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A Revised Classification of *Glossopetalon* (Crossosomataceae) Based on Restriction Site-Associated DNA Sequencing

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Abstract*—Glossopetalon* inhabits arid regions in the American west and northern Mexico on limestone substrates. The genus comprises four species: *G. clokeyi*, *G. pungens*, *G. texense*, and *G. spinescens*. Three of the species are narrow endemics. The fourth, *G. spinescens*, is a widespread species with six recognized varieties. All six varieties are intricately branched shrubs that have been difficult to identify due to a lack of clearly delineating morphological characters. Characters typically used to differentiate the varieties of *G. spinescens,* such as stem coloration, leaf blade size, and presence of stipules, are highly variable within and among populations. A custom protocol of double digest restriction-site associated DNA sequencing (ddRAD) was used to resolve the phylogeny of *Glossopetalon* and address if population genetic data analyses (such as STRUCTURE, SVDquartets, and phylogenetic networks) support the recognition of six varieties of *G. spinescens*. *Glossopetalon* was fully supported as monophyletic and *G. pungens* was resolved sister to the remaining taxa in the genus. The varieties of *G. spinescens* were resolved as two distinct lineages corresponding to their biogeography, one to the northwest (lineage 1) and one to southeast (lineage 2) of the species range. *Glossopetalon clokeyi* was resolved at the base of lineage 1 and *G. texense* was embedded within lineage 2 sister to *G. spinescens* var. *spinescens*. Taxonomic changes include the recognition of *G. texense* and *G. clokeyi* as varieties of *G. spinescens* and description of a unique population from northern Arizona as a new variety, *G. spinescens* var. *goodwinii*.

***Keywords*—**ddRAD, RADseq, STRUCTURE, SVDquartets.

Glossopetalon A.Gray (Gray 1853) is a genus of shrubby plants inhabiting the western United States and northern Mexico (St. John 1942). This genus is composed of four species: *G. clokeyi* (Ensign) H.St.John, *G. pungens* Brandegee, *G. spinescens* A.Gray, and *G. texense* (Ensign) H.St.John. In addition to the four species, there are six accepted varieties of *G. spinescens* for a total of nine taxa. The six accepted varieties include: *G. spinescens* var. *spinescens*, *G. spinescens* var. *microphyllum* N.H.Holmgren, *G. spinescens* var. *aridum* M.E.Jones, *G. spinescens* var. *meionandrum* (Koehne) Trelease, *G. spinescens* var. *planitierum* (Ensign) Yatskievych, and *G. spinescens* var. *mexicanum* (Ensign) H.St.John.

Formerly, the species of *Glossopetalon* were treated as *Forsellesia* Greene in Ensign's monograph (1942) because the Celastraceae also contained the genus *Glossopetalum* Schreber, which had priority. St. John (1942) disagreed with the use of *Forsellesia* and made all of the requisite combinations for Ensign's new taxa in *Glossopetalon*. The genus was transferred to the Crossosomataceae in 1978 based on morphological data (DeBuhr 1978; Thorne and Scogin 1978). Furthermore, additional studies using chloroplast barcoding markers have confirmed the placement of *Glossopetalon* in the Crossosomataceae (Sosa and Chase 2003; Oh 2010).

Glossopetalon is comprised of three endemic, morphologically distinct species: *G. texense,* which is distinguishable through its weakly spinescent stems and absence of stipules; *G. clokeyi* by its mucronate leaves and prostrate habit; and *G. pungens* by its terminal flowers, short stature, and spine-tipped leaves. *Glossopetalon texense* is endemic to the Nueces River drainage in Texas, *G. clokeyi* is endemic to the Spring Mountains of Nevada, and *G. pungens* is found in Clark, Lincoln, and Nye counties of Nevada in addition to San Bernardino County in California (Mason and Yatskievych 2014). All of these narrow endemics are species of conservation concern. Using the ranking system developed by NatureServe, two of the three taxa (*G. clokeyi* and *G. pungens*) have Imperiled G2 status while *G. texense* has a Critically Imperiled G1 status. Their status is due to the small number of known populations, substrate specific habitat, and a potential for disturbance (NatureServe 2017).

Glossopetalon spinescens is widespread from northern Mexico through the western United States. *Glossopetalon spinescens* is separated from the narrow endemics by its taller stature and strongly spinescent stem tips. Subtle morphological differences throughout its broad geographical range were previously treated as five distinct species in *Forsellesia: F. planitierum* Ensign, *F. meionandra* (Koehne) A. Heller, *F. nevadensis* Greene, *F. spinescens* (A.Gray) Greene and *F. stipulifera* (H.St.John) Ensign (Ensign 1942). Detailed morphological work by Holmgren (1988) and Yatskievych (2007) led to the inclusion of these five species in *G. spinescens* as four varieties with *F. nevadensis* and *F. stipulifera* placed in synonymy with *G. spinescens* var. *aridum*. The inclusion of *G. spinescens* var. *mexicanum* (St. John 1942) and *G. spinescens* var. *microphyllum* (Holmgren 1988) resulted in the six varieties currently recognized. In his treatment, Holmgren (1988) noted that these taxa did not "reveal adequate differences for species recognition." Furthermore, Shevock (1993) has questioned the validity of even recognizing subspecific taxa in *G. spinescens* stating that the taxa are highly variable and are delimited by weak characters.

Current keys to the varieties of *Glossopetalon spinescens* rely heavily on the morphology of the free portion of the stipules; however, these are poorly developed in *G. spinescens* var. *planitierium* and *G. spinescens* var. *meionandrum* and entirely absent in *G. spinescens* var. *spinescens* according to Mason and Yatskievych (2014). Published accounts of *G. spinescens* var. *mexicanum* conflict on whether stipules are present. Ensign (1942) originally described the type specimen as lacking stipules while Yatskievych's (2007) examination of the isotype indicated that it possessed "well developed stipules." Furthermore, stipules are "sometimes not observable when

leaves are fasciculate on short shoots" (Mason and Yatskievych 2014). *Glossopetalon spinescens* varieties *microphyllum* and *aridum* have both been described as having free portions of the stipules that are "sometimes difficult to observe" (Mason and Yatskievych 2014). In using the free portion of the stipules for the identification of the varieties of *G. spinescens*, it can be difficult to distinguish between absent free portions of stipules versus those that are difficult to observe.

Alternative characters used to distinguish the varieties of *G. spinescens* are stem coloration (Yatskievych 2007), leaf blade length (Ensign 1942), and coloration of swollen leaf bases that are comprised of the petiole and the adnate portion of the stipules (Mason and Yatskievych 2014). These characters are often variable within and among populations (Yatskievych 2007). In *G. spinescens* var. *mexicanum* second year stem coloration is green to yellowish versus orange/brown in *G. spinescens* var. *microphyllum* (Yatskievych 2007). Both of these varieties have smaller leaves but inhabit different areas of North America. *Glossopetalon spinescens* var. *microphyllum* is found in eastern Nevada, northern Arizona, and western Utah (Holmgren 1988) while *G. spinescens* var. *mexicanum* is found in Coahuila and Nuevo León, Mexico. Several varieties, namely *G. spinescens* var. *aridum*, *G. spinescens* var. *microphyllum*, and *G. spinescens* var. *planitierum* share swollen, dark-colored adnate portions of the stipules, which range from dark purple, red, to black (Mason and Yatskievych 2014). *Glossopetalon spinescens* varieties *mexicanum* and *spinescens* can have dark or whitish-brown adnate portions of the stipules instead. Due to the difficulty identifying the varieties of *G. spinescens* using the current morphological characters and a lack of information on seed morphology, a more detailed morphological comparison was undertaken in this study to assess the validity of the varieties and identify morphological character delimitations between the taxa.

The difficulty in using the current key to identify taxa became particularly obvious when a morphologically unique population of *Glossopetalon* was discovered on the Babbitt CO Bar Ranch in northern Arizona. This population did not fit into any of the published varieties of *G. spinescens* because of its short stature, intricate branching, and small, scabrous leaves. The population of *Glossopetalon* on the CO Bar Ranch is a low mounded, densely branched shrub 5–36 cm tall, with scabrous leaves 3–5 mm long, 0.8–1.5 mm wide, scabrous triangular free portions of the stipules, and dull green to yellowish green second year stem coloration (Fig. 1). The growth form is similar to that found in two endemic species, *G. clokeyi* and *G. pungens*, and is the key characteristic used to separate these taxa from *G. spinescens*. However, both of these species grow in crevices in vertical limestone cliff faces whereas the CO Bar Ranch population grows in crevices of horizontal limestone outcrops at the edge of cliffs. Furthermore, the two endemics do not have strongly spinescent growth forms or noticeably thickened dark purple-black adnate portion of their stipules like that of the CO Bar Ranch population. Given these discrepancies, we sought to identify this population using morphological and genomic data.

To date there has not been a robust study of the phylogeny of this genus. Sequence data for the barcoding markers *rbcL*, *atpB*, and *matK* exists only for an undetermined variety of *Glossopetalon spinescens* (Sosa and Chase 2003) and *G. spinescens* var. *aridum* (Oh 2010). Therefore, the goals of this study were to: 1) examine morphology to identify distinguishing characters of the varieties of *G. spinescens*; 2) identify the *Glossopetalon* population on the CO Bar Ranch; and 3) identify the species relationships within the genus and assess the varieties using molecular data. Due to the cryptic and variable morphological differences among the taxa of *Glossopetalon* and the possibility of gene flow, double digest restriction-site associated DNA sequencing (ddRAD) was employed to provide better phylogenetic resolution through generating genome-wide markers. Restriction-site associated DNAseq has been useful in clarifying unresolved phylogenetic relationships at or above the species level (Emerson et al. 2010; Eaton and Ree 2013; Hipp et al. 2014; Herrera and Shank 2016) and at the subspecific level (Reitzel et al. 2013; Xu et al. 2014; Shih et al. 2018).

Materials and Methods

Taxon Sampling—One hundred twenty-four samples representing 21 populations were collected for this study during 2018 and 2019. Eleven samples were obtained from herbarium specimens on loan from the following institutions: IEB, UNM, and TEX (Thiers 2020). Our molecular study incorporated representatives of all the genera comprising the Crossosomataceae. Each taxon of *Glossopetalon* was represented by a minimum of five samples (Table S1). Locality information and voucher numbers are also available in Table S1 (Allen and Ayers 2021). Collections were made from the type localities of each taxon except for *G. spinescens* var. *aridum*, which is the most widespread variety. These type locations were selected in an effort to capture the morphological divergence within *G. spinescens* while maintaining consistency in identifying taxonomic boundaries. Collections of *G. spinescens* from non-type localities such as the CO Bar Ranch population and collections from New Mexico were also made in order to identify populations through molecular methods. Collections were made under the following permit numbers: *Apacheria chiricahuensis* C.T.Mason CHIR-2019-SCI-0004; *Glossopetalon pungens* MOJA-2018-SCI-0029, and US Forest Service Region 3 RO-307.

DNA Extractions, Library Preparation, and Sequencing—Genomic DNA was isolated from silica dried leaf material and herbarium vouchers using an amended Sorbitol protocol (Štorchová et al. 2000) with the exception of *G. texense*, which was extracted using a CTAB protocol with the addition of pvp-40 (Doyle and Doyle 1987). Preliminary DNA quality was assessed with 1% agarose gel electrophoresis. DNA quantifications and purity determinations were conducted via a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Carlsbad, California) and PicoGreen quantification was conducted with a Synergy HTX Multi-Mode Microplate Reader (BioTek Instruments, Winooski, Virginia). All samples were normalized to 10ng/μL using 10mM Tris-Cl pH 8.0 before library preparation.

The libraries were prepared using an amended protocol of Peterson et al. (2012). Template DNA was digested with restriction enzymes *Msp*I and *Eco*RI (New England Biolabs, UK). Adapter ligation was simultaneously conducted during the same reaction. Preparation of the adapters (Eurofins Genomics, Louisville, KY) were as follows: the P1.1 *Eco*RI Adapter 5′-CCTATGTGGAGAGCCAGTAAGCGATGCTATGGT-3′ was annealed to P1.2 *Eco*RI Adapter 5′-[PHO]AATTACCATAGCATCGCTTACTGGCTCTCCACATAGG-3′ using a PTC-100 Programmable Thermal Cycler heated to 95°C for 5 minutes followed by a cool down to 25°C. Afterwards the *Eco*RI adapter was diluted to a concentration of 0.05 μM with sterile water. The P2.1-*Msp*I Adapter 5′-GTCAACGCTCACTACTGCGATTACCCAAGTCAG-3′ was likewise annealed to P2.2 Adapter 5′-[PHO]GCCCTGACTTGGGTAAGATAGCAC-3′,′ but subsequently diluted to a concentration of 0.5 μM using sterile water. The differences in concentration of the adapters were to account for the higher frequency of *Eco*R1 restriction enzyme sites. T4 DNA Ligase (New England Biolabs, UK) was employed to ligate the adapters to digested DNA fragments. Furthermore, the reagents utilized for this reaction were: BSA (100×), *Eco*R1 10× Buffer, T4 DNA Ligase 10× Buffer with 10 μM ATP, and sterile water. The reaction underwent 6 cycles of 37°C for 20 mins followed by 25°C for 20 mins and remained in the thermal cycler overnight at 10°C.

A 1:1 bead cleanup was performed with 25% PEG before the PCR indexing reaction. This amplification reaction consisted of Phusion HS II (Thermo Fisher Scientific, Waltham, MA), $MgCl_2$, custom primers, template DNA, and sterile water. Each sample was double indexed using distinctive forward and reverse indices. Indexing was performed over

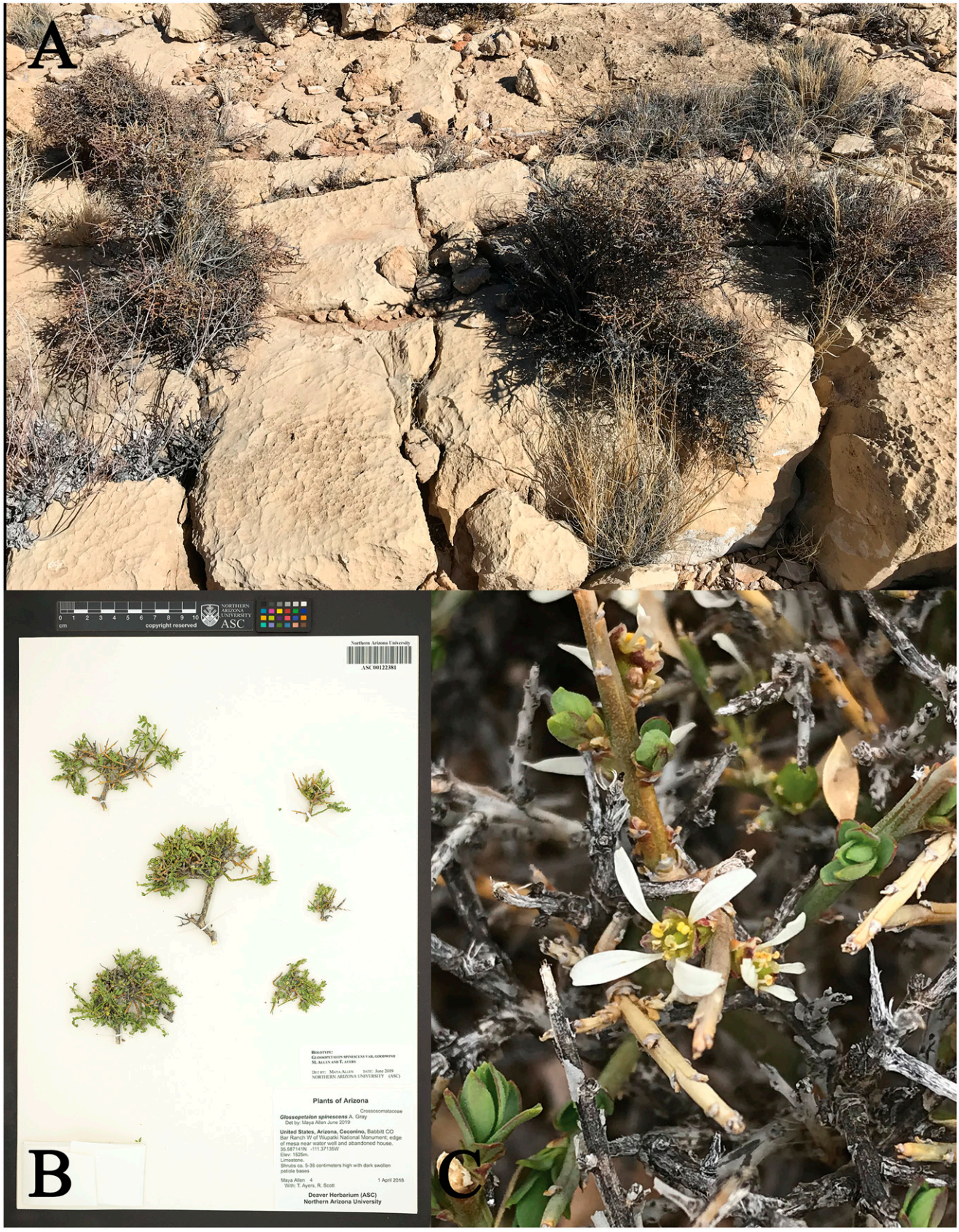

FIG. 1. Photographs of individuals of the CO Bar Ranch population. A. Limestone habitat. B. Holotype of *Glossopetalon spinescens* var. *goodwinii*. C. Flower, two- to three-year-old stems, and young leaves on new growth.

25 cycles of 95°C for 1 min, 35°C for 15 secs, 55°C for 15 secs, 72°C for 30 secs, and 72°C for 7 mins. Now that samples were indexed, all samples were pooled and underwent a 1:1 bead clean up with 18% PEG. Samples were subsequently quantified using a Nanodrop 1000 Spectrophotometer and analyzed on an Advanced Analytical Fragment Analyzer (Advanced Analytical Technologies GmbH, Heidelberg, Germany). Based on the high presence of the fragments from 200–550 base pairs, a size selection at that range was conducted using the Pippen Prep (Sage Science, Beverly, Massachusetts) for the ddRAD library. These libraries were sequenced on a single lane on a HiSeq 4000 instrument (Illumina, San Diego, California) at the University of Oregon's Genomics and Cell Characterization Core Facility using custom primers to produce single-end 150 base pair reads.

Sequence Data Preparation—Demultiplexing of raw data was conducted in accordance with akutils RADseq utility protocol using the module fastq-multx from EA-UTILS (Andrews 2019; Aronesty 2019). Demultiplexed data is publicly available in Dryad (Allen and Ayers 2021). Reads were cleaned via the processradtags unit of STACKS v. 1.37-gcc-5.2.0 and subsequent steps were conducted in v. 2.4 (Catchen et al. 2013). To assess the correct parameter setting for data assembly in ustacks and cstacks, a subset of 12 individuals from 12 populations were selected for parameter experimentation in the denovo_map.pl genotyping pipeline (Paris et al. 2017; Rochette and Catchen 2017). Parameters include M, which is the maximum number of mismatches that can be seen between stacks of the same sample; n, which is the mismatches of any two alleles of the population; and m, which is the minimum number of reads allowed per allele. Results of these tests were plotted in R v. 3.6.1 (R Core Team 2019) to visualize the number of loci vs. polymorphic loci in addition to the distribution of SNPs per locus. The parameters selected were contingent upon their effect on loci found in a minimum of 80% of samples (r80 loci). The parameter values applied to the full dataset were those that recovered the largest number of loci. The parameters selected were M = 5, m = 3, and n = 5, and were applied to module units ustacks and cstacks. The populations program in STACKS was executed to generate the phylip file dataset under the parameters of a 50% minimum percentage of individuals to process a locus (-r), the minimum percentage of individuals across populations required to process a locus (-R) was set to 25%, and the minimum number of populations a locus must be present in to be processed was kept at the default of 1 (Rochette and Catchen 2017). This filtered dataset of 5556 loci comprising 46,268 variant sites was utilized for downstream phylogenetic analysis (Table S2).

Phylogenetic Analysis—Maximum likelihood (ML) analyses were conducted in PhyML (Guindon et al. 2010). Smart model selection (Lefort et al. 2017) in PhyML identified the GTR substitution model as the optimal model and applied this model during the analysis. jModelTest v2.1 (Darriba et al. 2012) also indicated GTR was the best model for this dataset; therefore, it was utilized for Bayesian analysis as well. *Crossosoma* Nutt. and *Velascoa* Calderón & Rzed. were selected as outgroups based on previous studies (Sosa and Chase 2003; Oh 2010). The PhyML analysis was carried out with 1000 bootstrap replicates. MrBayes (Huelsenbeck and Ronquist 2001) through CIPRES v. 3.3 Science Gateway (Miller et al. 2010) was employed for Bayesian inference (BI). The BEAGLE library was enabled to perform the core Bayesian calculations (Ayres et al. 2012). A 50% majority rule consensus tree was constructed in six runs using the GTR model with four Markov chain Monte Carlo (MCMC) chains for one million generations with a 25% burn-in. SVDquartets (Chifman and Kubatko 2014) implemented in PAUP* v. 4.0 (Swofford 2003) was used to generate a 50% majority rule phylogenetic tree under the multispecies coalescent model (MSC) with 10,000 bootstrap replicates. All 5866 quartets were evaluated for the 21 taxa. This particular methodology was employed due to the recent evidence highlighting the need for MSC analyses in addition to ML. SVDQuartets, which estimates the species tree directly from variant site patterns, has proven to be accurate in resolving lineages with conflicting gene and species trees (Chou et al. 2015; Gonçalves et al. 2019). To assess incompatible or ambiguous signals in our data set such as incomplete lineage sorting and gene flow, a phylogenetic network was produced in SplitsTree4 using the neighbor-net method and GTR evolutionary model with 1000 bootstrap replicates for *Glossopetalon spinescens* (Huson and Bryant 2006).

Population Structure Analysis—STRUCTURE was utilized to assess the number of populations of *Glossopetalon spinescens* identifiable through Bayesian clustering (Pritchard et al. 2000; Falush et al. 2003; Hubisz et al. 2009). This analysis was implemented with a K-value range of 1 to 10 with 10 iterations for each K-value without assigning population membership a priori. The burn-in period constituted 5000 iterations and an MCMC of 100,000 repetitions with admixture and correlated allele frequencies were assumed. STRUCTURE HARVESTER (Earl and vonHoldt 2012) was used to identify the number of populations based on ΔK, which identifies the log likelihood changes between K values to determine the optimal number of clusters (Fig. S2; Evanno et al. 2005). Results were visualized using CLUMPAK, Cluster Markov Packager Across K (Kopelman et al. 2015).

Morphological Analysis—The following characters are considered important in the *Flora of North America* treatment (Mason and Yatskievych 2014) and were examined on 105 specimens: presence or absence of stipules, stipular length and shape, thickness of the petiole and adnate portion of stipules, and coloration, leaf blade length, persistent or early deciduous leaves, stem coloration at the end of second/third year, and shrub height. Based on field observations, leaf blade vestiture, seed color, and aril morphology were identified as potentially taxonomically informative and were also reviewed. Loans from the following institutions were obtained to supplement field collections and vouchers already present at ASC and NAVA: ARIZ, IEB, LL, RM, TEX, UNM, UNLV, and UTC (Thiers 2020).

Results

Data—A total of 391,475,863 reads were produced by Illumina sequencing, which averaged to 2.7×10^6 reads per sample. After demultiplexing, Illumina filtering, and discarding low quality reads, reads per sample averaged to 2.4×10^6 per sample; this cleaned data set was further processed through the STACKS core pipeline. After completion of the final program unit of the pipeline, 113,537 loci were removed that did not pass sample/population constraints from the initial 119,093 loci. Next, of the remaining 5556 loci, composed of 846,047 sites, 1335 of those sites were filtered, and 46,268 variant sites remained. Therefore, a dataset of 5556 loci comprising 46,268 variant sites was produced. Filtering statistics are further illustrated in Table S2 (Allen and Ayers 2021).

Phylogenetic Analysis—All analyses corroborated those of Oh (2010) in that *Glossopetalon* was found to be sister to the genus *Apacheria* rather than sister to a clade of *Apacheria* and *Velascoa* (Sosa and Chase 2003; Zhu et al. 2006). All populations of *Apacheria* formed a monophyletic clade (Figs. 2–3; Fig. S1). The two remaining genera, *Crossosoma* and *Velascoa,* were recovered as sister taxa in every analysis (Figs. 2–3; Fig. S1).

In all analyses *Glossopetalon* was fully supported as a monophyletic group (Figs. 2–3; Fig. S1). The species relationships of the genus were fully supported in all analyses with *G. pungens* as sister to the rest of the taxa within the genus (Figs. 2–3; Fig. S1). *Glossopetalon spinescens* was supported in all analyses to be paraphyletic with the inclusion of *G. clokeyi* and *G. texense* (Figs. 2–3; Fig. S1). The varieties of *G. spinescens* are divided into two well-supported distinct lineages (Figs. 2–3; Fig. S1).

Lineage 1 of *G. spinescens* is comprised of: *G. spinescens* var. *aridum*, *G. spinescens* var. *microphyllum*, *G. spinescens* var. *meionandrum,* and *G. clokeyi*. The population of low shrubs from the CO Bar Ranch was resolved to be sister to *G. spinescens* var. *aridum* sampled from Sedona, Arizona. The *G. spinescens* var. *aridum* specimens from the Cataract Ranch, Arizona were sister to CO Bar + *G. spinescens* var. *aridum* Sedona. *Glossopetalon spinescens* var. *microphyllum* was sister to the *G. spinescens* var. *aridum* clade in the BI and ML analyses (Fig. 2; Fig. S1); whereas in the SVDquartets analysis it was resolved to be sister to the *G. spinescens* var. *meionandrum* clade (Fig. 3). The *G. spinescens* var. *aridum* clade was well supported to be sister to var. *meionandrum* in every analysis (Figs. 2–3; Fig. S1). *Glossopetalon clokeyi* was recovered as sister to the rest of the taxa in this lineage in every analysis (Figs. 2–3; Fig. S1).

Lineage 2 was recovered with full support across all analyses (Figs. 2–3; Fig. S1) and is comprised of two clades: (*G. spinescens* var. *planitierum*) and (*G. spinescens* var.

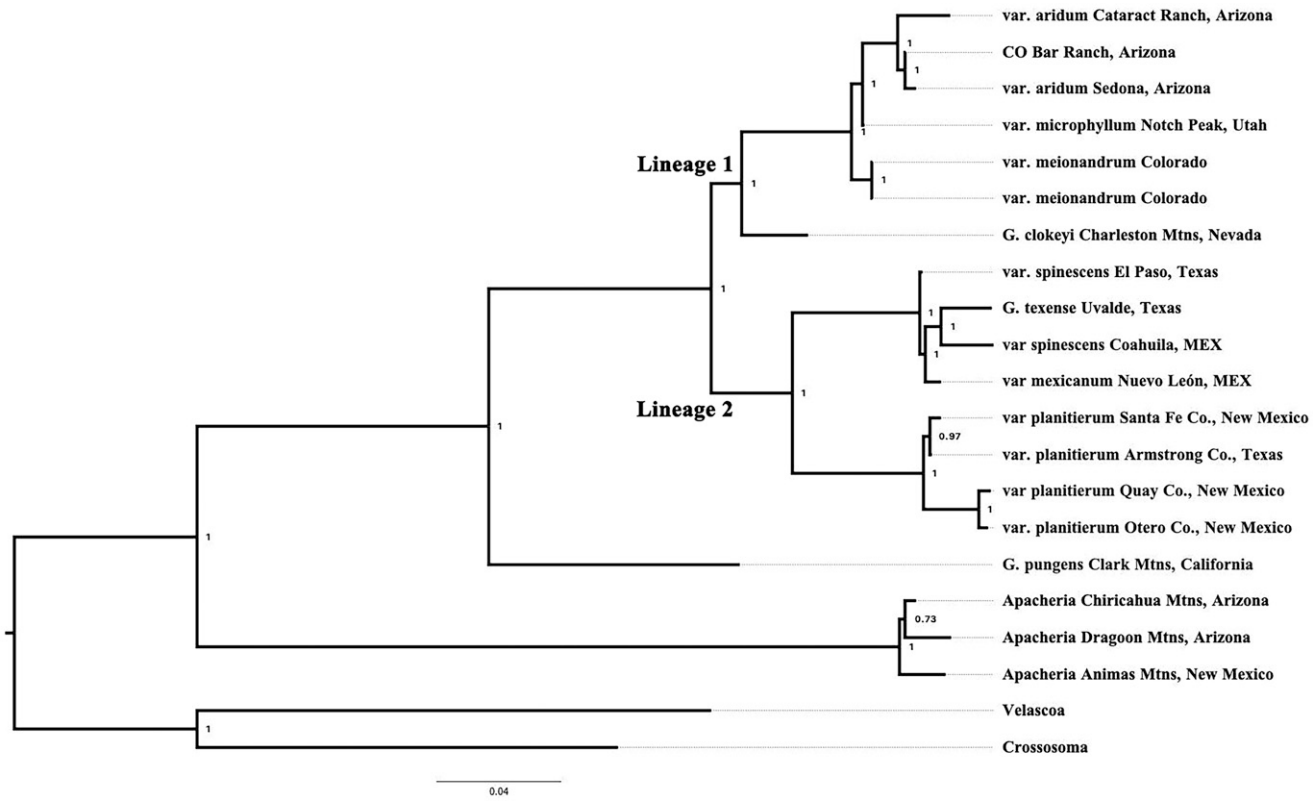

FIG. 2. A 50% majority rule Bayesian analysis consensus tree using the GTR evolutionary model with 4 Markov chain Monte Carlo (MCMC) chains for 1 million generations with a 25% burn-in. Posterior probability values are indicated at the nodes.

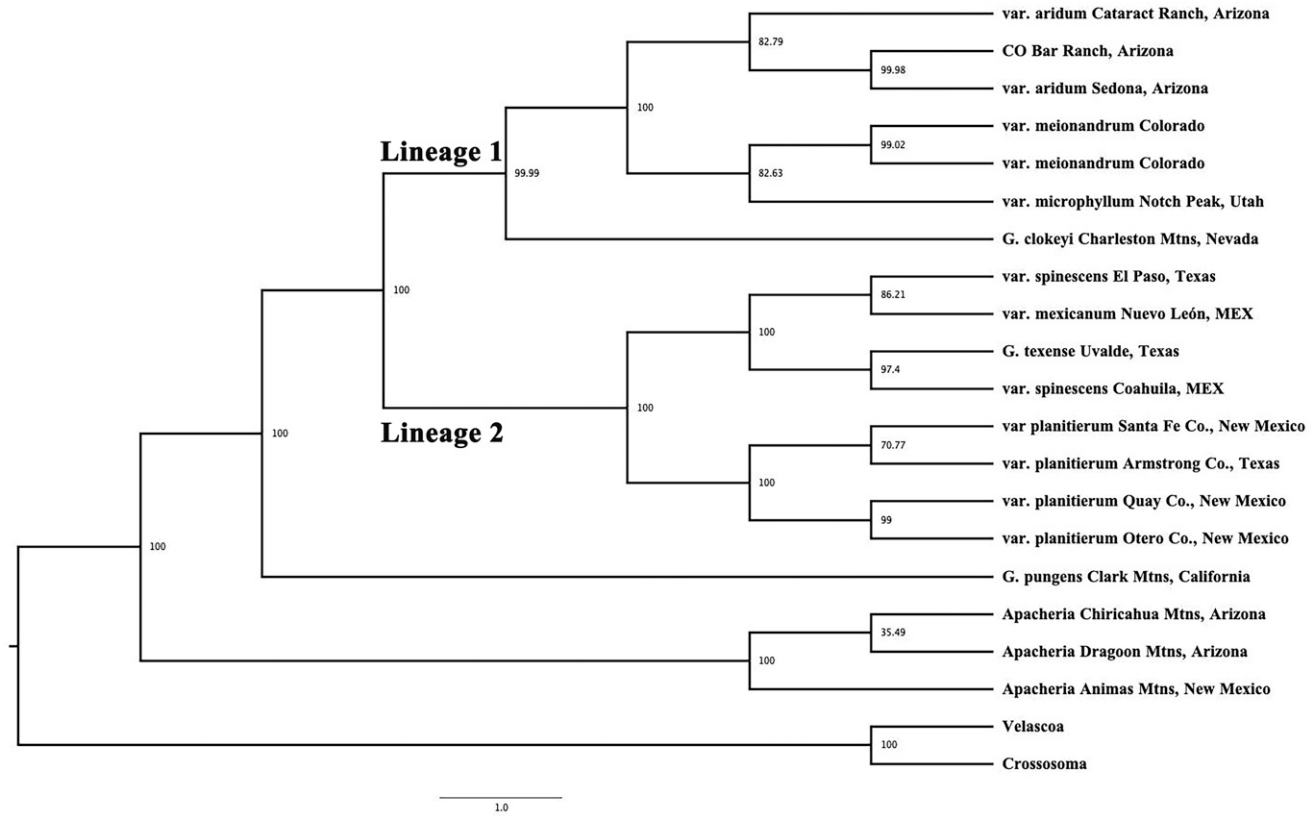

FIG. 3. Phylogenetic tree produced by SVDquartets under the multispecies coalescent model with 10,000 bootstrap replicates. Bootstrap support is indicated at each node.

spinescens + *G. texense* + var. *mexicanum*). The *G. spinescens* var. *planitierum* clade was represented by four populations with full support across all analyses for their monophyly (Figs. 2–3; Fig. S1). The clade of *G. spinescens* var. *spinescens* + *G. texense* + *G. spinescens* var. *mexicanum* was found to be monophyletic with full support in every analysis; interclade relationships, however, have conflicting results. SVDquartets with 86% support indicated *G. spinescens* var. *spinescens* is sister to the *G. spinescens* var. *mexicanum* Nuevo León population while *G. texense* is sister to var. *spinescens* Coahuila with 86% and 97% support, respectively (Fig. 3). These two clades' (*G. spinescens* var. *spinescens* + *G. spinescens* var. *mexicanum* Nuevo León and *G. texense* + *G. spinescens* var. *spinescens* Coahuila) sister relationship is fully supported (Fig. 3). Bayesian and ML analyses also resolved *G. texense* as sister to *G. spinescens* var. *spinescens* Coahuila (Fig. 2; Fig. S1) with the difference in topologies being that *G. spinescens* var. *mexicanum* Nuevo León and *G. spinescens* var. *spinescens* were resolved in a ladder and not as sister taxa. The BI analysis supported this topology with a posterior probability of 1, while ML has a 95% bootstrap value for *G. spinescens* var. *mexicanum* Nuevo León sister to (*G. texense* + *G. spinescens* var. *spinescens* Coahuila). The BI and ML analyses have full support for *G. spinescens* var. *spinescens* as the sister taxon to the rest of the clade (Fig. 2; Fig. S1).

Phylogenetic Network—In light of the conflicting relationships among analyses, namely, the position of *G. spinescens* var. *microphyllum*, and the relationships within the *G. spinescens* var. *spinescens* + *G. spinescens* var. *mexicanum* Nuevo León and *G. texense* + *G. spinescens* var. *spinescens* Coahuila clade, a phylogenetic network was created in SplitsTree4 to assess lineage histories that are not strictly bifurcating. Supplemental Figure 2 depicts the conflicting information that has contributed to a difference in topologies between the analyses (Allen and Ayers 2021).

Glossopetalon spinescens var. *microphyllum* was shown to have edges from *G. spinescens* var. *aridum* and *G. spinescens* var. *meionandrum* along with multiple splits in the phylogenetic network (Fig. S2). In assessing the relationships within the *G. spinescens* var. *spinescens* + *G. spinescens* var. *mexicanum* Nuevo León and *G. texense* + *G. spinescens* var. *spinescens* Coahuila clade, *G. spinescens* var. *spinescens* El Paso was recovered on its own edge arising from the split with the *G. spinescens* var. *planitierum* clade with a 100% bootstrap support. Along this same path a second node of *G. spinescens* variety *mexicanum* Nuevo León was fully supported. A final, fully supported edge terminated in a node that produced a split between *G. spinescens* var. *spinescens* Coahuila and *G. texense.* In sum, the phylogenetic network illustrates alternative splits and some gene tree discordance in the phylogenetic analyses for all mentioned populations in this clade (Fig S2).

Population Structure—STRUCTURE HARVESTER (Earl and vonHoldt 2012) identified two genetic clusters based on the ΔK graph (Fig. S3) (Evanno et al. 2005), which corresponded to the two spinescens lineages identified in other analyses (Figs. 2–4; Fig. S1). There was no admixture illustrated between the two lineages (Fig. 4). A second peak at K = 5 suggests further substructure within these two lineages (Fig. 4; Fig. S3).

Morphological Results—Morphological comparisons of *Glossopetalon texense, G. clokeyi,* and the varieties within *G. spinescens* are illustrated in Table S3 (Allen and Ayers 2021). Seed color is highly variable among the taxa with indications that as

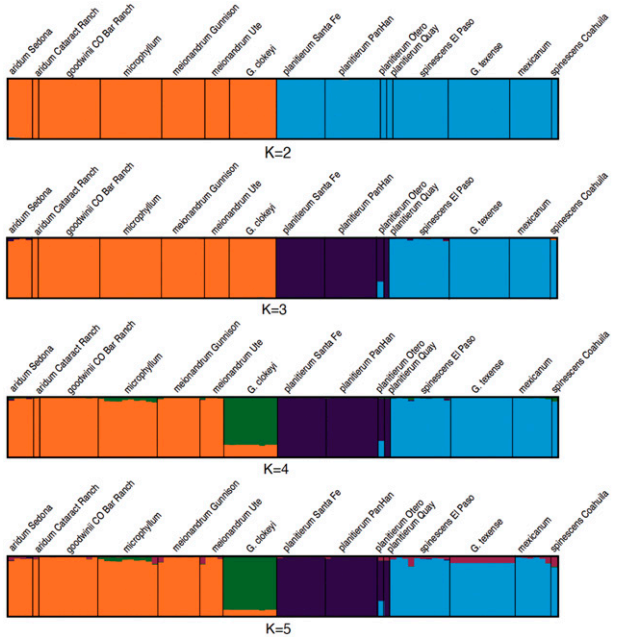

FIG. 4. CLUMPAK generated visualization of STRUCTURE analysis (K2–K5) of *Glossopetalon spinescens* with the optimal number of clusters K = 2 based on the ΔK graph generated by STRUCTURE HARVESTER, which corresponds to the two *spinescens* lineages. A second peak in the ΔK graph at K = 5 suggests further substructure within these two lineages.

seeds develop, they mature from cream to dark brown in color. *Glossopetalon clokeyi* collections consisted of cream seeds. Seed color is cream or dark brown in *G. spinescens* var. *microphyllum*, *G. spinescens* var. *planitierum*, and *G. spinescens* var. *mexicanum;* whereas in *G. spinescens* var. *aridum* seeds can be cream in color or light brown. *Glossopetalon texense* has light brown and dark brown seeds. The CO Bar Ranch population has only dark brown seeds. In *G. spinescens* varieties *meionandrum* and *spinescens*, seeds can be any of the three colors: cream, light brown, or dark brown. Seed size is generally around 2 mm for all the taxa and the seeds are micro-scabrate. In addition to identifying characters to differentiate the varieties, morphological characters were examined in light of the biogeographic distribution of the varieties of *G. spinescens* (Fig. 5). No characters were identified that can be used as morphological synapomorphies to separate the two lineages. However, scabrous leaf vestiture can differentiate the CO Bar Ranch population from *G. spinescens* var. *aridum*, which has glabrous leaf blades (Table S3). Pubescence of the CO Bar population's stipules were especially evident in the SEM images and is readily visible on the leaf blade under a dissecting microscope (Fig. 6). Vouchers of *G. spinescens* var. *spinescens* from El Paso and Coahuila, Mexico possess extremely minute free portions of the stipules ranging from 0.1–0.3 mm in length and these are possibly early deciduous, which is why they have been difficult to observe. *Glossopetalon spinescens* var. *mexicanum* also possessed well-developed free portions of the stipules confirming Yatskievych's (2007) examination of the isotype.

DISCUSSION

Phylogenetic Relationships—This study verified the generic relationships within the Crossosomataceae presented by Oh (2010). *Glossopetalon pungens* has been suggested to be

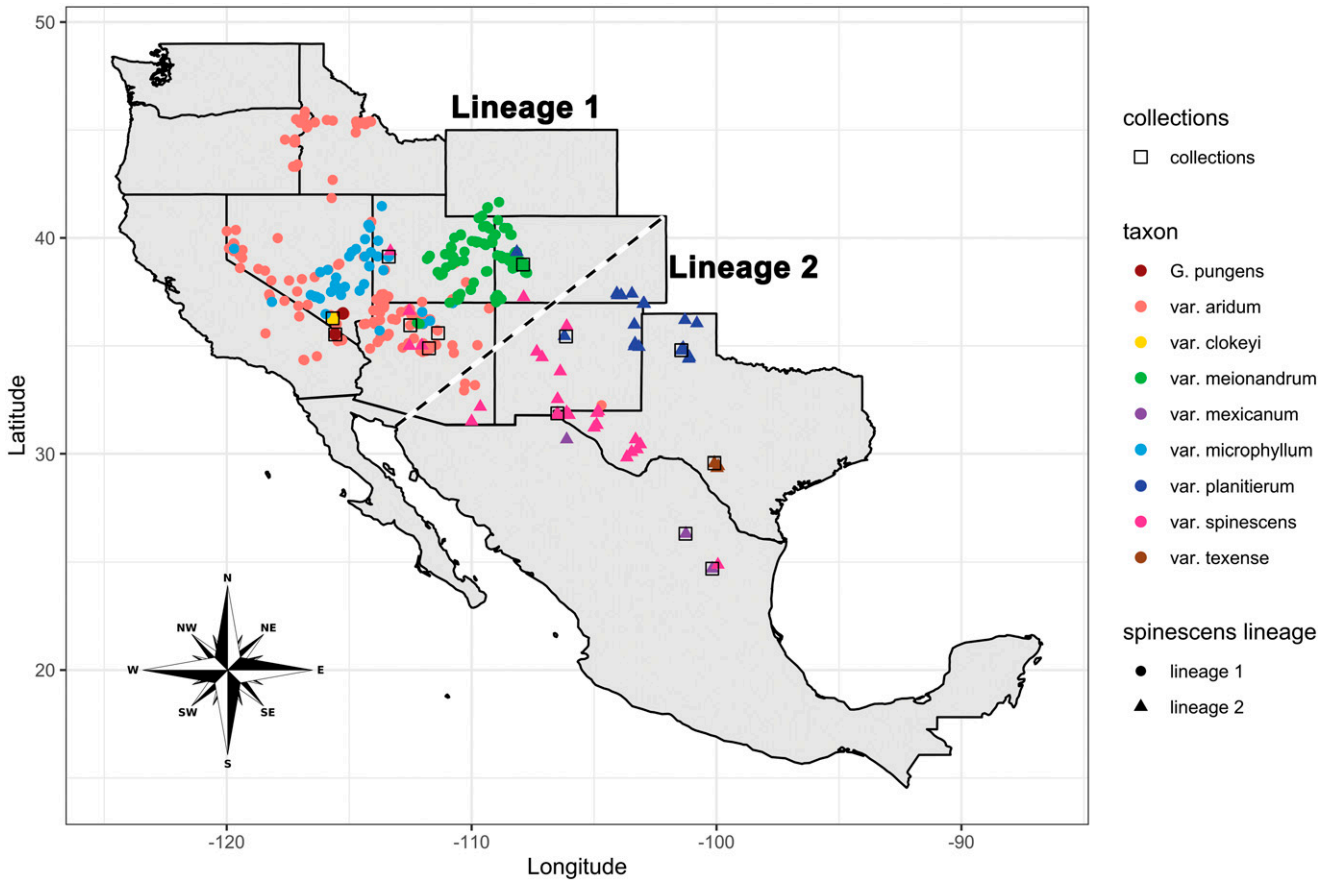

FIG. 5. Map of *Glossopetalon spinescens* collections illustrated by black open boxes and SEINet and MEXU herbarium occurrences (all other colors) with black dashed line showing separation of *Glossopetalon spinesens'* lineages. Taxa resolved in lineage 1 are indicated as circles and taxa resolved in lineage 2 are noted as triangles.

a member of the genus *Apacheria* due its similar spine-tipped leaves, prostrate habit, and terminal flowers like those seen in *Apacheria* (Ensign 1942). However, this study confirmed that *G. pungens* is sister to the rest of the taxa in the genus *Glossopetalon* and that the presence of alternate leaves, five sepals and petals, and five to ten stamens is a synapomorphy for the genus (versus opposite leaves, four sepals and petals and eight stamens of *Apacheria*). Shared morphological characters between *G. pungens* and *Apacheria* may be the result of shared pleisiomorphic characters that have been lost with subsequent speciation.

Glossopetalon clokeyi also has a prostrate habit and preference for vertical cliffs like *G. pungens* and *Apacheria.* Despite this shared morphology, *G. clokeyi* was sister to the rest of the taxa comprising lineage 1 of *G. spinescens* suggesting that prostrate habit is also a shared pleisiomorphy. These analyses in addition to the presence of stipules with slightly thickened adnate portions, axillary flowers, and acute leaf apices indicate that *G. clokeyi* should be treated as a variety of *G. spinescens* to accurately reflect the evolutionary history of this taxon.

Glossopetalon spinescens Lineages—*Glossopetalon spinescens* was identified as having two distinct lineages that reflect its geographical distribution (Figs. 2–5; Fig S1). Population STRUCTURE analysis indicated no admixture between the lineages when K = 2 (Fig. 4). Lineage 1 (*G. spinescens* var. *aridum*, *G. spinescens* var. *microphyllum*, *G. spinescens* var. *meionandrum*, and *G. clokeyi*) of *G. spinescens* occurs in the northwestern region of the species range from Arizona to Washington (Fig. 5). Lineage 2 (*G. spinescens* var. *planitierum* + *G. spinescens* var. *spinescens* + *G. texense* + *G. spinescens* var. *mexicanum*) occurs in the southeastern part of the species range from far eastern Arizona to northern Mexico (Fig. 5). Although *Glossopetalon spinescens* is composed of two distinct lineages, it lacks morphological characters to adequately describe the lineages as distinct species and thus must be retained a single species subdivided into its varieties.

Within lineage 1 analyses of *G. spinescens* var. *microphyllum* show discrepancies as to whether it is sister to *G. spinescens* var. *meionandrum* or *G. spinescens* var. *aridum* (Figs. 2–3; Fig S1). The reticulated phylogenetic network (Fig. S2) may be due to gene flow among both taxa with *G. spinescens* var. *microphyllum* or incomplete lineage sorting. The BI and ML analyses (Fig. 2; Fig. S1) also indicate that *G. spinescens* var. *microphyllum* is more closely related to *G. spinescens* var. *aridum* given the posterior probability of 1 and branch support of 97%, respectively. However, this taxon was also well-supported at 82% as being sister to *G. spinescens* var. *meionandrum* in the SVDquartets analysis (Fig. 3). *Glossopetalon spinescens* var. *microphyllum* is morphologically similar to *G. spinescens* var. *aridum* in that it is also has early deciduous leaves, similar shape of the free portions of the stipules, and swollen dark adnate portions of the stipules (Table S3). Since *G. spinescens* var. *microphyllum* occurs near documented *G. spinescens* var. *aridum* and *G. spinescens* var. *meionandrum* populations in Utah, there is the possibility of gene flow from either taxon with *G. spinescens* var. *microphyllum*.

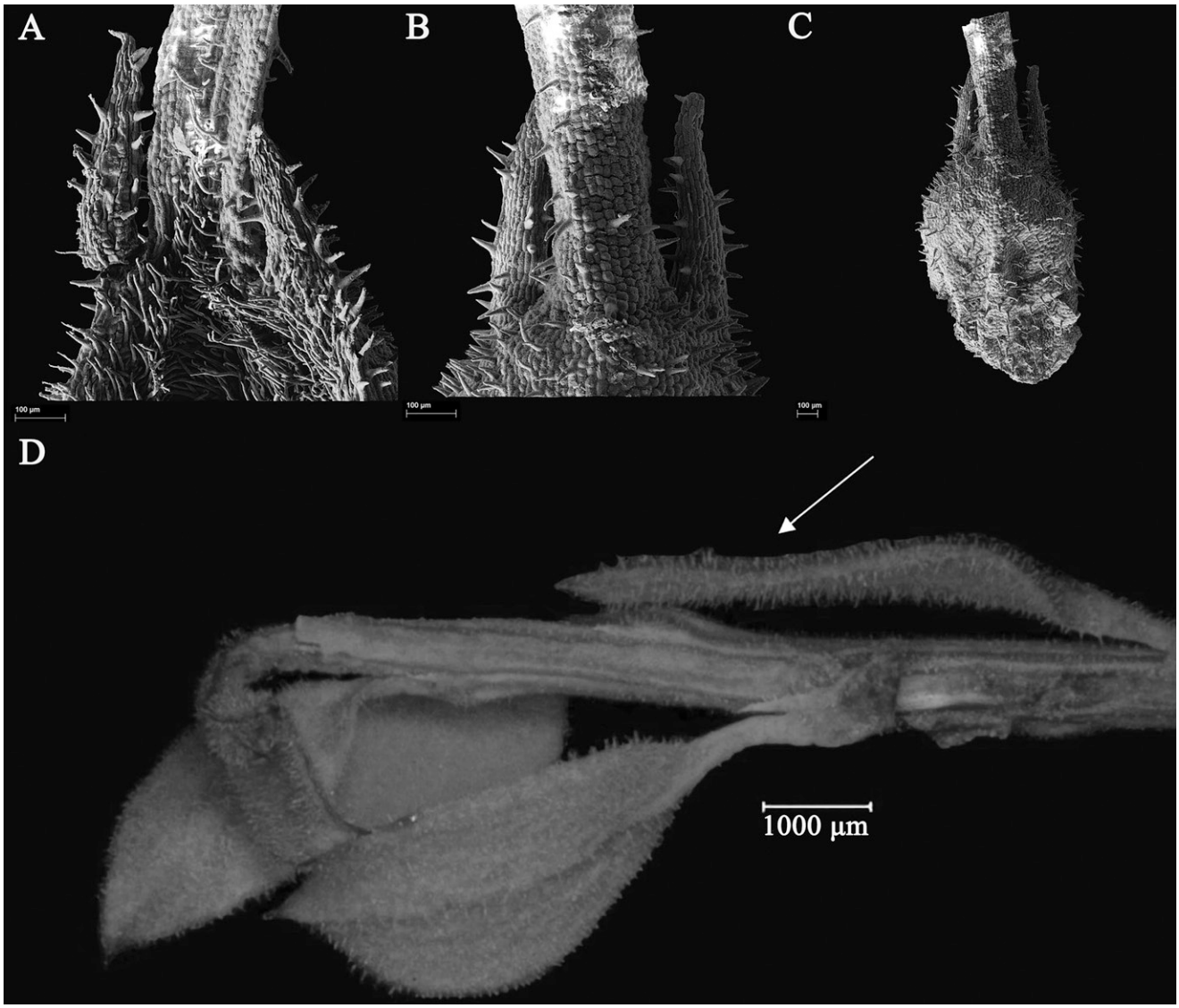

FIG. 6. Scanning electron microscopy illustrating the pubescent stipules of the CO Bar Ranch population. A. Adaxial view of free portion of the stipules. B. Abaxial view of free portion of the stipules. C. Abaxial view of adnate and free portion of the stipules. D. Digital microscope photographic image of pubescent vestiture of the CO Bar population. Arrow denotes a side view of a leaf showing distinctive scabrous vestiture on both surfaces.

The CO Bar Ranch population was identified as being closely related to *G. spinescens* var. *aridum*, the most widespread variety (Figs. 2–4; Fig. S1). Although embedded within the *G. spinescens* var. *aridum* clade, the population on the CO Bar Ranch differs from the *G. spinescens* var. *aridum* description in height (5–32 cm tall as opposed to the lowest limit of 25 cm for *G. spinescens* var. *aridum*). In addition to being significantly shorter, this population differs by being densely compact and intricately branched. The CO Bar Ranch population shares morphological characteristics with *G. spinescens* var. *aridum* in that it possesses darkened purple to black thickened adnate portions of the stipules and triangular free portions of the stipules (Table S3). In addition to scabrous stipules the CO Bar Ranch population also has scabrous leaves (Fig. 6); *G. spinescens* var. *aridum* is unique in having solely ciliolate leaf margins and the blade and stipules are glabrous. Due to the unique characteristics of the CO Bar Ranch population, it is described as a new variety to accurately reflect the morphological variation in this species.

Lineage 2 [*G. spinescens* var. *planitierum* (*G. spinescens* var. *spinescens* + *G. spinescens* var. *mexicanum* Nuevo León + *G. texense* + *G. spinescens* var. *spinescens* Coahuila)] primarily occurs in the southeastern region of the species range from eastern Arizona into northern Mexico (Fig. 5). All four populations of *G. spinescens* var. *planitierum* were supported as monophyletic with 100% bootstrap support in all phylogenetic analyses (Figs. 2–3; Fig. S1). Populations of *G. spinescens* var. *planitierum* also share the presence of free portions of the stipules, noticeably darkened and thickened adnate portions of the stipules, and tardily deciduous leaves (Mason and Yatskievych 2014) in contrast to the sister clade (*G. spinescens* var. *spinescens* + *G. spinescens* var. *mexicanum* Nuevo León + *G. texense*), which have minute or absent free portions of stipules, early deciduous leaves, and adnate portions of stipules not always thickened or darkened.

The sister clade to *G. spinescens* var. *planitierum* in lineage 2 is composed of *Glossopetalon texense*, two populations of *G. spinescens* var. *spinescens*, and a population of *G. spinescens*

var. *mexicanum* from Nuevo León. *Glossopetalon texense* lacks stipules, has stem tips that are not or weakly spinescent, and thickened leaf blade margins, a feature often seen in *G. spinescens* (Mason and Yatskievych 2014). Based on these results, *G. texense* is not recognized as a distinct species since it shares a common evolutionary history within *G. spinescens* and is sister to *G. spinescens* var. *mexicanum* populations in Nuevo León. As this taxon has unique characters unlike those of the current varieties of *G. spinescens*, *G. texense* should be treated as a variety (Table S3).

The clade *G. spinescens* var. *spinescens* + *G. spinescens* var. *mexicanum* Nuevo León + *G. texense* did not illustrate phylogenetic relationships that corresponded to geographical proximity. The Coahuila population of *G. spinescens* var. *spinescens* and *G. spinescens* var. *mexicanum* Nuevo León population were not resolved as sister taxa in SVDquartets (Fig. 3). Instead, *G. spinescens* var. *spinescens* Coahuila was found to be sister to *G. texense* from Uvalde County, TX (Fig. 3). This was also supported in the BI and ML analyses (Fig. 2; Fig. S1). The phylogenetic network illustrated a split between these taxa further supporting the sister relationship (Fig. S2). For the remaining two taxa in the clade, *G. spinescens* var. *mexicanum* Nuevo León was resolved sister to *G. spinescens* var. *spinescens* collected in El Paso, TX in the SVDquartets analysis, but this relationship was not exhibited in the BI and ML analyses (Fig. 2; Fig. S1). These two taxa in the phylogenetic network were fully supported as nodes on their own edges; however, these edges follow the same trajectory which eventually produces the split between *G. texense* and *var. spinescens* Coahuila with 100% bootstrap support (Fig. S2). More robust population sampling in Texas and throughout Mexico is necessary to resolve the population structure between *G. spinescens* var. *spinescens*, *G. spinescens* var. *mexicanum*, and *G. texense*.

Ensign (1942) originally described the type specimen of *G. spinescens* var. *mexicanum* from Sierra Pata Galana, Coahuila, Mexico as lacking stipules, but a more recent examination of the isotype and a second collection from Coahuila, Mexico indicated that this taxon possessed "well-developed stipules" (Yatskievych 2007). Our study confirmed that *G. spinescens* var. *mexicanum* possesses well-developed stipules (Table S3). Furthermore, Mason and Yatskievych (2014) suggested that *G. spinescens* var. *mexicanum* and *G. spinescens* var. *microphyllum* may be a single taxon as they both possess smaller leaves than the rest of the varieties. This suggestion is not supported as these taxa occur in two separate lineages of *G. spinescens* (Figs 2–4; Fig. S1). An additional outcome of this study is that specimens of *G. spinescens* var. *spinescens* were found to have very minute free portions of their stipules (0.1–0.3 mm in length) to well-developed stipules that fit the *G. spinescens* var. *aridum* description. As a result, *G. texense* is the only taxon in the "spinescens" lineage that does not possess the free portion of stipules (Table S3).

In summary, the phylogenetic relationships within the Crossomataceae identified by Oh (2010) were also resolved in this study. *Glossopetalon* was resolved as a monophyletic clade with *G. pungens* as the sister taxon to the rest of the taxa in the genus. *Glossopetalon spinescens,* was supported in all analyses to be paraphyletic with the inclusion of *G. clokeyi* and *G. texense* and represents two lineages. Seed color was not determined to be taxonomically informative. Taxonomic changes as a result of this research include the recognition of *G. texense* and *G. clokeyi* as varieties of *G. spinescens* and description of a unique population from northern Arizona as a new variety, *G. spinescens* var. *goodwinii*.

Taxonomic Treatment

Glossopetalon spinescens* var. *clokeyi (Ensign) M.L.Allen, comb. nov. *Glossopetalon clokeyi* (Ensign) H.St.John., Proc. Biol. Soc. Washington 55: 112 (1942). *Forsellesia clokeyi* Ensign. Amer. Midl. Naturalist 27: 504 (1942). Type: U.S.A. Nevada, Clark Co., Mt. Charleston Spring Mountains, Kyle Canyon, June 1940, *Clokey 8667* (holotype: UC; isotypes: A, CAS, GH, ILL, MICH, NY, PH, RENO, RSA, WIS).

Shrubs (10–)15–25 cm forming low mounds or mats, weakly spinescent. **Stems** dull green to yellowish green, transitioning to yellowish brown to gray after the second year; very slender 0.5 mm in diameter. **Leaves** early deciduous; stipules present, the free portion triangular to narrowly triangular or filiform, the adnate portion and petiole base not thickened or darkened; leaf blade oblanceolate, 4–6 mm × 1–1.5 mm, apex mucronate. **Flowers:** pedicels 1–2 mm; sepals 3–5, petals 3–5, oblanceolate, 2–4 mm long; stamens 4–6. F**ollicles** 2.5–4 mm long. **Seeds** cream, 1.7–2.1 mm, aril tan.

***Distribution and Habitat*—**The variety is found in crevices of vertical limestone cliffs in the Spring Mountains, Nevada.

***Specimens Examined*—USA. —**Nevada: Clark Co.: Spring Mountains, Robber Roost trail, [36.302293, -115.61083], 15 July 1993, *Frank J. Smith 3728* (UNLV 039067); Fletcher Canyon, [36.273686, -115.630360], 2363m, 8 July 2009, *P.J. Leary 6686* (UNLV 060966); canyon south of Robbers Roost, [36.301734, -115.611861], 2479m, 9 July 2009, *P.J. Leary 6696* (UNLV 060976); Mount Charleston, Kyle Canyon; trail to Mary Jane Falls, cliffs above trail circa 800 m SW of trail head, [36.27195, -115.67519], 2600 meters, 6 August 2018, *Maya L. Allen 15* (ASC00121534).

Glossopetalon spinescens* var. *texense (Ensign) M.L.Allen, comb. nov. *Glossopetalon texense* (Ensign) H.St.John. Proc. Biol. Soc. Washington 55:112 (1942). *Forsellesia texensis* Ensign. Amer. Midl. Naturalist 27: 510 (1942). Type: Texas, Uvalde Co., Montell, June 1917, *Palmer 12331* (holotype: CAS; isotypes: MO, LL, UC).

Shrubs 25–200 cm, upright with ascending branches, weakly spinescent. **Stems** laevigate, green to yellowish green transitioning to gray sometimes with black patches after the second year. **Leaves** persistent; stipules free portion absent; adnate portion of the stipules and petiole base whitened or light brown, rarely purple to nearly black; leaf blade oblanceolate, 6–20 mm × 3–5 mm, margins thickened, apex mucronate. **Flowers:** pedicels 4–7 mm; sepals 4 or 5, petals 4 or 5, oblanceolate, 5–7 mm long; stamens 7–9. F**ollicles** 4–5 mm long. **Seeds** dark brown or light brown, 2.9–3.2 mm, aril tan.

***Distribution and Habitat*—**The variety is found on ledges of limestone bluffs in the Nueces and Devil's River drainages.

***Specimens Examined*—USA.** —Texas: Uvalde Co. Ridge-top on divide between Sycamore Creek and Indian Creek watersheds, ca. 50–700 ft N to NNE of gate at Gap of Good Winds (on jeep trail marked on topo), ca. 1.6–1.7 airmiles SE to SSE of summit of Sycamore Mountain, ca. 2.0 airmiles W of Indian Creek Cave, on E 1/2 of Friday Ranch, 1680–1720 ft, [29.449722 -99.925833], 12 April 2000, *W. R. Carr 18819* (TEX); Montell Creek just E of County Road 415, ca 0.2 mi N of Machinery Hollow [29.574167, -100.085278], 450 meters, 5 June 2018, *T.J. Ayers 1934* (ASC00122244).

Glossopetalon spinescens* var. *goodwinii M.L.Allen, var. nov. Type: USA. Arizona. Coconino Co.: Babbitt CO Bar Ranch

NW of Wupatki National Monument; edge of mesa near water well and abandoned house. 35.587141N, -111.37135W, 1525 m, 1 April 2018, M. Allen 4 (holotype: ASC; isotypes: ARIZ, ASU).

Similar to *G. spinescens* var. *aridum* but plants 5–36 cm tall, stem coloration changing to orange or reddish brown after the second year, leaf blades, stipules, and adnate portion of the stipules and petiolar bases evenly scabrous.

Shrubs 5–36 cm, densely and intricately branched, spinescent. **Stems** dull green, pubescent, becoming orange or reddish brown in third to fourth year, older stems gray, glabrate. **Leaves** still developing at anthesis but early deciduous; stipules present and well developed, the free portion filiform, triangular, or linear, scabrous, mostly purple to black, the adnate portion and petiole base swollen and scabrous, purple to nearly black; leaf blade oblanceolate, scabrous, 2.31–7.76 mm × 0.9–1.7 mm, apex mucronate. **Flowers:** pedicels 1–2 mm; sepals 5, petals 6, linear, 3–5.2 mm long; stamens 5–10. **Follicles** 3–4 mm long. **Seeds** dark brown, 2 mm, aril tan. Figure 1.

***Distribution and Habitat*—**The variety has been found in crevices of horizontal Kaibab limestone outcrops at the edges of Marble Canyon or plateaus adjacent to the Little Colorado River drainage (Fig. S4).

***Phenology*—**The variety flowers from March to April (rarely with additional flowering in late September or October dependent on summer monsoons).

***Etymology*—**The variety is named in honor of the first collector, Greg Goodwin, a Forest Service biologist and avid plant collector in the southwestern US.

***Additional Specimens Examined*—USA.** —Arizona. Coconino Co.: Babbitt CO Bar Ranch east of Gray Mountain and north of Black Point. 35.43615, -111.24035, 1394 m, 4 June 2016, *G. Goodwin 5540* (ASC); Coconino Point [35.7954, -111.5800], 1564 m, 8 April 2014, *Marc A. Baker 18089* (ASC, NAVA); Navajo Nation, East Rim of Marble Canyon, just N of Sheep Springs Wash. 1545 m, 14 April 2014, *Daniela Roth 1767* (ASC, NAVA); Cape Solitude Quadrangle, Marble Canyon/Little Colorado River Gorge confluence, 1857 m, 3 May 2001, *Daniela Roth 1041* (ASC, NAVA); 3.3 miles south of Bitter Springs and 1.5 miles west of US highway 89A, 4.4 miles north of junction with US highway 89, 1524 m, 4 April 1991, *Bill Hevron 1052* (ASC, NAVA); Between Sheep Spring Wash and Tiger Wash east of Marble Canyon, 1584 m, 17 April 1991, *Bill Hevron 1094* (ASC, NAVA).

Revised Key to *Glossopetalon*

1. Stipules absent, leaf apices mucronate, mucro 0.6–1.2 mm, flowers terminal, stamens 10……*Glossopetalon pungens*
1. Stipules present but free portion sometimes difficult to observe (absent in var. *texense*), leaf apices acute to acuminate, rarely mucronate with mucro 0.1–0.4 mm, flowers axillary, stamens 5–10……*Glossopetalon spinescens*

Key to Varieties of *Glossopetalon spinescens* Based on Mason and Yatskievych (2014)

1. Free portion of stipules absent or extremely difficult to observe; leaf blade margins usually thickened
   2. Stem tips not or weakly spinescent; pedicels 4–7 mm long ..... *G. spinescens* var. *texense*
   2. Stem tips strongly spinescent; pedicels 1–2 mm. ..... *G. spinescens* var. *spinescens*
1. Free portion of stipules present; leaf blade margins usually not thickened
   3. Plants 5–36(–58) cm, forming low prostrate mounds or mats
      4. Plants (10–)15–25 cm, leaf blades and stipules glabrous. ..... *G. spinescens* var. *clokeyi*
      4. Plants 5–36 cm (58), leaf blades and stipules scabrous ..... *G. spinescens* var. *goodwinii*
   3. Plants 25–300 cm, forming relatively tall mounds or plants upright
      5. Leaf blades 3–7(8) × 1.2–2.7 mm ..... *G. spinescens* var. *microphyllum*
      5. Leaf blades 7–12(–17) × (1.5–)2–6 mm
         6. Free portion of stipules well developed, 0.5–1.7 mm; leaves often still developing at flowering and early deciduous; branches often appearing nearly leafless during much of growing season ..... *G. spinescens* var. *aridum*
         6. Free portion of stipules relatively poorly developed, 0.2–0.5(–0.8) mm; leaves mostly well developed at flowering and tardily deciduous; branches appearing leafy during most of the growing season.
            7. Stipule adnate portions yellowish or brownish tinged, often poorly developed, slightly thickened; petals mostly widest near apex, the apices rounded or abruptly acute to short-acuminate; stamens 5–7, equal or subequal ..... *G. spinescens* var. *meionandrum*
            7. Stipule adnate portions dark reddish purple to nearly black, usually well developed, noticeably thickened; petals mostly widest proximal to apex (sometimes nearly oblong), the apices rounded to gradually angled or acuminate; stamens usually 8, in 2 unequal series. ..... *G. spinescens* var. *planitierum*

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Author Contributions

MLA was responsible for most of the field work, gathering morphological and molecular data sets, analyzing all data, and writing the draft of the manuscript based on a Master's thesis submitted to Northern Arizona University; TJA proposed the project, helped with field work, provided guidance, and contributed to manuscript review and editing.

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