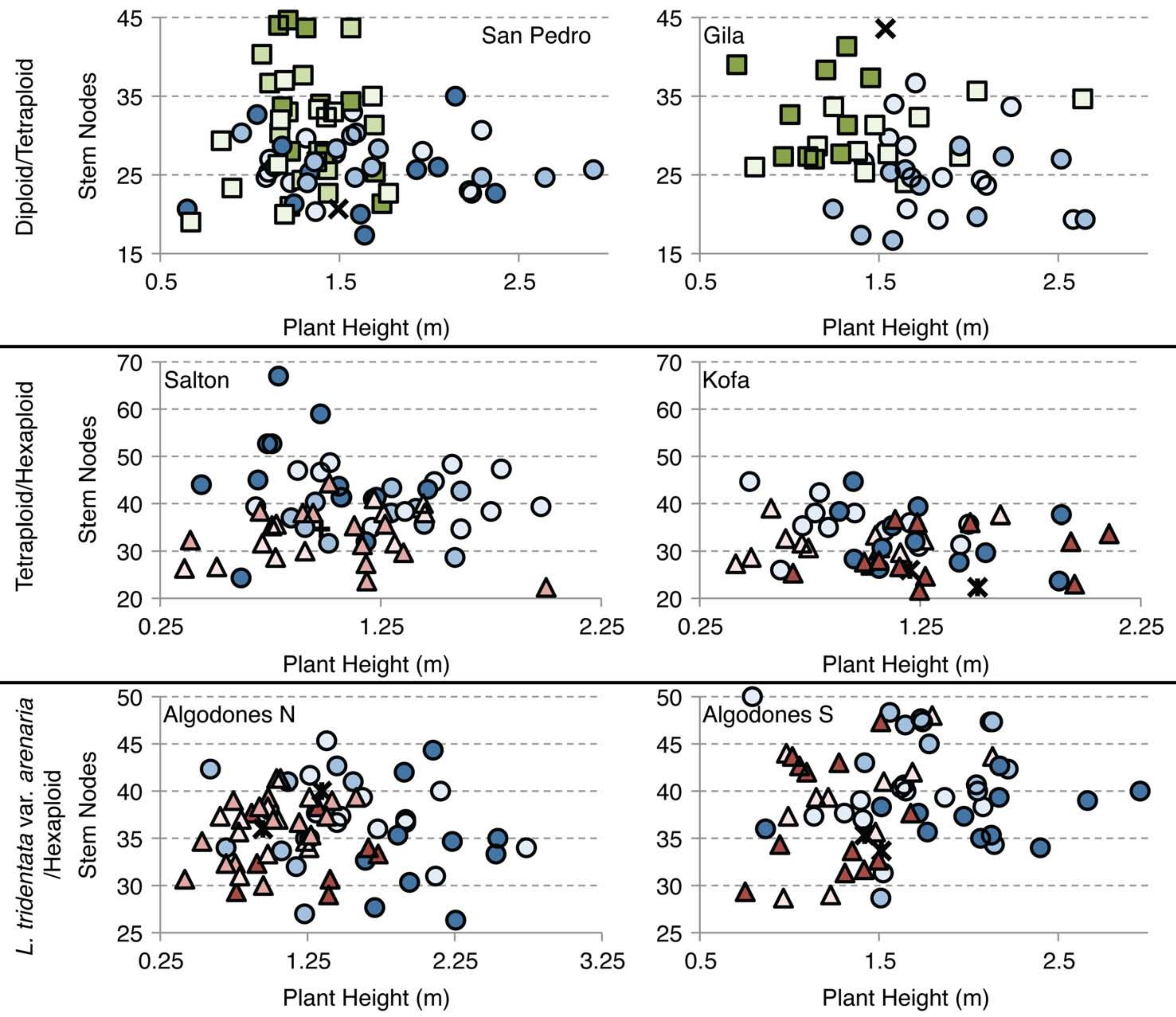
Fig. 4 Biplots showing character values of diploid (squares), tetraploid (circles), and hexaploid (triangles) plants for selected **a.** stem traits, **b.** floral traits, and **c.** leaf traits. Trait values for putative F1 hybrid triploids and pentaploids are shown by crosses and stars, respectively. All plants were measured within cytotype contact zones, and icon coloring indicates the spatial distribution of populations from which plants were sampled (Fig. 2). The darkly shaded icons represent plants from populations that are sympatric or nearly sympatric, while lighter shading indicates plants that are more allopatric.

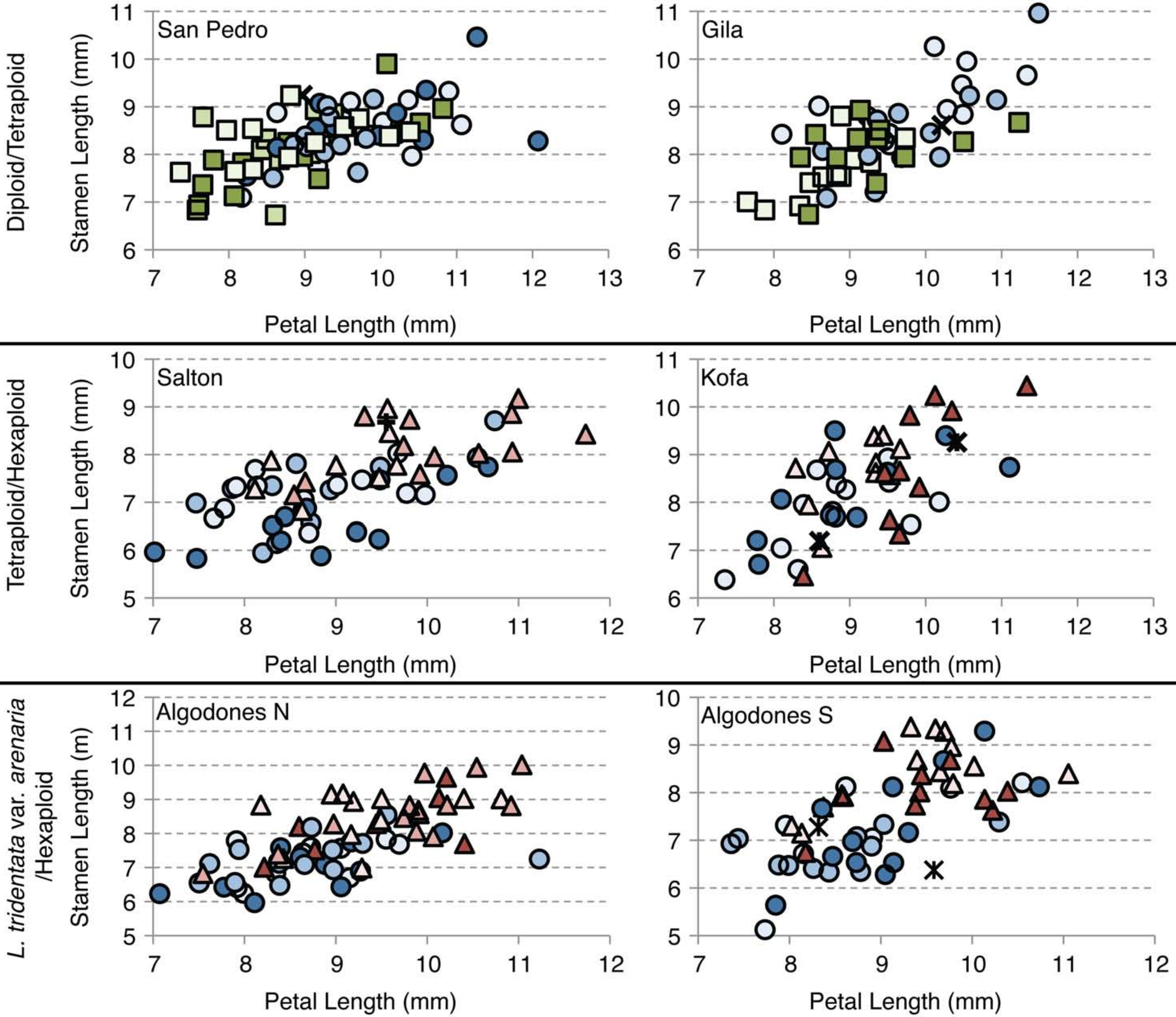
Fig. 5 Histograms displaying the diameter of pollen grains sampled from diploid, tetraploid, and hexaploid *L. tridentata*, and tetraploid *L. tridentata* var. *arenaria*.

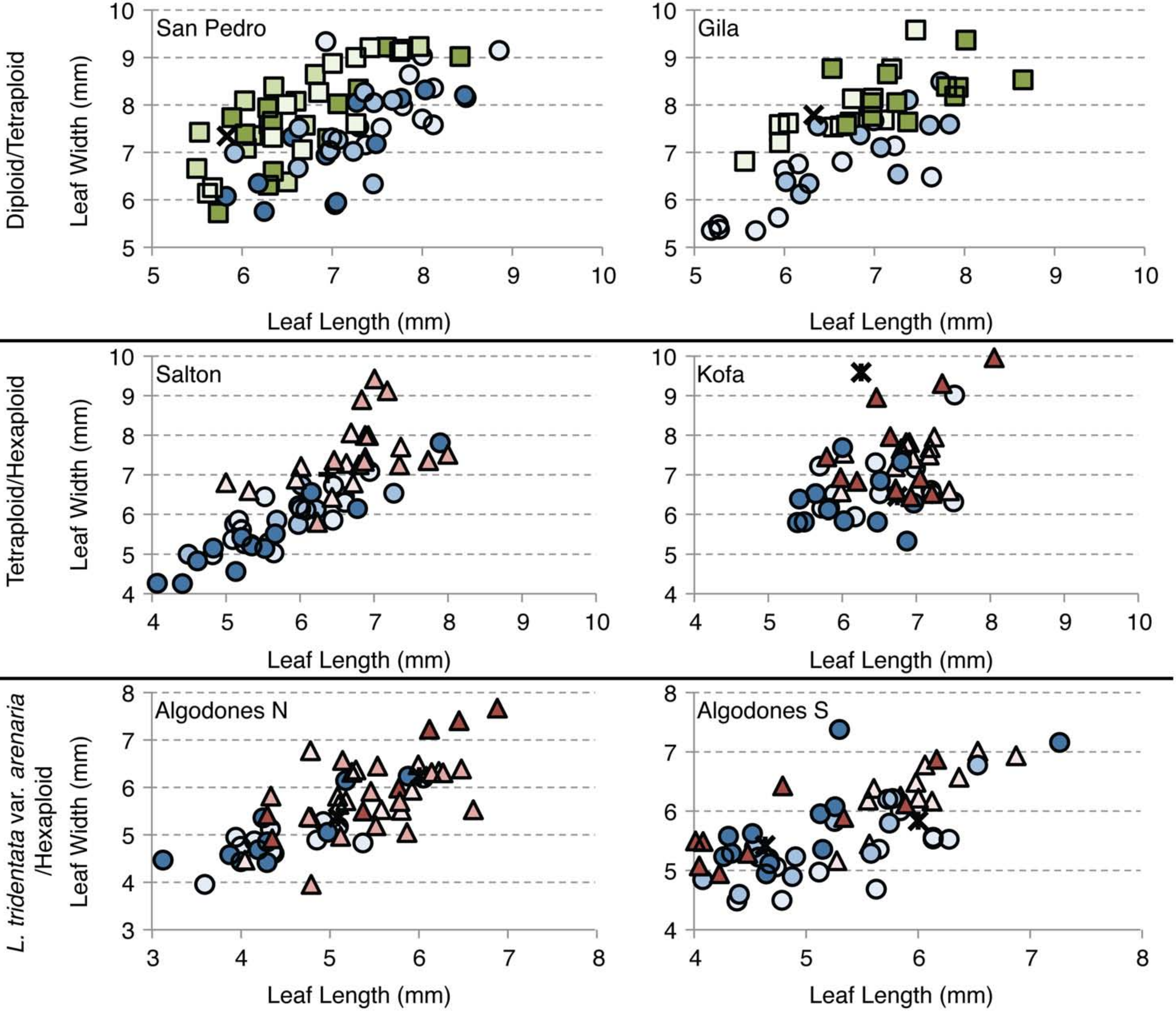

a
California
Arizona
Sonora
Phoenix
Yuma
Tucson
33°N
111°W
N
1
2
3
4
5
6
diploid □
tetraploid ●
hexaploid ▲
var. *arenaria* ▨
100 km
b
1 AlgodonesN
2 AlgodonesS
3 Salton
4 Kofa
5 San Pedro
6 Gila
c
50 m
Algodones N 4
40 m
Algodones S 3
50 m
Kofa 2
40 m
San Pedro 3

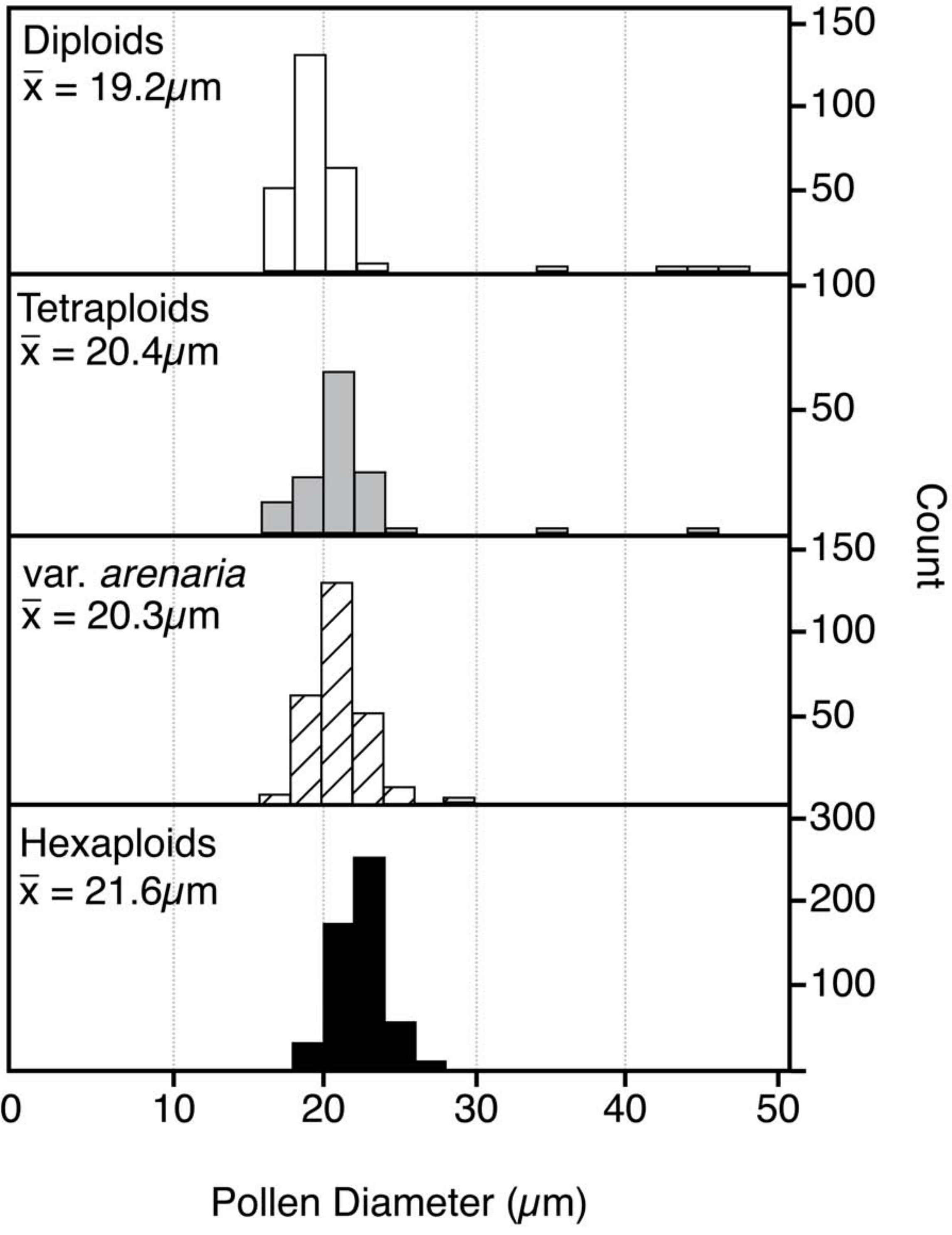

a
b
2
1
4
3
c
5
6
4
3
2
1
7


Gila
San Pedro
Kofa
Salton
Algodones S
Algodones N
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Estimated DNA content (pg)


Diploid/Tetraploid
Stem Nodes
San Pedro
Gila
45
35
25
15
0.5
1.5
2.5
Plant Height (m)
Tetraploid/Hexaploid
Stem Nodes
Salton
Kofa
70
60
50
40
30
20
0.25
1.25
2.25
Plant Height (m)
L. tridentata var. *arenaria* /Hexaploid
Stem Nodes
Algodones N
Algodones S
50
45
40
35
30
25
0.25
1.25
2.25
3.25
0.5
1.5
2.5
Plant Height (m)


Diploid/Tetraploid
San Pedro
Gila
Tetraploid/Hexaploid
Salton
Kofa
L. tridentata var. arenaria /Hexaploid
Algodones N
Algodones S
Stamen Length (mm)
Stamen Length (m)
Petal Length (mm)
5
6
7
8
9
10
11
12
13


Diploid/Tetraploid
Leaf Width (mm)
San Pedro
Gila
Leaf Length (mm)
Leaf Length (mm)
Tetraploid/Hexaploid
Leaf Width (mm)
Salton
Kofa
Leaf Length (mm)
Leaf Length (mm)
L. tridentata var. *arenaria* /Hexaploid
Leaf Width (mm)
Algodones N
Algodones S
Leaf Length (mm)
Leaf Length (mm)
10
9
8
7
6
5
4
3


Diploids
x̄ = 19.2μm
Tetraploids
x̄ = 20.4μm
var. *arenaria*
x̄ = 20.3μm
Hexaploids
x̄ = 21.6μm
150
100
50
100
50
150
100
50
300
200
100
Count
0
10
20
30
40
50
Pollen Diameter (μm)