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Phylogeny and Cyto geography of the North American Creosote Bush (*Larrea tridentata*, Zygophyllaceae)

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Abstract—The North American creosote bush (*Larrea tridentata*, Zygophyllaceae) is a widespread and ecologically dominant taxon of North American warm deserts. The species is comprised of diploid, tetraploid, and hexaploid populations, and touted as a classical example of an autopolyploid taxonomic complex. Here we use flow cytometry and DNA sequence data (non-coding cpDNA and nuclear ribosomal DNA) to evaluate spatial and evolutionary relationships among cytotype races, as well as the origins of the species from its South American ancestors. We find the geographic distribution of North American cytotypes to be highly structured, with limited co-occurrence within populations. Diploids reside only in the Chihuahuan Desert, as reported in previous biosystematic surveys, but tetraploid and hexaploid populations interdigitate along the margins of the Sonoran and Mojave Deserts. In phylogenetic analyses, North American plants comprise a monophyletic grouping that is sister to the South American diploid species, *L. divaricata*. North American populations exhibit genetic signatures of rapid demographic expansion, including a star-shaped genealogy, unimodal distribution of pairwise haplotype differences, and low genetic structure. Nonetheless, polyploid cytotypes are consistently distinguished from diploid cytotypes by a cpDNA indel character, suggesting a single origin of tetraploidy in the species. These findings suggest a recent origin of the North American creosote bush via long distance dispersal, with establishment of polyploid populations accompanying its rapid spread through the Northern Hemisphere.

Keywords—cpDNA, chromosome evolution, molecular systematics, nrDNA, phylogeography.

The genus *Larrea* comprises long-lived shrubs that are widespread across New World desert regions. Four species inhabit xeric regions of South America (*Larrea divaricata* Cav., *Larrea cuneifolia* Cav., *Larrea nitida* Cav., *Larrea ameghinoi* Cav.) with one species in North America (*Larrea tridentata* (DC.) Coville). *Larrea tridentata* (creosote bush) is the most widespread shrub in the American southwest and northern Mexico, often occurring in monospecific stands across thousands of square kilometers (Benson and Darrow 1944; Turner et al. 1995). Creosote bush is a food resource for many animals, including numerous specialist herbivores and pollinators, and regarded as both a defining element and a keystone species of North American warm deserts (Wells and Hunziker 1976; Mabry et al. 1977).

Larrea tridentata is also considered to be a classical example of a polyploid complex (Lewis 1980). Chromosome counts reveal that the species is composed of morphologically-similar diploid ($2n = 2x = 26$), tetraploid ($2n = 4x = 52$), and hexaploid ($2n = 6x = 78$) cytotypes (Yang 1967, 1968, 1970; Yang and Lowe 1968; Barbour 1969). Early sampling indicated that diploid populations occur in the Chihuahuan Desert (western Texas, southern New Mexico, southeastern Arizona, northeastern Mexico), tetraploid populations occur in the Sonoran Desert (south-central Arizona, southeastern California, north-central Mexico, and Baja California), and hexaploid populations occur in the Mojave Desert (northwestern Arizona, southeastern California, southern Nevada, and southwestern Utah). However, recent studies, which made use of guard cell measurements to infer ploidy level on living plants and fossils, suggest that cytotype distributions are geographically complex and temporally dynamic (Hunter et al. 2001). The occurrence of polyploidy in South America is limited to *L. cuneifolia*, a putative allopolyploid species (Hunziker et al. 1977).

Cytogenetic studies and isozyme analyses indicate that the North American creosote bush is autopolyploid (Sternberg 1976; Wells and Hunziker 1976; Hunziker et al. 1977; Poggio

et al. 1989; Cortes and Hunziker 1997). Although there has been little discussion of applying species or subspecies nomenclature to the autopolyploid “races” of *L. tridentata*, the species is sometimes regarded as conspecific to the morphologically similar species, and presumed progenitor, *L. divaricata*, which occurs in Argentina, Chile, and southwestern Peru (Raven 1963, 1972; Porter 1974; Hunziker et al. 1977). Moreover, a narrowly-distributed subspecific taxon, the dune creosote bush (*Larrea tridentata* var. *arenaria* L. D. Benson), has recently been recognized (Benson and Darrow 1981; Felger 2000). Unfortunately, there are limited molecular data available to evaluate taxonomic boundaries in the genus *Larrea* or to test biogeographical hypotheses regarding its unusual amphitropical distribution. Allozyme studies suggest recent divergence between *L. tridentata* and South American taxa but provide minimal resolution of species relationships (Cortes and Hunziker 1997). Similarly, non-coding cpDNA is consistent with the monophyly of North American creosote bush but provides little statistical support or resolution of species relationships (Sheahan and Chase 1996, 2000; Lia et al. 2001).

Here we present an in-depth analysis of cytotype distributions in *L. tridentata* based on flow cytometry, and perform phylogenetic and population genetic analyses based on non-coding cpDNA and nrDNA, to address the following questions: (1) What is the geographic distribution of cytotype races in relation to the Chihuahuan, Sonoran, and Mojave deserts? (2) Do cytotypes co-occur regionally and/or within populations? (3) Is the North American *L. tridentata* monophyletic and evolutionarily distinct from South American *Larrea* species? (4) Is the subspecific taxon, *L. tridentata* var. *arenaria*, distinguished phylogenetically from other North American creosote bush populations? And (5) is the population structure within and among North American populations consistent with recent demographic expansion and in situ polyploid formation?

MATERIALS AND METHODS

Sampling—We sampled individuals from 92 populations of *L. tridentata* throughout the southwestern U. S. A. (Texas, New Mexico, Arizona, California, and Nevada) and northern Mexico (Baja California, Chihuahua, Coahuila, and Sonora), a broad geographic region that encompasses diploid, tetraploid, and hexaploid populations (Appendix 1). The narrowly-distributed *L. tridentata* var. *arenaria* was sampled from the Algodones Dunes region of southern California (Appendix 1). Each population consisted of 5–50 randomly-selected, individually marked plants. To maximize the probability of sampling different genetic individuals (genets), samples were collected from plants that were at least 10 m apart. For each individual, GPS coordinates and elevation were recorded. Young vegetative tissues (leaves and stems) were harvested from each individual plant during the spring seasons of 2007–2009 and thereafter stored on silica gel. Outgroup sampling included several South American *Larrea* species (*L. divaricata*, *L. cuneifolia*, and *L. nitida*) as well as *Guaiaacum coulteri* A. Gray, which were provided by T. Van Devender (Arizona-Sonora Desert Museum, Tucson, Arizona) and J. Takara (Rancho Santa Ana Botanic Garden, Claremont, California). Voucher specimens for sampled populations are deposited at the Rancho Santa Ana Botanic Garden Herbarium, Claremont, California (Appendix 1).

Ploidy Determinations—We used flow cytometry to infer DNA content and ploidy of all collected specimens. For each sample, 10–15 leaves were placed in 1 mL of buffer (3.58 g HEPES, 2 mL of 0.5M solution of EDTA, 5.97 g KCl, 1.168 g NaCl, 102.7 g sucrose, 2 mL Triton X-100, 0.101 g spermine, 1 mL of β -mercaptoethanol in 1.0 L ddH₂O) and were chopped by hand for 1 min with a razor blade. The resulting slurry was pushed through a syringe filter (25 mm Millipore Swinnex filter holder, Fisher Scientific, Pittsburgh, Pennsylvania) that was fitted with a 48 μ m nylon mesh (Small Parts Inc., Miami Lakes, Florida) to remove debris. Samples were centrifuged for 1 min at 10,000 \times g and resuspended in 490 μ L of buffer with 10 μ L of 5 mg/ml propidium iodide solution and 0.1% RNase A (QIAGEN Inc., Valencia, California). Concurrent with resuspension, 2.5 μ L of prepared trout erythrocyte nuclei (BioSure Controls, Grass Valley, CA, USA) was added to each sample as an internal standard (2C DNA content of 5.2 pg). Stained samples containing conspicuous debris were passed through a second filter.

All samples were run on a FACSCalibur flow cytometer (B-D Biosciences, San Jose, California) in the Cell Sorting Facility at the University of Rochester School of Medicine, Rochester, New York. To determine ploidy level, we analyzed the relative fluorescence (FL2-A) of each sample, summarized as a frequency histogram using CellQuest Pro Software (version 5.2.1; B-D Biosciences). We calculated 2C DNA contents of all specimens by multiplying 5.2 pg by the ratio between the sample peak and control peak.

DNA Extraction, Amplification, and Sequencing—We isolated genomic DNA of 2–3 plants from 40 *L. tridentata* populations as well as outgroup specimens using the QIAGEN DNeasy plant kit (QIAGEN). For each sample, 10–20 mg of silica-preserved tissue was used. We amplified five non-coding chloroplast (cpDNA) regions (*psbA-trnH* intergenic spacer, *rpl32-trnL* intergenic spacer, *rpl16* intron, *rpoB-trnC* intergenic spacer, *petN-trnC* intergenic spacer) using universal primers (Shaw et al. 2005, 2007). We performed 25 μ L PCR reactions with 2 μ L genomic DNA, 0.125 μ L of each primer (100 mM stock, 0.5 μ M final concentration), 0.5 μ L dNTP mix (10 mM final concentration), 2.5 μ L standard *Taq* reaction buffer (10 \times) and 0.1 μ L standard *Taq* polymerase (5,000 units/mL; New England Biolabs, Ipswich, Massachusetts). Thermocycling and clean-up were performed as described by Ramsey et al. (2008). In addition to the cpDNA regions, we amplified the nuclear ribosomal external transcribed spacer using the primers 18S-ETS and Ast-1 (Markos and Baldwin 2001), and ITS using the primers Q1 and Q2 (Samuel et al. 1998), for a representative sample of 17 ingroup and outgroup specimens. We performed 25 μ L PCR reactions with 2 μ L genomic DNA, 0.125 μ L of each primer (100 mM stock, 0.5 μ M final concentration), 0.8 μ L dNTP mix (10 mM final concentration), 0.8 μ L 25 mM MgCl₂, 2.5 μ L standard *Taq* reaction buffer (10 \times) and 0.1 μ L standard *Taq* polymerase (5,000 units/mL; New England Biolabs). Thermocycling proceeded as follows: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 64°C for 1 min, 72°C for 1 min; completion of these cycles was followed by a final extension of 72°C for 10 min. Clean-up was performed as described by Ramsey et al. (2008).

Sequencing reactions were performed in a 12.5 μ L volume that included 1.5 μ L purified PCR product, 0.02 μ L of primer (100 mM stock, 0.2 μ M final concentration), 0.5 μ L 5M ultrapure betaine (USB, Cleveland, Ohio), 2.5 μ L Big Dye buffer (Applied Biosystems, Foster City, California) and 0.5 μ L Big Dye (version 3.1; Applied Biosystems). Sequencing

reactions were performed in forward and reverse directions. Products were purified using Montage™ vacuum plates (Millipore, Billerica, Massachusetts) and sequenced on an ABI 3730 capillary sequencer (University of Rochester Functional Genomics Center, Rochester, New York). High quality sequence data were obtained for cpDNA and nrDNA regions in all sampled taxa. Sequences were manually edited using Sequencher™ (version 4.1; Gene Codes, Ann Arbor, Michigan) and manually aligned using MacClade (Maddison and Maddison 2002). For ETS and ITS sequences, we occasionally observed secondary peaks beneath primary peaks in the DNA chromatograms. These secondary peaks could represent sequencing artifacts and/or DNA polymorphism. Because of the small number of base pair positions with secondary peaks, and the uncertain cause of their appearance, we always used the primary/dominant peak for calling base pairs. We retained indels for analysis when inserted/deleted regions did not contain microsatellites, which we defined as tandems of simple sequence repeated ten or more times (Hughes and Queller 1993). All DNA sequences used in this study can be found in GenBank (Appendix 1).

Phylogenetic Analyses—Phylogenetic analyses of cpDNA and nrDNA were performed with a representative sample of 17 ingroup and outgroup specimens, which included three ploidy levels of *L. tridentata* (DC.) Coville and *L. tridentata* var. *arenaria*. Sequence data were analyzed using maximum parsimony with PAUP* (version 4.0b10; Swofford 2003) and Bayesian approaches with MrBayes (version 3.1b; Ronquist and Huelsenbeck 2003). Partition homogeneity tests (incongruence length difference tests as implemented in PAUP*) did not suggest discordance among noncoding cpDNA regions ($p = 0.274$) or ETS and ITS ($p = 0.412$) so all analyses were based on the combined data sets. Parsimony analyses were conducted with 2,000 random addition tree-bisection-reconnection searches, with a starting tree generated by stepwise addition and gaps treated as fifth bases; bootstrap support for each node was evaluated with 10,000 replicates. Four runs of Metropolis-coupled Markov Chain Monte Carlo analyses were implemented in MrBayes with ten million generations, two runs of four chains each, sampling every 1,000 generations, and a heating factor of 0.2. Convergence was assessed when the average standard deviation in split frequencies between the two parallel runs was less than 0.01 (Glor and Laport 2010). A GTR + I model of evolution was fit to the cpDNA data and a GTR + G model was fit to the ETS/ITS data using hierarchical likelihood ratio test (hLRT, Felsenstein 1988) and Akaike information criterion (Akaike 1974) in MrModeltest version 1.1b (Nylander 2002). Nexus files and trees can be found in TreeBASE (study number S11545).

Network and Population Genetic Analyses—Network and population genetic analyses were conducted on 112 sequences from the 40 North American *L. tridentata* populations. Haplotype networks were constructed using TCS (version 1.21; Clement et al. 2000). We used the Arlequin software package (version 2.0; Schneider et al. 2000) to perform analysis of molecular variance (AMOVA) with two population grouping schemes: geographic (grouped by desert region) and cytotype (grouped by ploidy level). Desert designations were based on floristic analyses by Shreve (1942), Benson and Darrow (1944), and McLaughlin (1986). We performed 1,000 permutations to assess significance of variance components.

We evaluated the population history of North American *L. tridentata* using mismatch distributions, which are unimodal in evolutionary lineages undergoing demographic expansion (Slatkin and Hudson 1991; Rogers and Harpending 1992). Using Arlequin, we compared the observed frequency of pairwise differences to an expected distribution generated from a population expansion model with parametric bootstrapping (Schneider and Excoffier 1999). We also estimated demographic expansion parameters t (modal value of mismatch distribution), q_0 (2Nu before population expansion), and q_1 (2Nu after population expansion). Tajima's D , a statistic describing the frequency of alleles at a locus (Tajima 1989), was also calculated.

RESULTS

Flow Cytometry—Analyses of *L. tridentata* revealed a trimodal distribution of DNA contents that corresponded to values expected for a polyploid series (Fig. 1). The inferred mean 2C DNA values were 0.93 pg (diploid; range 0.76–1.19 pg), 1.84 pg (tetraploid; range 1.53–2.14 pg), and 2.79 pg (hexaploid; range 2.38–3.17 pg). Specimens of *L. tridentata* var. *arenaria* had DNA content values typical of tetraploid *L. tridentata* (mean = 1.79 pg, range 1.61–1.98 pg). There was

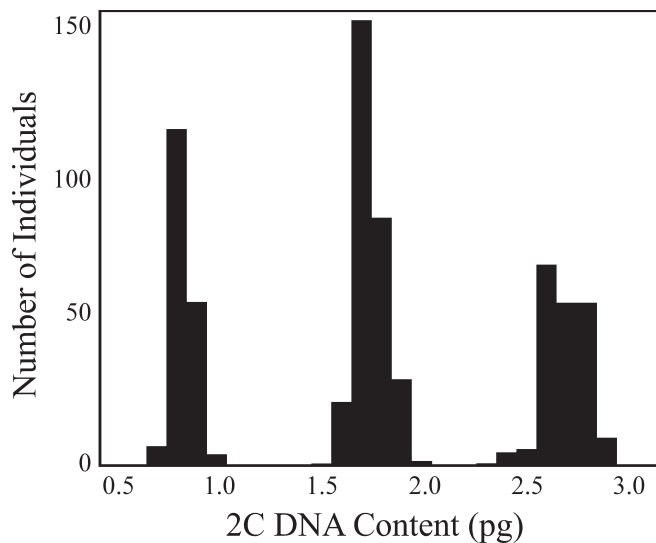


FIG. 1. 2C DNA content of *L. tridentata* collections as inferred by flow cytometry.

no overlap of DNA content values between diploid, tetraploid, and hexaploid cytotypes, suggesting that flow cytometry can unambiguously determine the ploidy level of the North American creosote bush (Fig. 1).

Cytotype Distribution—Diploid cytotypes occurred primarily in the eastern portion of the study area (Chihuahuan

Desert), tetraploid cytotypes in the central portion (Sonoran Desert), and hexaploid cytotypes in the western portion (Mojave Desert) (Figs. 2, 3). The boundary between diploid and tetraploid *L. tridentata* distributions corresponded closely to the traditionally defined boundary between the Chihuahuan and Sonoran Deserts, based on floristic affinities. In contrast, tetraploid and hexaploid cytotypes exhibited spatially complex distributions in Arizona and eastern California. Several hexaploid populations were found in central Arizona, a region considered typical of the Sonoran Desert floristic province (Figs. 2, 3). The average elevation differed significantly between diploid populations (mean = 1,045 m, range 658–1,540 m), tetraploid populations (mean 513 m, range -52–1,078 m), and hexaploid populations (mean = 234 m, range -54–658 m) (ANOVA, $F_{3,80} = 58.627$, $p < 0.001$). Populations of *L. tridentata* var. *arenaria* were found to be geographically limited to low elevation areas in the Algodones Dunes of southeastern California (mean = 55 m, range 10–124 m) (Figs. 2, 3).

Despite the large number of diploid, tetraploid, and hexaploid plants sampled over the study region ($N = 775$ specimens in 92 populations), only two populations were found where cytotypes co-occurred, the vast majority of populations were comprised entirely of individuals of a single cytotype. In two tetraploid populations, a single hexaploid was identified out of 25 sampled individuals (populations AZ08-AA and AZ08-AE, Appendix 1). We did not find plants with DNA content values expected for F1 intercytotype hybrids (i.e. triploids and pentaploids; Fig. 1).

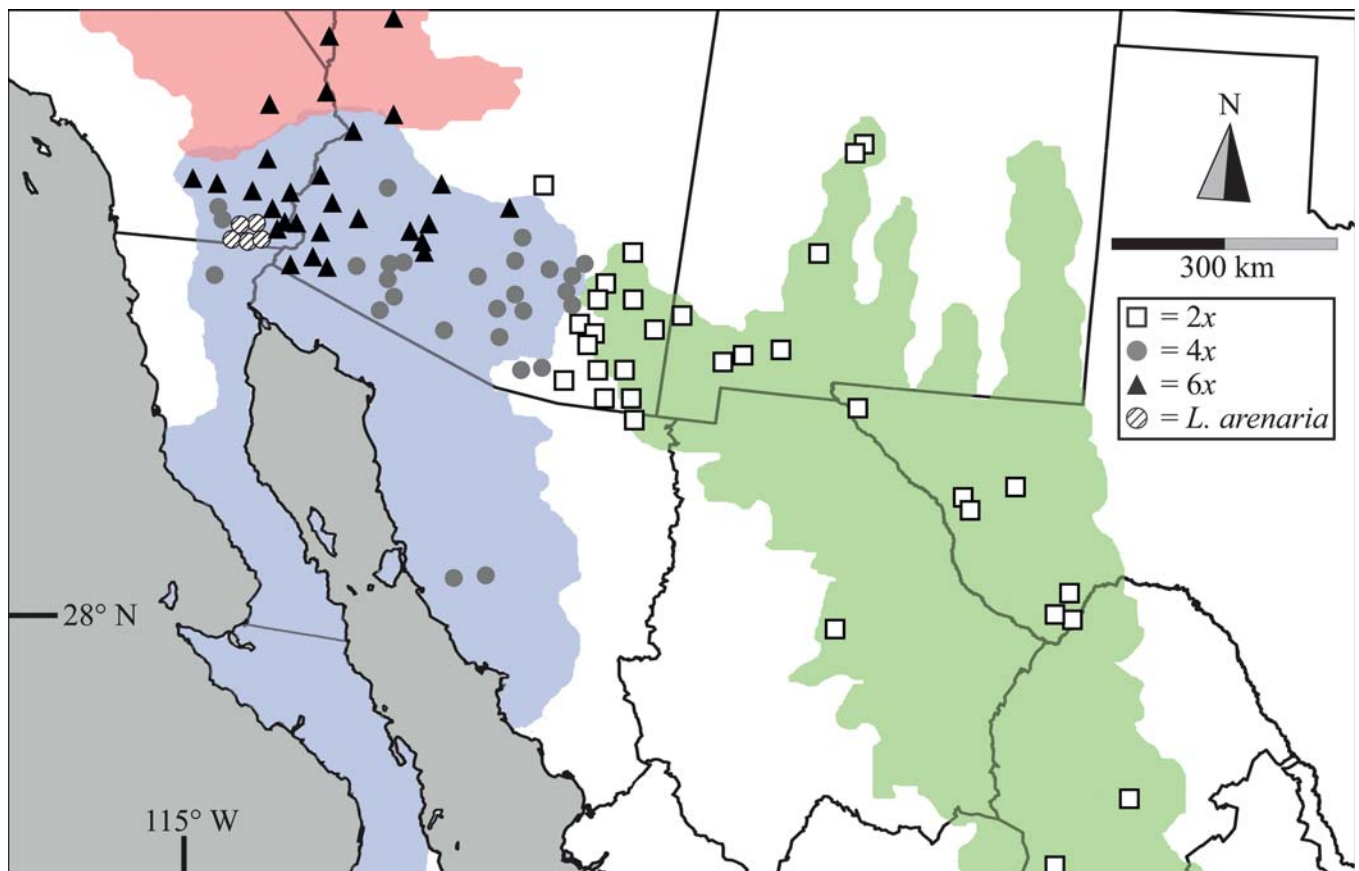


FIG. 2. Geographic distribution of diploid, tetraploid, and hexaploid *L. tridentata* as found in this study. Colored areas identify the approximate boundaries of the Chihuahuan (green), Sonoran (blue), and Mojave (red) desert regions, as commonly delineated by floristic studies.

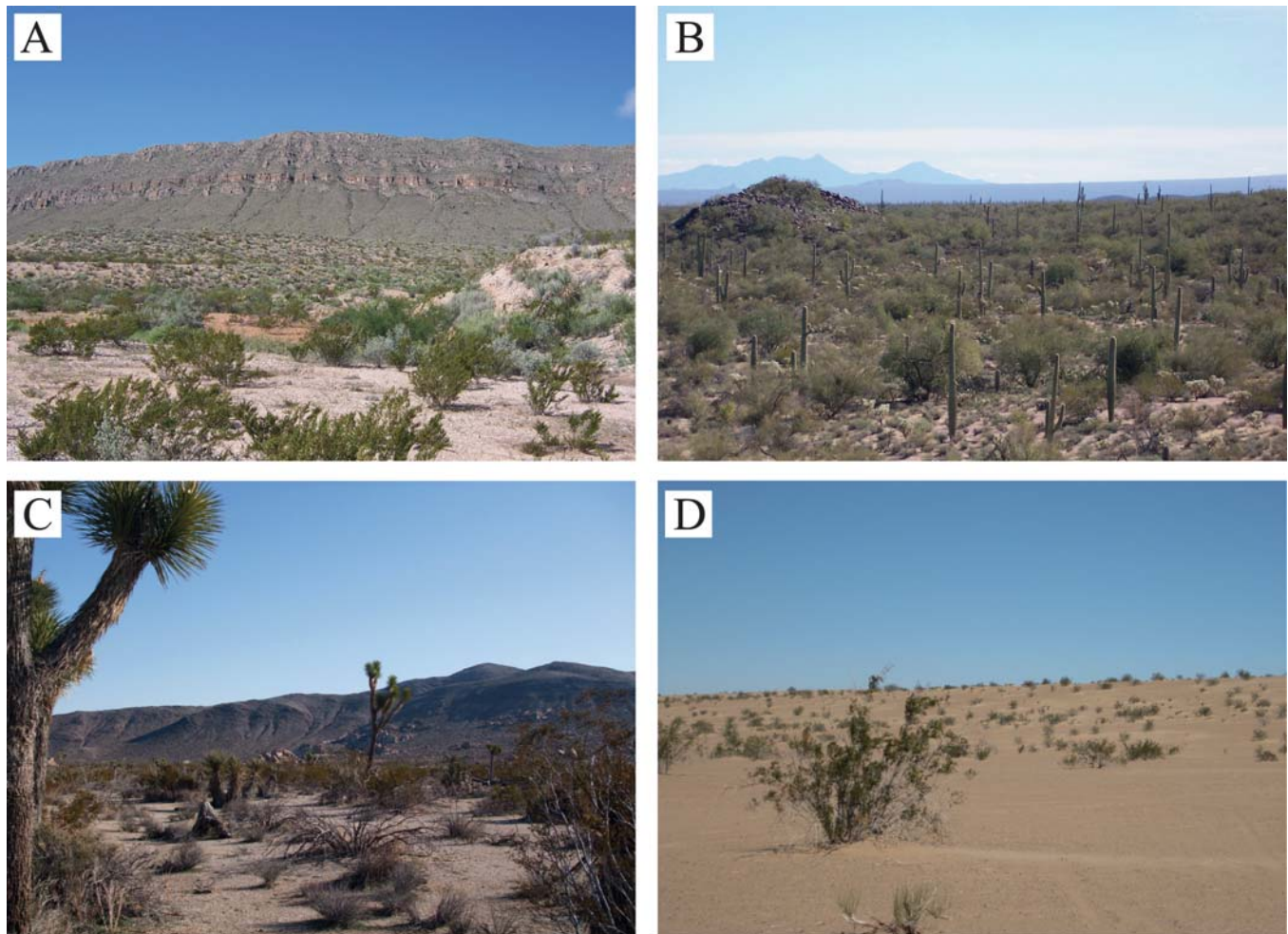


FIG. 3. Photographs of *L. tridentata* cytotypes in representative habitat. A. Diploid plants in the Chihuahuan Desert, east of Big Bend National Park and near the Rio Grande, southern Texas. B. Tetraploid plants in the Sonoran Desert, west of Tucson, Arizona in the Ironwood Forest National Monument. C. Hexaploid plants in the Mojave Desert, near Joshua Tree National Park in southern California. D. *Larrea tridentata* var. *arenaria* on the west flank of the Algodones Dunes in southeastern California.

Phylogenetic Analyses—The combined cpDNA dataset totaled 3,211 base pairs, including 56 variable and 23 parsimony-informative sites. Most of the five non-coding regions had relatively similar numbers of informative sites (aligned base pairs, variable sites, and informative sites: *psbA-trnH*, 449, 6, 3; *rpl32-trnL*, 674, 20, 8; *rpl16* intron, 875, 13, 7; *rpoB-trnC*, 841, 14, 4; *petN-trnC*, 374, 1, 1). Fifty-one unique haplotypes were recovered from the 112 aligned *L. tridentata* sequences, with two major haplotypes shared by 44 individuals (Table 1). The most common observed haplotype differences involved single nucleotide polymorphisms; however, 21 indels of varying size were also observed, including a three base pair indel at position 648 in *rpoB-trnC* that distinguished polyploid cytotypes from diploids (Table 1; Figure 4). Inclusion of cpDNA data from outgroup taxa added 107 parsimony-informative sites and five unique haplotypes.

The ETS/ITS dataset contained 946 nucleotide base pairs, including 24 variable and 10 parsimony informative sites. The ETS and ITS had numbers of informative sites similar to the cpDNA dataset (aligned base pairs, variable sites, and informative sites; ETS, 343, 7, 2; ITS, 603, 17, 8). Inclusion of ETS and ITS data from outgroup taxa added 78 parsimony-informative sites and five unique haplotypes.

Heuristic search of the cpDNA dataset generated a single most parsimonious tree (length = 613 steps; CI = 0.992; RI = 0.974); Bayesian analysis produced an identical tree topology (Fig. 5). There was strong statistical support for the monophyly of North American *L. tridentata* (congruent nodes with $\geq 99\%$ bootstrap support and $\geq 99\%$ posterior probabilities), which was sister to the South American diploid species *L. divaricata* (0.313% sequence divergence = 10 indel and base pair differences among 3,247 characters). Heuristic search of the ETS/ITS dataset generated 184 most parsimonious trees (length = 331 steps; CI = 0.936; RI = 0.860); Bayesian analysis produced a tree topology consistent with the heuristic consensus. There was modest statistical support for the monophyly of the North American *L. tridentata* (convergent nodes with 66% bootstrap support and 55% posterior probabilities; 1.27% sequence divergence = 12 indel and base pair differences among 946 characters). However, the monophyly of *L. divaricata* and *L. tridentata* was strongly supported (Fig. 5).

Population Genetic Analyses—Network analysis of *L. tridentata* cpDNA haplotypes revealed complex genealogical relationships involving two major haplotypes (separated by a single mutational step) and 49 minor haplotypes (separated from major haplotypes by one or a few mutational

TABLE 1. Chloroplast DNA haplotypes recovered from North American *L. tridentata*.

Haplotype	psbA-trnH				rpl32-trnL										rpl16-intron										rpoB-trnC				petN-trnC						
	0	1	2	4	0	1	2	3	4	5	6	7	8	9	4	5	6	7	8	9	4	5	6	7	8	9	0	1	0	1					
a	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A	
b	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
c	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
d	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
e	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
f	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
g	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
h	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
i	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
j	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
k	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
l	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
m	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
n	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
o	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
p	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
q	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
r	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
s	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
t	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
u	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
v	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
w	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
x	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
y	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
z	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
a'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
b'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
c'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
d'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
e'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
f'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
g'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
h'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
i'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
j'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
k'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
l'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
m'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
n'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
o'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
p'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
q'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
r'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
s'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
t'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
u'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
v'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
w'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
x'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A
y'	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	C	C	A	A	A	A

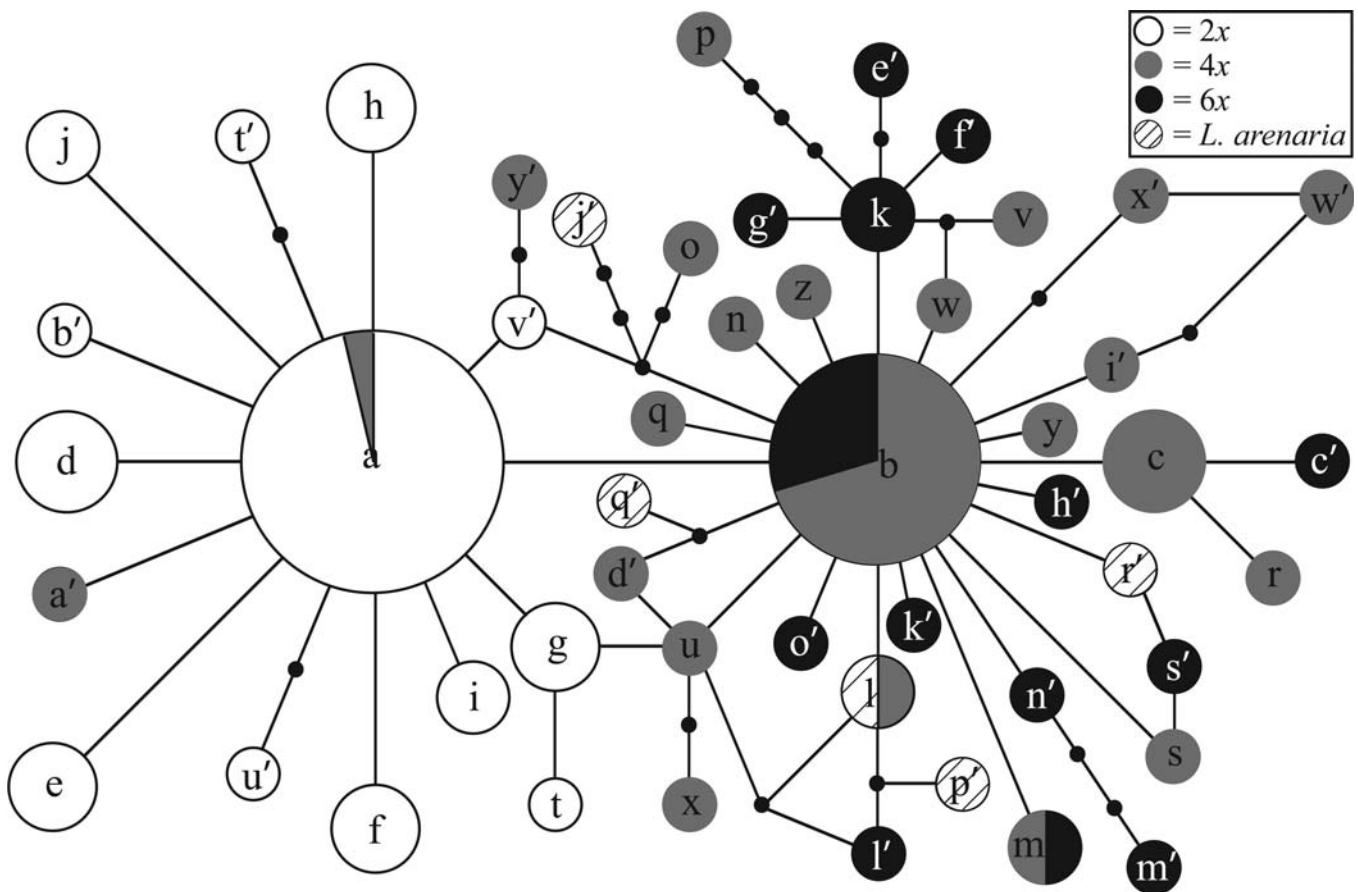


FIG. 4. Network analysis of cpDNA haplotypes recovered from North American *L. tridentata* populations. Circles are sized to reflect haplotype frequency and shaded to distinguish ploidy levels; small unlabeled dots represent inferred mutational steps among haplotypes. Diploid plants are distinguished from polyploid plants by possession of a three base-pair insertion in the *rpoB-trnC* intergenic spacer (Table 1).

steps) (Fig. 4). The major haplotypes (a, b) occurred at frequencies of 24.1% and 15.2%, respectively; frequencies of minor haplotypes were 3.6% (c, d), 2.7% (e–h), 1.8% (i–m), and 0.9% (n–y') (Table 1; Fig. 4). While haplotypes were widely shared among tetraploid and hexaploid populations (major haplotype b and associated minor haplotypes), diploid populations harbored a distinct group of haplotypes (major haplotype a and associated minor haplotypes) (Table 1; Fig. 4). Haplotypes recovered from the sand-dune endemic *L. tridentata* var. *arenaria* were broadly distributed throughout the network, and similar or identical to those of tetraploid and hexaploid cytotypes of *L. tridentata* (Fig. 4).

AMOVA indicated that the majority of cpDNA sequence variation in *L. tridentata* was found within populations (65.8 and 67.1%) rather than among populations (4.5 and 10.9%) (Table 2). Variance was nonetheless associated significantly with population groupings defined by ploidy or desert province (22.0 and 29.7%; $p < 0.001$) (Table 2). Pairwise differences among cpDNA haplotypes exhibited a unimodal distribution (mean = 2.3 differences) that did not differ significantly from a stepwise population expansion model for a haploid locus ($p < 0.001$) (Fig. 6). Estimates of demographic parameters and confidence intervals ($\alpha = 0.05$) were as follows: modal value, t , 2.383 [1.510–2.799]; $2N\mu$ before expansion, q_0 , 0 [0–1.201]; $2N\mu$ after population expansion, q_1 , 7,730.000 [32.281–9,097.188]. The observed value of Tajima's D was -2.432. Coalescent simulations based on a neutral model (no recombination, constant population size) had a 95% confidence interval

of [-1.595, 1.863]. Thus, the excess occurrence of minor cpDNA haplotypes in North American creosote bush was unlikely to have been recovered by chance ($p < 0.001$).

Mismatch distributions and estimates of demographic parameters and confidence intervals ($\alpha = 0.05$) were also calculated for each cytotype individually. For diploids, the mean number of pairwise differences = 1.3, Tajima's D was -2.226 [-1.724, 1.921], $p = 0.002$; for tetraploids the mean number of pairwise differences = 2.4, Tajima's D was -2.362 [-1.763, 1.836], $p < 0.001$; for hexaploids the mean number of pairwise differences = 2.4, Tajima's D was -1.917 [-1.772, 1.739], $p = 0.016$.

DISCUSSION

Cytogeography—The cytotypes of *L. tridentata* span large areas of western North America, and occur in topographically complex terrain (Figs. 2, 3). We found that the distribution of diploid, tetraploid, and hexaploid populations generally correspond to the Chihuahuan, Sonoran, and Mojave Deserts, as reported in classical studies (Yang 1967, 1968, 1970; Yang and Lowe 1968; Barbour 1969). The boundary between diploid and tetraploid cytotypes seems particularly well defined, only two populations of diploids were found further west than the easternmost populations of tetraploids, for example. Populations of tetraploids and hexaploids, however, were broadly mixed throughout central and western Arizona. Hexaploid populations were found further east than previously reported, and

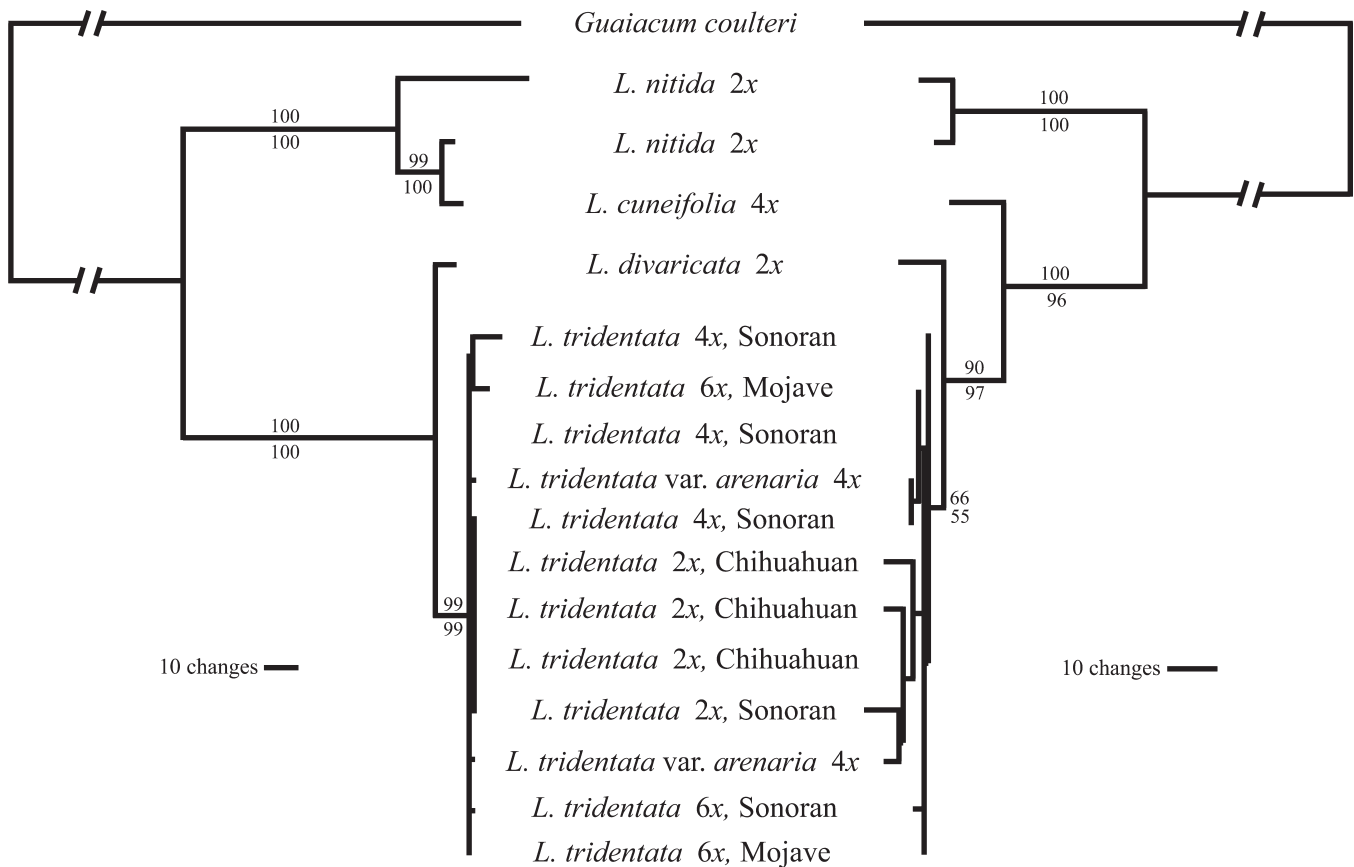


FIG. 5. Consensus phylogeny of North and South American *Larrea* based on five non-coding cpDNA regions (left) and nrDNA (right). Numbers indicate statistical support for nodes based on bootstrapping of parsimony analyses (top) and posterior probabilities of Bayesian analyses (bottom).

we observed sympatric occurrence of tetraploid and hexaploid cytotypes in two study sites (Fig. 2).

Cytotype distributions may reflect historical barriers that formerly separated the chromosomal races, but have since been obscured. For example, the floristic endemism of the Chihuahuan and Sonoran Deserts suggests their formation has occurred independently and over an extended evolutionary period (Betancourt et al. 1990). In contrast, there is considerable disagreement about the floristic distinctiveness of the Mojave and Sonoran Deserts (McLaughlin 1986; Turner 1994). Even in the western portion of the range, however, co-occurrence of cytotypes was rarely observed within single populations. This finding may reflect minority cytotype exclusion, a frequency-dependent selection caused by reproductive barriers between ploidy levels (Hagberg and Ellerström 1959; Levin 1975, 1983). It is also proposed, how-

ever, that the cytotypes of *L. tridentata* have alternate phenological, life-history, and physiological attributes, and thus may be “pre-adapted” to occur in different environmental conditions (Lumaret 1988; Thompson and Lumaret 1992; De Soyza et al. 1997; Husband and Schemske 1998; Ignace and Huxman 2009; Maherali et al. 2009).

Origin of North American *Larrea*—Phylogenies generated from cpDNA and nrDNA support the monophyly of *L. tridentata* (Fig. 5). North American *Larrea* is sister to the South American *L. divaricata*, confirming the close evolutionary relationship of these taxa inferred from morphological, cytogenetic, and allozyme studies. Colonization of North America appears to have happened on a single occasion, or perhaps on a few occasions from closely-related South American sources, but further sampling of South American populations is needed to resolve these possibilities. The

TABLE 2. Analysis of molecular variance for cpDNA haplotypes recovered from North American *L. tridentata*, with population groupings based on geography (desert) and ploidy level.

Analysis	Percentage of variation	Fixation index	Statistical significance
Groups defined by geography			
Among groups	22.0	$\phi_{CT} = 0.22$	$p < 0.001$, df = 2
Among populations within groups	10.9	$\phi_{SC} = 0.14$	$p < 0.001$, df = 37
Within populations	67.1	$\phi_{ST} = 0.33$	$p < 0.001$, df = 72
Groups defined by ploidy level			
Among groups	29.7	$\phi_{CT} = 0.30$	$p < 0.001$, df = 2
Among populations within groups	4.5	$\phi_{SC} = 0.06$	$p < 0.001$, df = 37
Within populations	65.8	$\phi_{ST} = 0.34$	$p < 0.001$, df = 72

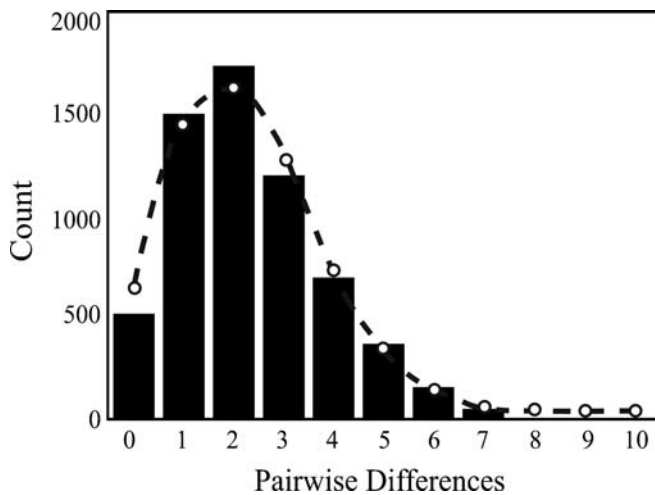


FIG. 6. Pairwise mismatch distribution for cpDNA haplotypes recovered from North American *L. tridentata*. The dashed line indicates values expected by a model of population expansion.

taxonomic status of *L. tridentata* vs. *L. divaricata* is complicated by the species' allopatric distributions and partial reproductive compatibility in experimental crosses (Yang et al. 1977, 2000). However, because of the DNA sequence differences observed between *L. tridentata* and *L. divaricata*, and the absence of polyploid populations in *L. divaricata* (Hunziker et al. 1977), we favor recognition of the taxa as separate species rather than as conspecific. Nonetheless, sequence divergence between *L. tridentata* and *L. divaricata* is low, approximately 0.313% (10 changes among 3,198 base pairs) for cpDNA and 1.27% (12 changes among 946 base pairs) for ETS/ITS.

Because of its ecological dominance in North American deserts, there has been longstanding interest in dating the arrival of creosote bush to northern latitudes (Barbour 1969; Hunziker et al. 1972, 1977; Betancourt et al. 1990; Lia et al. 2001). Individual clones in the Mojave Desert have been estimated to be several thousand years old (Sternberg 1976; Vasek 1980), and macrofossils recovered from packrat (*Neotoma*) middens date *L. tridentata* to the last glacial maximum (ca. 18,700 ybp; Betancourt et al. 1990; Hunter et al. 2001), while palynological evidence in lake sediments of Death Valley dates to 47,000–109,000 ybp (Woolfenden 1996; Bader 2000). Most botanists suspect that creosote bush has occurred in North American deserts far longer than is documented by the fossil record, however, and have turned to molecular data to estimate divergence time between *L. tridentata* and *L. divaricata* (Hunter et al. 2001; Lia et al. 2001).

DNA substitution rates in flowering plants are on the order of 0.1–0.3% per million years for non-coding cpDNA (Wolfe et al. 1987; Muse 2000) and 0.1–0.8% per million years for non-coding nrDNA (ITS; Kay et al. 2006). Sequence divergence reported here suggests that colonization of North America occurred in the Pleistocene or late Pliocene (cpDNA pairwise divergence = 0.313%, 0.52–1.57 mya; ITS pairwise divergence = 0.672%, 0.42–3.36 mya). This timeframe is younger than suggested by Lia et al. (2001; 4.2–8.4 mya) based on the chloroplast gene *rbcL* and similar to that of Cortes and Hunziker (1997; 0.6–1.2 mya) based on allozymes. An alternate approach to dating the amphitropical dispersal of *Larrea* focuses on North American herbivore and pollina-

tor groups that specialize on creosote bush as a food resource. For example, gall midges of the *Asphondylia auripila* group (Cecidomyiidae) are *L. tridentata* specialists (Waring and Price 1989) and were recently shown by Joy and Crespi (2007) to represent an adaptive radiation via divergence in host feeding location. Based on a mtDNA divergence rate of 1.1–1.2% per million years (Tamura 1992; Brower 1994), the observed COI pairwise divergence between *Larrea*-specialist and non-*Larrea* specialists of *Asphondylia* (9.3%) puts a lower bound of 3.88–4.23 mya for the arrival of creosote bush in North America (Joy and Crespi 2007).

Rates of molecular evolution are too variable to provide precise estimates of divergence times in the absence of fossil calibrations, and botanists may never know the exact time that *Larrea* arrived in North America. Nonetheless, estimates based on a variety of data sources and criteria suggest a relatively recent origin between the mid-Pleistocene and late Pliocene. This conclusion is also supported by population genetic analyses (see below).

Phylogeography and Genetic Structure—The phylogeography of *L. tridentata* is suggestive of recent and rapid demographic expansion from a small founding population. We found only two common cpDNA haplotypes (a and b) that together comprised 39.3% of all recovered sequences (Fig. 4). Most cpDNA haplotypes were unique or shared by a few individuals (Table 1) and, consistent with prior work in diploid *L. tridentata* (Duran et al. 2005), there was little genetic structure associated with either geography or population boundaries (Table 2). Genealogical relationships of cpDNA haplotypes were star-shaped (Figs. 4, 5) and exhibited the unimodal DNA mismatch distribution (Fig. 6) that is characteristic of expanding populations (Rogers and Harpending 1992).

The clearest evidence of population genetic structure was associated with ploidy, as a tri-nucleotide indel in the *rpoB-trnC* intergenic spacer distinguished diploid cytotypes from tetraploid and hexaploid cytotypes (Fig. 4). In contrast, we found no consistent sequence differences between tetraploid and hexaploid cytotypes. These findings suggest that tetraploidy evolved a single time among the North American creosote bush, but that hexaploids either (i) have formed recurrently in tetraploid populations; (ii) have extensively hybridized with their tetraploid progenitors; or (iii) have had insufficient time to develop characteristic DNA sequences (Soltis and Soltis 1999; Soltis et al. 2007). The rate of hexaploid formation in tetraploid populations far exceeds the rate of tetraploid formation in diploid populations, and as a rule, pentaploid hybrids are more fertile than triploid hybrids (Ramsey and Schemske 1998, 2002; Ramsey 2007). Thus, the paucity of sequence differences between tetraploid and hexaploid cytotypes is not unexpected.

One complication to the aforementioned interpretation is that the *rpoB-trnC* indel appears to be a derived insertion: South American species (*L. cuneifolia*, *L. divaricata*, and *L. nitida*) and polyploid cytotypes of the North American *L. tridentata* share the deletion (Table 1). It is unclear if the occurrence of the *rpoB-trnC* deletion in polyploid *L. tridentata* represents a character reversion, i.e. backwards mutation to the ancestral state, as exhibited by the South American *L. divaricata*, or if the indel was polymorphic in ancestral populations of *L. tridentata* with the insertion subsequently fixing only in the diploid lineage. Furthermore, two tetraploid individuals have the insertion (Fig. 4). One of these

individuals (haplotype a', Fig. 4) was sampled near diploid populations (AZ07-S, Appendix 1) and was probably generated by intercytotype gene flow or neopolyploidy. However, the second individual (haplotype a, Fig. 4) was sampled near Hermosillo, Mexico (SN07-B, Appendix 1), several hundred kilometers from known diploid populations. Backward mutation may be the best explanation for the existence of this individual. Additional population sampling of *L. divaricata* and diploid *L. tridentata* may clarify the origin of the *rpoB-trnC* indel character. Regardless of its history, the indel may be a useful marker for evaluating hybridization and introgression among modern-day diploid and polyploid creosote bush populations.

Larrea tridentata var. *arenaria*—This subspecific taxon was defined on the basis of morphology and endemism to the Algodones Dunes, there was no prior investigation of its ploidy level or evolutionary history (Benson and Darrow 1981; Felger 2000). Our analyses reveal var. *arenaria* to be tetraploid and to harbor cpDNA and ETS/ITS haplotypes identical to those of tetraploid and hexaploid *L. tridentata* (DC.) Coville (Table 1; Fig. 4). *Larrea tridentata* var. *arenaria* resides at the western range boundary of tetraploid *L. tridentata* (DC.) Coville, and moreover, the nearest populations of *L. tridentata* (DC.) Coville to the Algodones Dunes are hexaploid (Fig. 2). In a few sites, tetraploid *L. tridentata* var. *arenaria* and hexaploid *L. tridentata* (DC.) Coville occur in sympatry (R. Laport unpubl. data). Nonetheless, specimens of *L. tridentata* var. *arenaria* are distinguished from *L. tridentata* (DC.) Coville by a combination of stem, leaf, and floral traits, and are readily identifiable in the field (R. Laport pers. obs.). Based on these findings, we suggest that *L. tridentata* var. *arenaria* had a recent origin as a dune-adapted ecotype of the more widespread *L. tridentata* (DC.) Coville, and warrants continued recognition at the subspecific level.

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APPENDIX 1. List of taxa used in the present study with collection information, voucher numbers, and GenBank accessions. Voucher specimens were prepared from field-collected plants in 2006–2009 and are deposited at the Rancho Santa Ana Botanic Gardens, Claremont, California (RSA). Data are presented by taxon per paragraph in the following sequence: ploidy; field collection location; population identifier and GPS coordinates (from WGS 84 datum); collecting individual and voucher accession; and GenBank accession numbers for the *psbA-trnH* intergenic spacer, *rpL32-trnL* intergenic spacer, *rpL16* intron, *rpoB-trnC* intergenic spacer, *petN-trnC* intergenic spacer, and ribosomal ETS and ITS sequences. “–” indicates no sequence data was generated for the indicated region.

Larrea tridentata (DC.) Coville (2x); U. S. A.: Arizona: Cochise Co.: AZ07-B (31° 48' N, 110° 09' W), R. Laport 766171;--;--;-- (2x); U. S. A.: Arizona: Cochise Co.: AZ07-J (32° 15' N, 110° 20' W), R. Laport 766172;--;--;-- (2x); U. S. A.: Arizona: Cochise Co.: AZ07-K (32° 09' N, 110° 17' W), R. Laport 766173; JF266714, JF266715, JF266716; JF266948, JF266949, JF266950; JF266831, JF266832, JF266833; JF267065, JF267066, JF267067; JF267182, JF267183, JF267184; JF267308. (2x); U. S. A.: Arizona: Cochise Co.: AZ07-L (32° 02' N, 110° 19' W), R. Laport 766174; JF266717, JF266718, JF266719; JF266951, JF266952, JF266953; JF266834, JF266835, JF266836; JF267068, JF267069, JF267070; JF267185, JF267186, JF267187;-- (2x); U. S. A.: Arizona: Cochise Co.: AZ07-U (32° 16' N, 110° 21' W), R. Laport 766175; JF266744, JF266745, JF266746; JF266978, JF266979, JF266980; JF266861, JF266862, JF266863; JF267095, JF267096, JF267097; JF267212, JF267213, JF267214;-- (2x); U. S. A.: Arizona: Cochise Co.: AZ08-AI (31° 54' N, 109° 44' W), R. Laport 766176;--;--;-- (2x); U. S. A.: Arizona: Gila Co.: AZ07-G (33° 20' N, 110° 35' W), R. Laport 766177;--;--;-- (2x); U. S. A.: Arizona: Gila Co.: AZ07-O (33° 10' N, 110° 31' W), R. Laport 766178; JF266726, JF266727, JF266728; JF266960, JF266961, JF266962; JF266843, JF266844, JF266845; JF267077, JF267078, JF267079; JF267194, JF267195, JF267196;-- (4x); U. S. A.: Arizona: Gila Co.: AZ07-F (33° 01' N, 110° 44' W), R. Laport 766179;--;--;-- (4x); U. S. A.: Arizona: Gila Co.: AZ07-P (33° 09' N, 110° 48' W), R. Laport 766180; JF266729, JF266730, JF266731; JF266963, JF266964, JF266965; JF266846, JF266847, JF266848; JF267080, JF267081, JF267082; JF267197, JF267198, JF267199;-- (4x); U. S. A.: Arizona: Gila Co.: AZ07-Q (33° 05' N, 110° 42' W), R. Laport 766181; JF266732, JF266733, JF266734; JF266966, JF266967; JF266968; JF266849, JF266850, JF266851; JF267083, JF267084, JF267085; JF267200, JF267201, JF267202;-- (2x); U. S. A.: Arizona: Graham Co.: AZ07-H (32° 50' N, 109° 37' W), R. Laport 766182;--;--;-- (2x); U. S. A.: Arizona: Graham Co.: AZ07-M (33° 09' N,

- 110° 24' W), *R. Laport 766183*; JF266720, JF266721, JF266722; JF266954, JF266955, JF266956; JF266837, JF266838, JF266839; JF267071, JF267072, JF267073; JF267188, JF267189, JF267190;-. (6x); U. S. A.: Arizona: La Paz Co.: AZ07-E (33° 36' N, 114° 28' W), *R. Laport 766184*;-. (6x); U. S. A.: Arizona: La Paz Co.: AZ07-I (34° 17' N, 114° 05' W), *R. Laport 766185*;-. (6x); U. S. A.: Arizona: La Paz Co.: AZ08-AM (33° 31' N, 114° 13' W), *R. Laport 766186*;-. (4x); U. S. A.: Arizona: Maricopa Co.: AZ07-C (32° 32' N, 112° 52' W), *R. Laport 766187*; JF266711, JF266712, JF266713; JF266945, JF266946, JF266947; JF266828, JF266829, JF266830; JF267062, JF267063, JF267064; JF267179, JF267180, JF267181;-. (4x); U. S. A.: Arizona: Maricopa Co.: AZ07-W (33° 36' N, 113° 18' W), *R. Laport 766188*; JF266750, JF266751, JF266752; JF266984, JF266985, JF266986; JF266867, JF266868, JF266869; JF267101, JF267102, JF267103; JF267218, JF267219, JF267220;-. (4x); U. S. A.: Arizona: Maricopa Co.: AZ08-AP (32° 50' N, 112° 46' W), *R. Laport 766189*;-. (4x); U. S. A.: Arizona: Maricopa Co.: AZ08-AQ (32° 41' N, 112° 51' W), *R. Laport 766190*;-. (4x); U. S. A.: Arizona: Maricopa Co.: AZ08-AU (32° 55' N, 112° 44' W), *R. Laport 766191*;-. (6x); U. S. A.: Arizona: Maricopa Co.: AZ07-V (33° 03' N, 112° 38' W), *R. Laport 766192*; JF266747, JF266748, JF266749; JF266981, JF266982, JF266983; JF266864, JF266865, JF266866; JF267098, JF267099, JF267100; JF267215, JF267216, JF267217; JF267303. (6x); U. S. A.: Arizona: Maricopa Co.: AZ08-AF (33° 32' N, 111° 25' W), *R. Laport 766193*;-. (6x); U. S. A.: Arizona: Maricopa Co.: AZ08-AG (33° 52' N, 112° 35' W), *R. Laport 766194*;-. (6x); U. S. A.: Arizona: Maricopa Co.: AZ08-AK (33° 11' N, 112° 38' W), *R. Laport 766195*;-. (6x); U. S. A.: Arizona: Maricopa Co.: AZ08-AL (33° 17' N, 112° 38' W), *R. Laport 766196*;-. (6x); U. S. A.: Arizona: Maricopa Co.: AZ08-AT (32° 58' N, 112° 40' W), *R. Laport 766197*;-. (6x); U. S. A.: Arizona: Mohave Co.: AZ07-X (34° 43' N, 113° 36' W), *R. Laport 766198*; JF266753, JF266754; JF266987, JF266988; JF266870, JF266871; JF267104, JF267105; JF267221, JF267222; JF267309. (6x); U. S. A.: Arizona: Mohave Co.: AZ08-AR (36° 07' N, 114° 00' W), *R. Laport 766199*;-. (2x); U. S. A.: Arizona: Pima Co.: AZ07-T (32° 29' N, 110° 29' W), *R. Laport 766200*; JF266741, JF266742, JF266743; JF266975, JF266976, JF266977; JF266858, JF266859, JF266860; JF267092, JF267093, JF267094; JF267209, JF267210, JF267211;-. (2x); U. S. A.: Arizona: Pima Co.: AZ08-AX (31° 56' N, 110° 39' W), *R. Laport 766201*;-. (4x); U. S. A.: Arizona: Pima Co.: AZ07-A (32° 04' N, 111° 15' W), *R. Laport 766202*; JF266878, JF266879, JF266880; JF266942, JF266943; JF266944, JF266945, JF266946, JF266827; JF267059, JF267060, JF267061; JF267176, JF267177, JF267178; JF267298. (4x); U. S. A.: Arizona: Pima Co.: AZ07-Z (31° 59' N, 112° 50' W), *R. Laport 766203*; JF266758, JF266759, JF266760; JF266992, JF266993, JF266994; JF266875, JF266876, JF266877; JF267109, JF267110, JF267111; JF267226, JF267227, JF267228;-. (4x); U. S. A.: Arizona: Pima Co.: AZ08-AA (32° 25' N, 111° 21' W), *R. Laport 766204*;-. (4x); U. S. A.: Arizona: Pima Co.: AZ08-AB (32° 27' N, 111° 28' W), *R. Laport 766205*;-. (4x); U. S. A.: Arizona: Pima Co.: AZ08-AC (32° 20' N, 111° 27' W), *R. Laport 766206*;-. (4x); U. S. A.: Arizona: Pima Co.: AZ08-AH (31° 57' N, 110° 55' W), *R. Laport 766207*;-. (4x); U. S. A.: Arizona: Pima Co.: AZ08-AV (32° 01' N, 111° 58' W), *R. Laport 766208*;-. (4x); U. S. A.: Arizona: Pima Co.: AZ08-AW (31° 57' N, 110° 43' W), *R. Laport 766209*;-. (2x); U. S. A.: Arizona: Pinal Co.: AZ07-N (33° 10' N, 110° 30' W), *R. Laport 766210*; JF266723, JF266724, JF266725; JF266957, JF266958, JF266959; JF266840, JF266841, JF266842; JF267074, JF267075, JF267076; JF267191, JF267192, JF267193;-. (4x); U. S. A.: Arizona: Pinal Co.: AZ07-R (32° 44' N, 110° 38' W), *R. Laport 766211*; JF266735, JF266736, JF266737; JF266969, JF266970, JF266971; JF266852, JF266853, JF266854; JF267086, JF267087, JF267088; JF267203, JF267204, JF267205;-. (4x); U. S. A.: Arizona: Pinal Co.: AZ07-S (32° 38' N, 110° 33' W), *R. Laport 766212*; JF266738, JF266739, JF266740; JF266972, JF266973, JF266974; JF266855, JF266856, JF266857; JF267089, JF267090, JF267091; JF267206, JF267207, JF267208;-. (4x); U. S. A.: Arizona: Pinal Co.: AZ08-AD (32° 49' N, 111° 12' W), *R. Laport 766213*;-. (4x); U. S. A.: Arizona: Pinal Co.: AZ08-AE (33° 18' N, 111° 22' W), *R. Laport 766214*;-. (4x); U. S. A.: Arizona: Pinal Co.: AZ08-AJ (32° 50' N, 112° 08' W), *R. Laport 766215*;-. (2x); U. S. A.: Arizona: Yavapai Co.: AZ08-AY (34° 39' N, 111° 45' W), *R. Laport 766216*;-. (4x); U. S. A.: Arizona: Yuma Co.: AZ08-AO (32° 50' N, 113° 32' W), *R. Laport 766217*;-. (6x); U. S. A.: Arizona: Yuma Co.: AZ07-D (32° 39' N, 114° 21' W), *R. Laport 766218*;-. (6x); U. S. A.: Arizona: Yuma Co.: AZ07-Y (32° 38' N, 114° 08' W), *R. Laport 766219*; JF266755, JF266756, JF266757; JF266989, JF266990, JF266991; JF266872, JF266873, JF266874; JF267106, JF267107, JF267108; JF267223, JF267224, JF267225;-. (6x); U. S. A.: Arizona: Yuma Co.: AZ08-AN (32° 52' N, 114° 22' W), *R. Laport 766220*;-. (6x); U. S. A.: Arizona: Yuma Co.: AZ08-AS (33° 10' N, 114° 01' W), *R. Laport 766221*;-. (6x); U. S. A.: Arizona: Yuma Co.: AZ09-AZ (32° 32' N, 114° 34' W), *R. Laport 766222*;-. (4x); U. S. A.: California: Imperial Co.: CA07-F (33° 07' N, 115° 52' W), *R. Laport 766224*; JF266770, JF266771, JF266772; JF267004, JF267005, JF267006; JF266887, JF266888, JF266889; JF267121, JF267122, JF267123; JF267238, JF267239, JF267240; JF267305. (4x); U. S. A.: California: Imperial Co.: CA09-R (33° 06' N, 115° 49' W), *R. Laport 766225*;-. (6x); U. S. A.: California: Imperial Co.: CA07-B (33° 20' N, 116° 00' W), *R. Laport 766515*;-. (6x); U. S. A.: California: Imperial Co.: CA07-G (33° 24' N, 115° 49' W), *R. Laport 766226*; JF266773, JF266774, JF266775; JF267007, JF267008, JF267009; JF266890, JF266891, JF266892; JF267124, JF267125, JF267126; JF267241, JF267242, JF267243;-. (6x); U. S. A.: California: Imperial Co.: CA07-I (32° 53' N, 114° 50' W), *R. Laport 766227*; JF266779, JF266780, JF266781; JF267013, JF267014, JF267015; JF266896, JF266897, JF266898; JF267130, JF267131, JF267132; JF267247, JF267248, JF267249;-. (6x); U. S. A.: California: Imperial Co.: CA08-M (33° 01' N, 114° 37' W), *R. Laport 766228*;-. (6x); U. S. A.: California: Imperial Co.: CA08-N (33° 17' N, 114° 44' W), *R. Laport 766229*;-. (6x); U. S. A.: California: Imperial Co.: CA08-S (33° 06' N, 114° 54' W), *R. Laport 766230*;-. (6x); U. S. A.: California: Imperial Co.: CA08-T (32° 54' N, 114° 39' W), *R. Laport 766231*;-. (6x); U. S. A.: California: Riverside Co.: CA07-E (33° 40' N, 115° 48' W), *R. Laport 766237*; JF266767, JF266768, JF266769; JF267001, JF267002, JF267003; JF266884, JF266885, JF266886; JF267118, JF267119, JF267120; JF267235, JF267236, JF267237; JF267304. (6x); U. S. A.: California: San Bernardino Co.: CA07-C (34° 43' N, 114° 30' W), *R. Laport 766238*; JF266763, JF266764; JF266997, JF266998; JF266880, JF266881; JF267114, JF267115; JF267231, JF267232;-. (6x); U. S. A.: California: San Bernardino Co.: CA07-D (34° 25' N, 115° 42' W), *R. Laport 766239*; JF266765, JF266766; JF266999, JF267000; JF266882, JF266883; JF267116, JF267117; JF267233, JF267234;-. (6x); U. S. A.: California: San Diego Co.: CA09-Q (33° 18' N, 116° 13' W), *R. Laport 766240*;-. (6x); U. S. A.: Nevada: Clark Co.: NV08-A (35° 11' N, 114° 36' W), *R. Laport 766244*;-. (2x); U. S. A.: New Mexico: Bernalillo Co.: NM07-C (34° 55' N, 106° 43' W), *R. Laport 766245*;-. (2x); U. S. A.: New Mexico: Bernalillo Co.: NM07-F (34° 54' N, 106° 43' W), *R. Laport 766246*; JF266792, JF266793; JF267026, JF267027; JF266909, JF266910; JF267143, JF267144; JF267260, JF267261; JF267307. (2x); U. S. A.: New Mexico: Doña Ana Co.: NM07-B (32° 38' N, 107° 13' W), *R. Laport 766247*;-. (2x); U. S. A.: New Mexico: Hidalgo Co.: NM07-G (32° 38' N, 109° 01' W), *R. Laport 766248*; JF266794, JF266795, JF266796; JF267028, JF267029, JF267030; JF266911, JF266912, JF266913; JF267145, JF267146, JF267147; JF267262, JF267263, JF267264;-. (2x); U. S. A.: New Mexico: Luna Co.: NM07-A (32° 19' N, 107° 40' W), *R. Laport 766249*;-. (2x); U. S. A.: New Mexico: Luna Co.: NM07-D (32° 20' N, 107° 38' W), *R. Laport 766250*; JF266786, JF266787, JF266788; JF267020, JF267021, JF267022; JF266903, JF266904, JF266905; JF267137, JF267138, JF267139; JF267254, JF267255, JF267256;-. (2x); U. S. A.: New Mexico: Socorro Co.: NM07-E (33° 37' N, 107° 08' W), *R. Laport 766251*; JF266789, JF266790, JF266791; JF267023, JF267024, JF267025; JF266906, JF266907, JF266908; JF267140, JF267141, JF267142; JF267257, JF267258, JF267259;-. (2x); U. S. A.: Texas: Brewster Co.: ADARA (29° 25' N, 102° 53' W), *R. Laport 766255*;-. (2x); U. S. A.: Texas: Brewster Co.: TX07-B (29° 29' N, 102° 53' W), *R. Laport 766256*; JF266811, JF266812, JF266813; JF267045, JF267046, JF267047; JF266928, JF266929, JF266930; JF267162, JF267163, JF267164; JF267279, JF267280, JF267281; JF267302. (2x); U. S. A.: Texas: Brewster Co.: TX07-C (29° 34' N, 102° 57' W), *R. Laport 766257*; JF266814, JF266815, JF266816; JF267048, JF267049, JF267050; JF266931, JF266932, JF266933; JF267165, JF267166, JF267167; JF267282, JF267283, JF267284;-. (2x); U. S. A.: Texas: Culberson Co.: VANHRN (30° 46' N, 104° 44' W), *R. Laport 766258*;-. (2x); U. S. A.: Texas: El Paso Co.: TX07-A (31° 41' N, 106° 16' W), *R. Laport 766259*; JF266808, JF266809, JF266810; JF267042, JF267043, JF267044; JF266925, JF266926, JF266927; JF267159, JF267160, JF267161; JF267276, JF267277, JF267278;-. (2x); U. S. A.: Texas: Jeff Davis Co.: SKENT (31° 00' N, 104° 11' W), *R. Laport 766260*; JF266797, JF266798, JF266799, JF266800; JF267031, JF267032, JF267033, JF267034; JF266914, JF266915, JF266916, JF266917; JF267148, JF267149, JF267150, JF267151; JF267265, JF267266, JF267267, JF267268;-. (2x); U. S. A.: Texas: Jeff Davis Co.: TX07-D (30° 44' N, 104° 41' W), *R. Laport 766261*; JF266817, JF266818, JF266819; JF267051, JF267052, JF267053; JF266934, JF266935, JF266936; JF267168, JF267169, JF267170; JF267285, JF267286, JF267287;-. (4x); Mexico: Baja California: Mup. Mexicali: LAGSA (32° 20' N, 115° 47' W), *R. Laport 766223*; JF266784, JF266785; JF267018, JF267019; JF266901, JF266902; JF267135, JF267136; JF267252, JF267253;-. (2x); Mexico: Chihuahua: Mup. Chihuahua: CHICITY (28° 31' N, 105° 56' W), *R. Laport 766241*;-. (2x); Mexico: Coahuila de Zaragoza: Mup. Cuatrociénegas: CUATR (26° 52' N, 102° 08' W), *R. Laport 766242*; JF266782, JF266783; JF267016, JF267017; JF266899, JF266900; JF267134, JF267135; JF267250, JF267251; JF267306. (2x); Mexico: Coahuila de Zaragoza: Mup. San Pedro: SPDLIC (25° 52' N, 102° 54' W), *R. Laport 766243*;-. (2x);

