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EVOLUTION OF THE CHOLLAS (CACTACEAE)

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ABSTRACT

Cacti with the common name cholla are classified into several genera of the subfamily Opuntioideae (Cactaceae), and appear to be a monophyletic group. Although recent studies have provided strong resolution of the base of this group, assessment of evolutionary processes within genera have been limited due to taxon sampling. Analysis of four spacer and intron sequences of the chloroplast genome reveals evolutionary patterns and trends that are largely congruent with other recent studies. We concur with recent calls for the recognition of *Micropuntia pulchella* as monotypic and distinct from *Grusonia*, the name we use to refer to all species sometimes classified under *Marenopuntia* and *Corynopuntia*. *Grusonia* is monophyletic, and *Cylindropuntia* is borne on two clades of differing branch lengths. By incorporating the elements of ploidy, reproduction, and sequence divergence, we provide new insight into allopolyploid ancestry, the adaptive impact of ploidal-level variation in species, and the role of reproductive mode in diversification rates in the chollas.

Key Words: *Cylindropuntia*, chloroplast DNA, *Grusonia*, phylogeny, polyploidy.

The common name cholla, in combination with a great range of adjectives (club, dwarf, pencil, jumping, etc.) has been applied to mat-forming, shrubby, to tree-like species of cacti with segmented, cylindrical stems in North America. They are a familiar feature of the Chihuahuan, Sonoran, and Mohave deserts, and vary in distribution from narrow endemics to species with ranges that span multiple states on both sides of the U.S.-Mexican border. And although recent work has included the chollas in broader analyses of cactus phylogeny and taxonomy, treatment of evolutionary trends and interspecific relationships in this group have been less common. The present study aims to add to our understanding of the natural history of the chollas.

Recent classifications of the 70 or so cholla species have distributed them among a variety of genera: *Cylindropuntia* (Engelm.) F.M.Knuth, *Corynopuntia* F.M.Knuth, *Grusonia* Rchb.f. ex Britton & Rose, *Marenopuntia* Backeberg, or *Micropuntia* Daston. Contemporary phylogenetic analyses portray the chollas as monophyletic within subfamily Opuntioideae (Griffith and Porter 2009). The work of Barcenas et al. (2011) and Ritz et al. (2012) generated a proposal for three tribes in the Opuntioideae: Opuntieae, Tephrocacteae, and Cylindropuntieae, the latter of which would include the traditional chollas plus *Pereskiopsis* Britton & Rose and *Quiabentia* Britton & Rose. Recent work also resolves this pattern (Majure et al. 2019; Köhler et al. 2020).

Cylindropuntia is the largest genus of chollas; it comprises approximately 40 species, displays the broadest geographic range, and its circumscription is not currently debated. *Cylindropuntia* species are distinguished from the other chollas primarily by smooth spines that are fully covered by deciduous, papery sheaths. The remaining chollas have at one extreme all been assigned to *Grusonia* Rchb.f. ex Britton & Rose (*sensu* Stuppy 2002) or split among *Grusonia*, *Corynopuntia*, *Marenopuntia*, and *Micropuntia* in various treatments. These species contrast with *Cylindropuntia* by exhibiting mostly flattened, rough spines, with a spine sheath lacking or covering just the tip of the spine. The history of taxonomic revisions involving chollas has been summarized elsewhere (Barcenas 2016).

Diversification of the chollas has been tied to the expansion of the deserts of North America and the climatic fluctuations of the Pleistocene epoch (Arakaki et al. 2011; Hernandez-Hernandez et al. 2014; Majure et al. 2019). Adaptation and speciation were likely facilitated by hybridization and polyploidy, both of which are common in the chollas (Pinkava 2002). Polyploidy, following hybridization (allopolyploidy) or not (autopolyploidy), has resulted in chromosome count variation among and sometimes within species of *Cylindropuntia* ranging from $2n = 22$ to 88 (Pinkava 2002).

Despite solid recent work revealing the main clades of the chollas, there is still need for resolution of the patterns within these clades, incorporating both diploid and polyploid species. In the present study, we assess sequence variation in spacer regions and introns of the chloroplast genome to provide phylogenetic insight. Prior work has shown the utility of non-coding chloroplast DNA to phylogenetic studies both across vascular plants (Shaw et al. 2005, 2007) and among cholla species specifically (Wallace and Dickie 2002; Griffith and Porter 2009;

Barcenas et al. 2011; Barcenas 2016). For the purposes of interpreting allopolyploid ancestry, we generally assume maternal inheritance of the chloroplast genome, although biparental inheritance has been noted in some cactus species (Corriveau and Coleman 1988, Zhang et al. 2003). Here we present a denser sampling of both diploid and polyploid species than other recent studies of the Cylindropuntieae, with the goal of bringing new insights into the natural history of the chollas.

MATERIALS AND METHODS

Taxon Sampling

All sequences analyzed in the present study were generated from specimens we collected in the field or obtained from the Desert Botanical Garden (Phoenix, AZ). We aimed for exhaustive sampling of the species, regardless of ploidy, with multiple representatives included in the analysis where sample supply and PCR success allowed. *Opuntia littoralis* (Engelm.) Cockerell served to root the trees. A total of 86 exemplars representing 51 species were analyzed; taxon authorship and reported chromosome numbers are provided in Table 1.

DNA Extraction and Analysis

DNA extraction was performed on lyophilized stem tissue following the protocol of Pepper and Norwood (2001). DNA amplification primer sequences for the *trnH*$^{(GUG)}$-*psbA* intergenic spacer, the *rpL16* intron, and the *rpS16* intron followed Shaw et al. (2005), and for the *trnQ*$^{(UUG)}$-5′*rpS16* intergenic spacer, Shaw et al. (2007). Amplification of all regions used the following parameters: 80°C, 5 min; 35X (95°C, 1 min; 50°C, 1 min with a ramp of 0.3°C/s; 65°C, 4 min); 65°C, 5 min. Sequencing of purified PCR products (PureLink kit: Invitrogen, San Diego, CA) utilized the same primers as PCR, and was carried out by Retrogen, Inc. (San Diego, CA). Alignment of resulting sequences (Genbank accession numbers: Table 1) was done using MUSCLE (service of EMBL-EBL: ebi.ac.uk/Tools/msa/muscle/) and by eye; gaps were treated as missing data. Except for the Bayesian and parsimony analyses, indels were added to the data set as binary characters following a simple indel coding strategy (Simmons and Ochoterena 2000). Highly variable microsatellite loci (combinations of mostly uni-, di-, and trinucleotide repeats) were excluded from the analyses.

Phylogenetic analyses using maximum likelihood (ML) and Bayesian inference (BI) were performed on the CIPRES Science Gateway (Miller et al. 2010). ML analysis (RAxML 8.2.12 on XSEDE, Stamatakis 2014) employed the GTRGAMMA model of nucleotide substitution to estimate the best tree and calculate bootstrap support for the branches. Automated model selection in PAUP* (version 4.0a build 162, Sinauer Associates, Sunderland, MA) identified sequence evolution model HKY + I + G, which was employed for the BI analysis (MrBayes 3.2.6 on XSEDE, Ronquist et al. 2012). Four Markov Chain Monte Carlo (MCMC) chains were run for 1,500,000 generations, with sampling every 100 generations. Assessment of stationarity of -*ln*L values of these trees informed a 20% burn-in, and the remaining trees were used to construct a majority-rule consensus tree. Parsimony bootstrap values were generated using the PAUP* "fast" option and 100,000 replicates.

RESULTS

We generated all 339 sequences analyzed in the present study (Table 1); five sequences are missing due to amplification or sequencing failure. The aligned and concatenated sequence data set totaled 3147 bases (*trnH*($^{GUG)}$-*psbA* =481; *rpL16* =1188; *rpS16* =858; *trnQ*$^{(UUG)}$-5′*rps16* =620), of which 162 were excluded due to sequence ambiguity or the hypervariability associated with microsatellite loci; the sequences of this refined set yielded 24, 81, 34, and 27 parsimony informative characters, respectively. Thirty binary characters were added to the analysis to account for indel variation, for use in the Bayesian and parsimony bootstrap analyses.

There was no conflict among the Maximum Likelihood Best Tree (= ML best tree), the Bayesian Consensus Tree, and the Parsimony Bootstrap Tree, allowing us to use the ML Best Tree to show topology, branch length, and clade support from all analyses (Fig. 1). At the base of all trees, a poorly supported (60% Maximum Likelihood Bootstrap–MB, 52% Bayesian Posterior Probability–BP, 56% Parsimony bootstrap–PB) clade bears *Micropuntia pulchella* (Engelm.) M.P.Griff. and *Pereskiopsis* and is sister to the strongly supported (100%, 100%, 98%) clade of *Cylindropuntia* and remaining *Grusonia* (including *Corynopuntia* and *Marenopuntia)* species (Fig. 1). This echoes previous studies (Barcenas 2016; Griffith and Porter 2009; Majure et al. 2019) that showed substantial distinction of *Micropuntia pulchella* from any other species of *Grusonia*.

All trees exhibit the *Grusonia* + *Cylindropuntia* trichotomy, which is supported by eight synapomorphies, including a large deletion. Although this creates uncertainty about the monophyly of *Cylindropuntia* (Fig. 1), these same clades were resolved by Barcenas (2016) and Majure et al. (2019), but with *Grusonia* sister to a monophyletic *Cylindropuntia*, supported by 79% MB, 57% BP, and 100% PB (Barcenas 2016), and by 100% MB and PB (Majure et al. 2019). The three clades are labeled Cylindropuntia I, Cylindropuntia II, and Grusonia and are supported by three, eleven, and six apomorphies, respectively, with high support from each measure (Fig. 1). Thirty indels were coded and used only in the Bayesian analysis, and included 0, 5, and 1

TABLE 1. NOMENCLATURAL GUIDE TO THE SPECIMENS INCLUDED FOR DNA SEQUENCE ANALYSIS. Chromosome counts (in brackets; obtained from Pinkava 2002, Baker and Pinkava 2018) refer to the taxon, not these samples. Collection and voucher information: Desert Botanical Garden (DES), San Diego Natural History Museum (SD), Sul Ross University (SRSC); the remaining collections display collection and accession numbers referring to living specimens at the Desert Botanical Garden or the San Diego Botanic Garden (marked SBG). Genbank accession numbers are provided separately for the chloroplast introns and spacers examined: *rp*L16, *rp*S16, *trn*H, and *trn*Q; dashes note missing sequences. An asterisk (*) indicates collection information only.

Taxon	Terminal (Fig. 1)	Collection	*rp*L16	*rp*S16	*trn*H	*trn*Q
***Cylindropuntia* (Engelm.) F.M.Knuth**						
C. abyssi (Hester) Backeb. [22]	abyssii26505	Hodgson 26505 2020-0313-01	MW419436	MW419520	MW419604	MW419690
	abyssi2652	Hodgson 26520 2020-0314-01	MW419437	MW419521	MW419605	MW419691
C. acanthocarpa (Engelm. & Bigelow) F.M.Knuth [22]						
var. *acanthocarpa*	acanthocarpa0238	Hodgson 8115 1994-0238-0101	MW419438	MW419522	MW419606	MW419692
	acanthocarpa569	Baker 7878 1990-0569-0101	MW419439	MW419523	MW419607	MW419693
	acanthocarpa170	Quirk s.n. 1985-0170-0102	MW419440	MW419524	MW419608	MW419694
C. alcahes (F.A.C.Weber) F.M.Knuth [22, 33]						
var. *alcahes*	alcahes0064	Trelease s.n. 2009-0064-0101	MW419441	MW419525	MW419609	MW419695
	alcahes4835	SD: Rebman 4835	MW419442	MW419526	MW419610	MW419696
var. *burrageana* (Britton & Rose) Rebman	alcahesburr024	Baker s.n. 2010-0024-0101	MW419443	MW419527	MW419611	MW419697
	alcahesburr671	Rebman 2865 1994-0671-1001	MW419444	MW419528	MW419612	MW419698
C. anteojoensis (Pinkava) E.F. Anderson [44]	anteojoensis0089	Lott s.n. 1976-0089-0101	MW419445	MW419529	MW419613	MW419699
C. arbuscula (Engelm.) F.M. Knuth [66]	arbuscula201	Baker s.n. 1995-0201-0101	MW419446	MW419530	MW419614	MW419700
C. bernardina (Engelm. ex Parish) M.A. Baker et al.) [22]	bernardina624	SD: Mayer 624	MW419447	MW419531	MW419615	MW419701
C. bigelovii (Engelm.) F.M. Knuth [22, 33]						
var. *bigelovii*	bigelovii620	SD: Mayer 620	MW419448	MW419532	MW419616	MW419702
	bigelovii4956	SD: Rebman 4956	MW419449	MW419533	MW419617	MW419703
var. *ciribe* (Engelm. Ex J.M.Coult.) Rebman [22]	bigeloviiciribe5173	SD: Rebman 5173	MW419450	MW419534	MW419618	MW419704
C. californica (Torrey & A.Gray) F.M.Knuth [22]						
var. *californica*	californica575	Baker s.n. 1993-0575-0101	MW419451	MW419535	MW419619	MW419705
var. *rosarica* (G.E.Linds.)Rebman	californicaros917	unknown 1987-0917-0101	MW419452	MW419536	MW419620	MW419706
C. calmalliana (J.M.Coulter) F.M.Knuth [66]	calmalliana599	Baker s.n. 1993-0599-0101	MW419453	MW419537	MW419621	MW419707
	calmalliana673	Rebman 2822 1994-0673-1001	MW419454	MW419538	MW419622	MW419708

TABLE 1. CONTINUED

Taxon	Terminal (Fig. 1)	Collection	*rp*L16	*rp*S16	*trn*H	*trn*Q
C. caribaea (Britton & Rose) F.M.Knuth [?]	caribaea0356	Huntington Gardens s.n. 2004-0356-0109	MW419455	MW419539	MW419623	MW419709
C. cedrosensis Rebman [?]	cedrosensis2495	SD: Rebman 2495	———	———	MW419624	MW419710
C. cholla (F.A.C.Weber) F.M.Knuth [22, 33, 44]	cholla189	Thompson Arboretum s.n. 1939-0189-0101	MW419456	MW419540	MW419625	MW419711
	cholla4501	SD: Rebman 4501	MW419457	MW419541	MW419626	MW419712
C. delgadilloana J.Rebman & D.Pinkava [22]	delgadilloana712	Rebman 2560 1994-0712-1002	MW419458	MW419542	MW419627	MW419713
C. echinocarpa (Engelm. & Bigelow) F.M.Knuth [22]	echinocarpa626	SD: Mayer 626	MW419459	MW419543	MW419628	MW419714
	echinocarpa7728	Baker 7728 1990-0564-01	MW419460	MW419544	MW419629	MW419715
C. fosbergii C.B.Wolf [33]	fosbergii606	SD: Mayer 606	MW419461	MW419545	MW419630	MW419716
C. fulgida (Engelm.) F.M.Knuth [22, 33]	fulgida342	Hodgson 17454 2003-0342-0101	MW419462	MW419546	MW419631	MW419717
	fulgida568	Baker 7880 1990-0568-0101	MW419463	MW419547	MW419632	MW419718
	fulgida5281	SD: Rebman 5281	MW419464	MW419548	MW419633	MW419719
C. gander (C.B.Wolf) Rebman & Pinkava [22]						
subsp. *ganderi*	ganderi4077	SD: Rebman 4077	MW419465	MW419549	MW419634	MW419720
	ganderi4957	SD: Rebman 4957	MW419466	MW419550	MW419635	MW419721
subsp. *catavinensis* (Rebman) Rebman	gandericata166	Baker s.n. 2011-0166-0101	MW419467	MW419551	MW419636	MW419722
C. imbricata (Haw.) F.M.Knuth [22]	imbricata0048	Puente 4103 2009-0048-0103	MW419468	MW419552	MW419637	MW419723
	imbricata0059	Ward 363 2008-0059-0101	MW419469	MW419553	MW419638	MW419724
C. kleiniae (DC.) F.M.Knuth [44]	kleiniae0886	Leon s.n. 1993-0886-1001	MW419470	MW419554	MW419639	MW419725
C. leptocaulis (DC.) F.M.Knuth [22, 33, 44]	leptocaulis190	Kaumen s.n. 1978-0190-0201	MW419471	MW419555	MW419640	MW419726
	leptocaulis196	Kaumen s.n. 1978-0196-0201	MW419472	MW419556	MW419641	MW419727
C. lindsayi (Rebman) Rebman [44]	lindsayi127	Ward s.n. 2011-0127-0101	MW419473	MW419557	MW419642	MW419728
	lindsayi165	Baker s.n. 2011-0165-0101	MW419474	MW419558	MW419643	MW419729
C. molesta (Brandegee) F.M.Knuth [88]	molesta0244	Pinkava 8786 1973-0244-0102	MW419475	MW419559	MW419644	MW419730
C. munzii (C.B.Wolf) Backeb. [22, 33]	munzii4970	SD: Rebman 4970	MW419476	MW419560	MW419645	MW419731
C. prolifera (Engelm.) F.M.Knuth [22, 33, 66]	prolifera638	SD: Mayer 638	MW419477	MW419561	MW419646	MW419732
	prolifera3951	SD: Rebman 3951	MW419478	MW419562	MW419647	MW419733
C. ramosissima (Engelm.) F.M.Knuth [22, 33]	ramosissima28	Ward s.n. 2007-0028-0101	MW419479	MW419563	MW419648	MW419734

TABLE 1. CONTINUED

Taxon	Terminal (Fig. 1)	Collection	*rp*L16	*rp*S16	*trn*H	*trn*Q
	ramosissima909	Gass s.n. 1985-0909-2101	MW419480	MW419564	MW419649	MW419735
C. sanfelipensis (Rebman) Rebman [66]	sanfelipensis42	Rebman s.n. 1999-0042-0101	MW419481	MW419565	MW419650	MW419736
C. santamaria (E.M.Baxter) Rebman [22]	santamaria2785	SD: Rebman 2785	MW419482	———	MW419651	MW419737
C. spinosior (Engelm.) F.M.Knuth (= C. imbricata subsp. spinosior, Baker et al. 2019) [22]	spinosior0178	unknown 2003-0178-0101	MW419483	MW419566	MW419652	MW419738
C. tesajo (Engelm. ex Coult.) F.M.Knuth [22]	tesajo35	Lindsay s.n. 1939-0035-0102	MW419484	MW419567	MW419653	MW419739
	tesajo53	Rebman 4972 1999-0053-0101	MW419485	MW419568	MW419654	MW419740
C. tunicata (Lehmann) F.M.Knuth [22, 33]	tunicata345	Puente s.n. 2004-0345-0101	MW419486	MW419569	MW419689	MW419741
	tunicata568	Baker 6255 1985-0568-0108	MW419487	MW419570	MW419655	MW419742
C. versicolor (Engelm. ex Coult.) F.M.Knuth (= *C. thurberi* subsp. *versicolor* , Baker et al. 2019) [22]	versicolor0035	Hodgson 16449b 2003-0035-0103	MW419488	MW419571	MW419656	MW419743
C. whipplei (Engelm. & Bigelow) F.M.Knuth [22, 44]	whipplei0001	Hodgson s.n. 2007-0001-0101	MW419489	MW419572	MW419657	MW419744
	whipplei25441	DES: Hodgson 25441	MW419490	MW419573	MW419658	MW419745
C. wolfii (L.D.Benson) M.A.Baker [66]	wolfii594	SD: Mayer 594	MW419491	MW419574	MW419659	MW419746
	wolfii3820	SD: Rebman 3820	MW419492	MW419575	MW419660	MW419747
***Grusonia* Rchb.f. ex Britton & Rose**						
G. aggeria (Ralston & Hilsenb.) E.F.Anderson [22]	aggeria2314	SRSC: Fenstermacher 2314	MW419493	MW419576	MW419661	MW419748
	aggeria3495	Marshall s.n. 1952-3495-01-1	MW419494	MW419577	MW419662	MW419749
G. bradtiana (J.M.Coult.) Britton & Rose [22]	bradtiana141	Evans s.n. 1978-0141-01-3	MW419495	MW419578	MW419663	MW419750
	bradtiana5660	Mieg 42 1956-5660-0101	MW419496	MW419579	MW419664	MW419751
G. bulbispina (Engelm.) H.Robinson [44]	bulbispina804	Dodson et al. s.n. 1990-0804-02-2	MW419497	MW419580	MW419665	MW419752
G. clavata (Engelm.) H.Robinson [22]	clavata6404	Russell s.n. 1959-6404-01-1	MW419498	MW419581	MW419666	MW419753
G. densispina (Ralston & Hilsenb.) Pinkava [44]	densispina0124	Zimmerman 2859 2010-0124-01-2	MW419499	MW419582	MW419667	MW419754
	densispina2306	DES*: Fenstermacher 2306	MW419500	MW419583	MW419668	MW419755
G. emoryi (Engelm.) Pinkava [44]	emoryi0112	Felger 88-11 1988-0112-02-1	MW419501	MW419584	MW419669	MW419756
	emoryi10897	Baker 10897 1995-0204-01	MW419502	MW419585	MW419670	MW419757
G. grahamii (Engelm.) H.Robinson [44]	grahamii0705	Zimmerman 2546A 1988-0705-02-1	MW419503	MW419586	MW419671	MW419758

TABLE 1. CONTINUED

Taxon	Terminal (Fig. 1)	Collection	*rp*L16	*rp*S16	*trn*H	*trn*Q
	grahamii1142	SRSC: Fenstermacher 1142a	MW419504	MW419587	MW419672	MW419759
	grahamii2442	DES*: Fenstermacher 2442	MW419505	MW419588	MW419673	MW419760
G. invicta (Brandegee) E.F.Anderson [22]	invicta0033	Tegelberg s.n. 1939-0033-01-1	MW419506	MW419589	MW419674	MW419761
	invicta75	Voss s.n. 75.0060P (SBG)	MW419507	MW419590	MW419675	MW419762
G. marenae (S.H.Parsons) E.F.Anderson [?]	marenae7265	McGill s.n. 1962-7265-01-2	MW419508	MW419591	MW419676	MW419763
	marenae8491	Gentry s.n. 1966-8491-01-1	MW419509	MW419592	MW419677	MW419764
G. moelleri (A. Berger) E.F.Anderson [22]	moelleri9412	Quirk s.n. 1968-9412-02-6	———	MW419593	MW419678	MW419765
G. parishii (Orcutt) Pinkava [22]	parishii027	Wolf 8296 2007-0027-01	MW419510	MW419594	MW419679	MW419766
	parishii0565	Baker 7550.1 1990-0565-01-1	MW419511	MW419595	MW419680	MW419767
G. schottii (Engelm.) Robinson [66]	schottiiJFSN	DES*: Fenstermacher s.n.	MW419512	MW419596	MW419681	MW419768
G. vilis (Rose) H. Robinson [?]	vilis12834	Baker 12834 2019-0031-01	MW419513	MW419597	MW419682	MW419769
G. wrightiana (E.M.Baxter) E.M.Baxter [44]	wrightiana17601	Baker 17601.1 2019-0351-01	MW419514	MW419598	MW419683	MW419770
***Micropuntia* Daston**						
Micropuntia pulchella (Engelm.) M.P.Griff. [22]	pulchella17770	DES*: Baker 17770.1	MW419515	MW419599	MW419684	———
	pulchella17776	DES*: Baker 17776.3	MW419516	MW419600	MW419685	MW419771
Opuntia littoralis (Engelm.) Cockerell	Opuntialittoralis	SD: Mayer 639	MW419517	MW419601	MW419686	MW419772
Pereskiopsis porteri (Brandegee) Britton & Rose	porterii109	DBG s.n. 1975-0109-21-1	MW419518	MW419602	MW419687	MW419773
	porteri537	Quirk s.n. 1984-0537-01-2	MW419519	MW419603	MW419688	MW419774

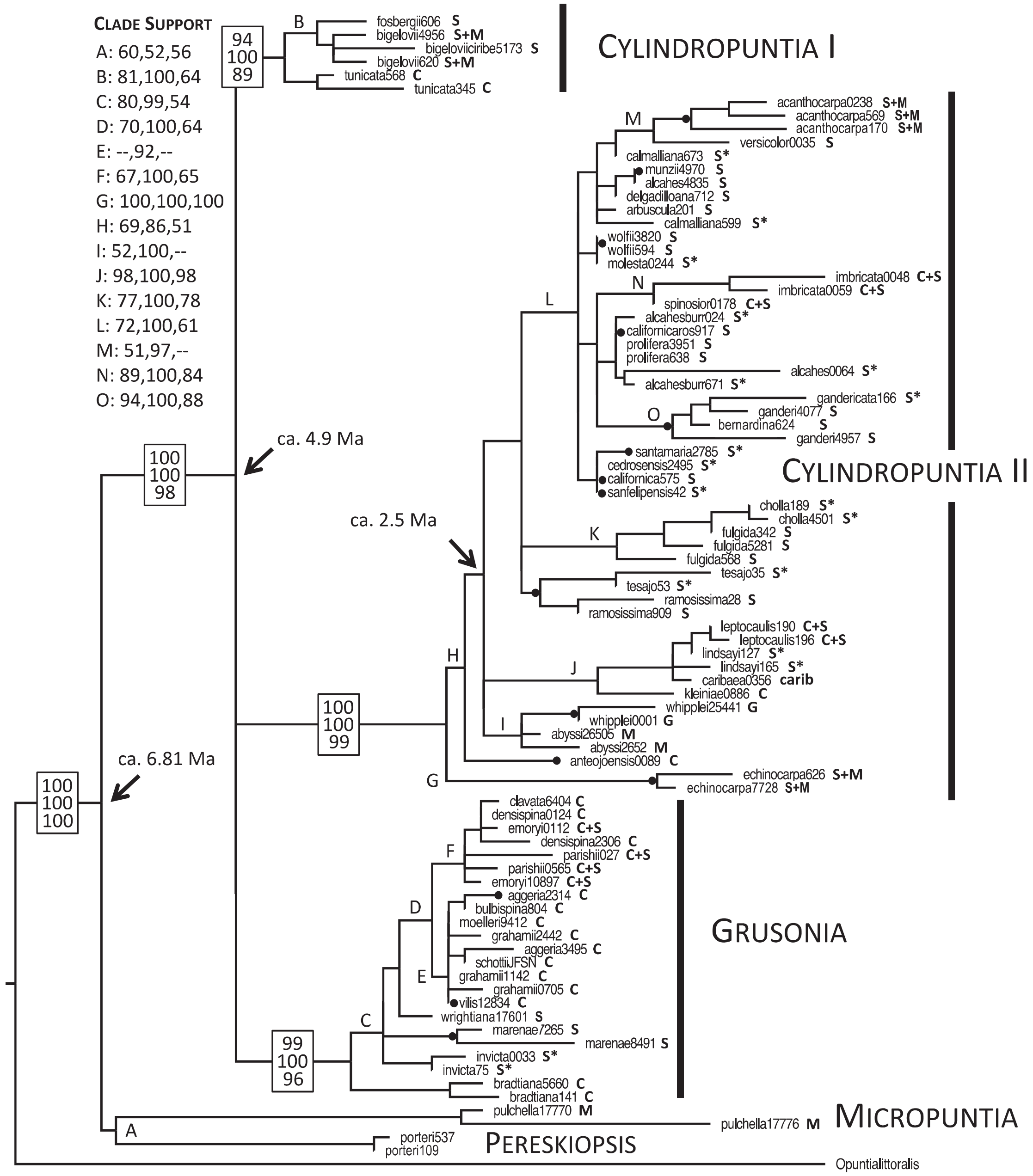

FIG. 1. Best tree resulting from Maximum Likelihood analysis of cpDNA sequence data, presented as a phylogram. Support noted for clades include Maximum Likelihood Bootstrap percentage, Bayesian Posterior Probability, and Parsimony Bootstrap percentage, respectively. Black dots mark clades or species exhibiting dry fruit; after the specimen identifier, C, M, and S refer to species range in Chihuahuan, Mohave, or Sonoran deserts, respectively. The arrows connect the estimated origin date (Hernandez-Hernandez et al. 2014) to selected nodes.

synapomorphies, respectively, for the aforementioned clades.

On the Grusonia clade, *G. bradtiana* (J.M.Coult.) Britton & Rose, the type species of *Grusonia* and native of the Chihuahuan desert (Mexican state of Coahuila), is shown as sister to the rest of the Grusonia clade, which corresponds to *Corynopuntia* F.M.Knuth (Fig. 1). The trichotomy at the base of this clade features the lineages of *G. invicta* (Brandegee) E.F.Anderson (central Baja California, Mexico) and *G. marenae* (S.H.Parsons) E.F.Anderson (Sonora, Mexico), the latter species having formerly been described as a monotypic genus (*Marenopuntia marenae* Backeberg). *Grusonia wrightiana* (E.M.Baxter) E.M.Baxter (Sonoran desert and Baja California) is sister to the larger remaining clade, which is split into two lineages (Fig. 1): a more southern-centered clade of U.S. and Mexican Chihuahuan desert species (*G. aggeria* (Ralson & Hilsenb.) E.F.Anderson, *G. bulbispina* (Engelm.) H.Robinson, *G. grahamii* (Engelm.) H.Robinson, *G. moelleri* (A.Berger) E.F.Anderson, *G. schottii* (Engelm.) Robinson, and *G. vilis* (Rose) H. Robinson) and a more northern- and western-centered clade of Chihuahuan, Sonoran and Mohave desert species of U.S. and Mexico (*G. clavata* (Engelm.) H.Robinson, *G. densispina* (Ralston & Hilsenb.) Pinkava, *G. emoryi* (Engelm.) Pinkava, and *G. parishii* (Orcutt) Pinkava). Both clades include species of a group called the *Grusonia schottii* complex (*G. aggeria, G. emoryi, G. grahamii, G. schottii, G. densispina*; Ralston 1987; Ralston and Hilsenbeck 1989, 1992), revealing paraphyly of this group unless it is expanded to also include at least *G. clavata, G. parishii, G. bulbispina*, and *G. vilis*. The two representatives of *G. emoryi* do not appear as sisters in this northwestern clade, which may indicate divergence that warrants reconsideration of *G. stanlyi* Engelm. (represented by specimen Baker 10897), which had been synonymized with *G. emoryi* by Pinkava (1999).

Cylindropuntia I, comprising *C. bigelovii* (Engelm.) F.M.Knuth, *C. tunicata* (Lehmann) F.M.Knuth, and *C. fosbergii* C.B.Wolf, combines species of series *Bigelovianae* and *Imbricatae* (Britton and Rose 1919; Hunt 2006), and is thus surprising from a morphological perspective. The close relationship of *Cylindropuntia bigelovii*, a common species from Baja California to the Mojave and Sonoran desert of Mexico, CA, AZ, NV, and NM., and *C. tunicata*, which is widespread in the Chihuahuan desert of central and northern Mexico to southern Texas, has been revealed only recently (Barcenas 2016; Majure et al. 2019). But despite their differences, both species produce stem segments that are easily detached, have prominent tubercules, similar numbers of spines per areole, and similar size and colors of glochids. Both produce fleshy, spineless fruits, and both species have the unusual distinction of producing roots of medicinal value to indigenous people, *C. bigelovii* as a laxative and *C. tunicata* as a diuretic (Anderson 2001).

Cylindropuntia II bears the majority of *Cylindropuntia* species, and features a basal branch that bears *C. echinocarpa* (Engelm. & Bigelow) F.M.Knuth, which is widespread in the Mohave and Sonoran deserts of Baja California and Sonora, as well as in California, Nevada, and Arizona.

Cylindropuntia anteojoensis (Pinkava) E.F.Anderson is sister to the remainder of this clade; trees with lower support (not shown) commonly placed this species on a clade with *C. ramosissima* (Engelm.) F.M.Knuth and *C. tesajo* (Engelm. ex Coult) F.M.Knuth, which form a more derived clade (Fig. 1)—a pattern also shown by Barcenas et al. (2016) and Majure et al. (2019). All three species exhibit narrow "pencil-like" stems and dry fruit; *C. anteojoensis* is eastern-most, in the Chihuahuan desert of Coahuila, Mexico; *C. ramosissima* is widespread in the Sonoran and Mohave deserts and northeastern Baja California, and *C. tesajo* appears to be an endemic derivative of the former species in Baja California.

Cylindropuntia cholla (F.A.C.Weber) F.M.Knuth and *C. fulgida* (Engelm.) F.M.Knuth form a clade; both species produce fleshy, proliferating fruits, and are similar in stature and flower color. *Cylindropuntia cholla* is a species of Baja California, whereas *C. fulgida* ranges from Arizona south into the states of Sonora and Sinaloa in Mexico.

Another clade bears the widespread *Cylindropuntia leptocaulis* (DC.) F.M.Knuth, whose range spans the breadth of northern and central Mexico, as well as the southern U.S., from Arizona to Oklahoma and Texas. Sharing this clade are *C. lindsayi* (Rebman) Rebman, endemic to southern Baja California, *C. kleiniae*, of the Chihuahuan desert of the U.S. and Mexico, and *C. caribaea* (Britton & Rose) F.M.Knuth, the sole cholla of the Caribbean, native to Hispaniola. It is not clear whether dispersal or vicariance are responsible for the isolation and speciation of *C. lindsayi* and *C. caribaea*. The species of this clade share tall stature (1.8–3 m) slender (pencil-like) stem segments, and fleshy fruit. The presence of the tetraploid *C. kleiniae* and *C. lindsayi* on this clade with *C. leptocaulis*, which exists in diploid, triploid, and tetraploid forms, suggests one or more of these species could be an allopolyploid, and at least one of the others could be a parent.

The more northern and upland species *Cylindropuntia whipplei* (Engelm. & Bigelow) F.M.Knuth shares a clade with the narrow endemic *C. abyssi* (Hester) Backeb., which is suspected to be of hybrid origin, possibly involving *C. bigelovii* or *C. acanthocarpa* (Engelm. & Bigelow) F.M.Knuth (Pinkava 2003; Hunt 2006). If *C. abyssi* is indeed a hybrid, the present study provides evidence that the maternal parent is *C. whipplei*.

A large, well-supported lineage comprises the remaining species of this clade. Although morphologically diverse, these species share a distribution that is limited to the more western and northern deserts (Sonoran, Mohave, and Baja California). Much of the structure revealed in the analysis of this clade is not robust, but some patterns merit consideration.

Cylindropuntia acanthocarpa and *C. versicolor* (Engelm. ex Coult.) F.M.Knuth overlap in range in the Sonoran Desert of Arizona and Mexico. Note that recent work has reduced *C. versicolor* to a subspecies of *C. thurberi* (Engelm.) F.M.Knuth (Baker et al. 2019), which could not be represented in the present study.

Besides sharing a clade, *Cylindropuntia imbricata* (Haw.) F.M.Knuth and *C. spinosior* (Engelm.) F.M.Knuth also have overlapping ranges in the deserts of Arizona, New Mexico, and northern Mexico. Baker et al. (2019) have also reduced *C. spinosior* to a subspecies of *C. imbricata*.

Three narrow endemics of Baja California: *C. cedrosensis* Rebman (Isla de Cedros), *C. santamaria* (E.M.Baxter) Rebman (Isla Magdalena), and *C. sanfelipensis* (Rebman) Rebman (San Felipe Desert), share a clade with the more widespread *C. californica* var. *californica* (Torrey & A.Gray) F.M.Knuth, suggesting a possible progenitor role for the latter species. Due to its similarity to *C. wolfii* (L.D.Benson) M.A.Baker, we expected that *C. sanfelipensis* would show a close relationship with that species. However, the hexaploid genome of *C. sanfelipensis* may indicate an allopolyploid origin that might have included paternal contributions from *C. wolfii*, also a hexaploid.

Cylindropuntia ganderi (C.B.Wolf) Rebman & Pinkava) has been segregated from *C. acanthocarpa* (Rebman 2001), a change that is entirely justified by the distinction these taxa exhibit in the tree. The position of *C. bernardina* (Engelm. ex Parish) M.A.Baker et al., formerly *C. california* var. *parkeri* (J.M.Coult.) Pinkava, as a sister to specimens of *C. ganderi,* rather than the other varieties of *C. californica,* affirms its recent recognition (Baker and Pinkava 2018). *Cylindropuntia bernardina* is found at the northern edge of the range of *C. ganderi* in southern California.

The presence of *C. alcahes* (F.A.C.Weber) F.M.Knuth and *C. prolifera* (Engelm.) F.M.Knuth together on a clade is expected, as the latter probably originated via hybridization of the former with *C. cholla* (Mayer et al. 2000). The placement of a representative of *C. californica* var. *rosarica* (G.E.Linds.) Rebman on this clade, isolated from *C. californica* var. *californica,* is another indicator that this taxon needs additional study.

The union of *C. molesta* (Brandegee) F.M.Knuth and *C. wolfii* on a clade is unexpected and may be spurious. Alternatively, the hexaploid *C. wolfii* and the octoploid *C. molesta* may share genomes through allopolyploid ancestry.

Discussion

Our work concurs with previous studies that propose reviving *Micropuntia* to recognize a monotypic *M. pulchella* (Griffith 2002; Griffith and Porter 2009; Barcenas 2016; Majure et al. 2019). *Grusonia* (*Micropuntia*) *pulchella* does not share a clade with the other members of the Grusonia clade, which in fact share a more recent ancestor with *Cylindropuntia*. The morphological and ecological case for *Micropuntia pulchella* is also strong, including unique features of the stem tuber, spine angle, areole wool, habit, and geography (Griffith 2002).

The topology of the Grusonia clade supports either a monotypic *Grusonia* (= *G. bradtiana* Britton and Rose) and monophyletic *Corynopuntia* F.M.Knuth, or a *Grusonia sensu lato* (Stuppy 2002), which includes the entire clade. The former taxonomic proposal, however, seems to overemphasize the distinction of *G. bradtiana* from the remaining species of the Grusonia clade, either morphologically or genetically (Fig. 1; see Majure et al. (2019) for a thorough examination of the options for *Grusonia* taxonomy). Consequently, we concur with those (Robinson 1973; Majure et al. 2019) promoting subsuming all *Corynopuntia* species into a more inclusive *Grusonia*.

History of the Chollas

Ancestral elements of subfamily Opuntioideae originated in the central Andes of South America, and with other lineages of Cactaceae extended their range into North America (Raven and Axelrod 1978; Wallace and Dickie 2002). The northward migration and radiation of the chollas appears tied to the expansion of the North American deserts beginning in the mid-Miocene (Arakaki et al. 2011; Hernandez-Hernandez et al. 2014), approximately 8-15 mya (Van Devender and Brusca 2015). The emergence of tribe Cylindropuntieae has been estimated to be 9.05 mya (no credibility interval provided; Hernandez-Hernandez et al. 2014), although a more recent estimate places the origin at approximately 17.9 mya (14.8-20.4 mya; Majure et al. 2019). Cylindropuntieae probably originated in South America and expanded north, evidenced by the South American endemic *Quiabentia*: opuntioids with large, persistent leaves and other plesiomorphic features. *Pereskiopsis*, the other leafy, non-cholla genus of the tribe is native to southern Mexico; therefore the "cholla" elements of tribe Cylindropuntieae are products of the North American deserts, ranging from central Mexico to the southern and western United States.

Divergence time analyses of Hernandez-Hernandez et al. (2014) calculated a date of 4.9 mya (ca. 2.7-7.7 mya) for the common ancestor of *Grusonia* + *Cylindropuntia*. This suggests a late Miocene-Pliocene time frame for divergence of the three major clades of chollas; an earlier date of 12 mya (9.3-14.5 mya) has also been suggested (Majure et al. 2019). However, both sets of estimates suggest a great deal of cholla speciation occurred during the Pleistocene, <2.5 mya.

The Hernandez-Hernandez et al. (2014) study of 224 species, representing ca. 85% of the generic diversity of the Cactaceae, also detected a significantly heightened diversification rate for the *Grusonia* + *Cylindropuntia* clade—which is most evident in the branch lengths of clade Cylindropuntia II (Fig. 1). Studies of diversification times in *Cereus* (Silva et al. 2017) and *Pilosocereus* (Lavor et al. 2019) support the hypothesis that the multiple glacial and interglacial episodes of the Pleistocene drove the radiation of

TABLE 2. COMPARISON OF CHROMOSOME NUMBER, RANGE SIZE, AND SISTER SPECIES STATUS FOR SELECTED PAIRS OF *CYLINDROPUNTIA* SISTER SPECIES.

Sister species	Chromosome number	Range (W=widespread, N=narrow)	Isolation
C. leptocaulis	22, 33, 44	W: across northern Mexico and southern U.S.	Sea of Cortez
C. lindsayi	44	N: Baja California Sur	
C. ramosissima	22, 44	W: Mojave and Sonoran deserts of U.S. and Mexico, and extreme northeastern Baja California	Sea of Cortez
C. tesajo	22	N: Central Baja California	
C. whipplei	22, 44	W: high desert, grassland, sagebrush, and pine forest of AZ, CO, NM, and UT, U.S.	Peripatric?
C. abyssi	22	N: Mohave Co., AZ, U.S.	
C. bigelovii	22, 33	W: Sonoran desert of U.S. and Baja California	Peripatric?
C. fosbergii	33	N: one population in eastern San Diego Co., CA	
C. cholla	22, 33, 44	W: throughout Baja California	Sea of Cortez
C. fulgida	22, 33	W: Sonoran desert of U.S. and northern Mexico	

these genera. Expansion and contraction of the North American deserts occurred repeatedly through the Pleistocene, with the most recent expansion of the Sonoran desert into southern Arizona and California occurring just 9000 years ago (Van Devender and Brusca 2015). The formation of the Gulf of California (Sea of Cortez) may have had a role in the divergence of at least three sister-species pairs: *C. cholla*/*C. fulgida, C. leptocaulis*/*C. lindsayi*, and *C. ramosissima*/*C. tesajo* (Table 2); the Gulf started opening approximately 10 mya and is still widening (Moore and Buffington 1968).

The increase in diversification rate noted in the chollas may have been facilitated not only by desertification and climate fluctuation, but also by intrinsic genetic and reproductive processes of these taxa. Hybridization is common among chollas, and can lead to the origin of fertile allopolyploid species, but autopolyploidy is also common (Pinkava 2002). Moreover, the ability of chollas to reproduce vegetatively via stem segment propagules can allow sterile odd-ploidy auto- and allopolyploids to not only survive, but possibly benefit from the dosage effects of extra sets of chromosomes (Baker and Pinkava 1987, 2018; Pinkava 2002). Six *Cylindropuntia* species (*C. alcahes, C. bigelovii, C. fulgida, C. munzii* (C.B.Wolf) Backeb., *C. ramosissima*, and *C. tunicata*) exist as diploids or triploids, and in at least one species, *C. bigelovii*, the triploids exhibit a greater number of plants and size of range than the diploids (Pinkava 2002). In general, polyploidy in subfamily Opuntiodeae has been detected in 64.3% of species, a rate that far exceeds the other subfamilies (Cactoideae = 12.5%, Pereskioideae = 0.0%) (Pinkava 2002). Our calculation of polyploidy rates, omitting hybrids, is 60% for *Cylindopuntia* and 47% for *Grusonia* (see Baker and Pinkava 2018). Moreover, in twelve of forty C*ylindropuntia* species, autopolyploidy has resulted in at least two ploidal levels, in *Grusonia* it is eight of 17 species (Table 1). And among the clades bearing *Cylindropuntia* species (Fig. 1), in every case where there are clear sister-species pairs that differ in numbers of documented ploidy levels, the species with greater ploidal variation have larger ranges, usually much larger (Table 2). This pattern may simply indicate a correlation between population size and potential for ploidy variation, or it may indicate the potential niche diversity that accompanies ploidal level diversity, a consequence of the myriad ways polyploidy stokes the evolutionary potential of plants (Stebbins 1950; Soltis and Soltis 2000; Baker and Pinkava 2018).

Cylindropuntia I and II. A monophyletic *Cylindropuntia* did not achieve sufficient support in our analyses, although this resolution has been found in other studies (Barcenas 2016; Majure et al., 2019). The cholla trichotomy suggests a rapid divergence of these three clades during the formation of the deserts of North America. Subsequent to this divergence, however, there are differences in the stem and crown branch lengths of these three clades, with the starkest contrast shown between Cylindropuntia I and Cylindropuntia II (Fig. 1). Cylindropuntia II exemplifies the diversification rate increase (Hernandez-Hernandez et al. 2014) discussed above, but could the absence of a similar level of diversification and divergence in Cylindropuntia I to some degree reflect mode of reproduction? Excluding the triploid specimens (Table 1), normal levels of fertility are reported for the species of Cylindropuntia II, except *C. cholla*, *C. fulgida*, and *C. molesta.* In contrast, the taxa of Cylindropuntia I, *C. tunicata*, *C. bigelovii*, and *C. fosbergii*, show an almost complete reliance on asexual reproduction. The range of *C. bigelovii* var. *bigelovii* consists mostly of sterile triploid populations, with just a few known diploid populations; interestingly, the narrow Baja California endemic, *C. bigelovii* var. *ciribe* is diploid and fertile (Rebman 2001; Pinkava 2002). *Cylindropuntia fosbergii* exists only as sterile triploids in a single diffuse population in southern California (Mayer et al. 2011; Baker et al. 2012). Some consider *C. fosbergii* a hybrid between *C. bigelovii* and an unidentified species, but Mayer et al. (2011) found insufficient evidence for a hybrid origin of *C. fosbergii* and hypothesize a sister relationship for it and *C. bigelovii. Cylindropuntia tunicata* exists in diploid and triploid forms, but the fruits of diploids are mostly sterile (Anderson 2001). *Cylindropuntia rosea* (DC.) Backeb., not sampled in this study, is the only other species of this clade and while its fertility in its native range (states of Hidalgo and Puebla, Mexico) is unclear, it reportedly produces inviable seeds in introduced populations in Australia (Hosking et al. 2007).

Asexual reproduction is a key reproductive and survival strategy in chollas (Pinkava 2002), and asexual lineages in general may possess a theoretical advantage over sexual lineages in diversification rate (Barraclough et al. 2003; Fontaneto et al. 2012). But under the rapidly changing selective pressures of the Pliocene through the Pleistocene, sexuality may have proven the superior mechanism for divergence and speciation of the chollas. Comparisons of genetic variation within and among populations of sexual and asexual cholla species would provide valuable insight into the genetics of adaptation and divergence.

Evolutionary Trends in the Chollas

Among *Grusonia* species, there is a general trend in plant stature and stem segment length from taller and longer, respectively, in the basal clades, to prostrate and shorter in the more derived clades. This could be construed as a response to the increasing desertification of the region. Most *Cylindropuntia* species, in contrast, exhibit an erect, shrubby to tree-like habit, but include seven species with a "pencil"-like stem segment morphology. These species, except *C. anteojoensis*, are concentrated on two different clades (*C. tesajo*/*C. ramosissima* and *C. kleiniae*/*C. caribaea*/*C. lindsayi*/*C. leptocaulis*; Fig. 1), a convergence suggesting the adaptive advantage to slender stems during the diversification of these lineages.

Adaptation to a drying climate could also account for the multiple occurrences of dry fruit production among the chollas: 3 species in Grusonia and 13 in Cylindropuntia I (Fig. 1), the latter showing at least five clades with dry fruit-fleshy fruit sister taxa. This suggests not only convergence among lineages, but a more recent (Pleistocene) shift towards a dry, bur-like fruit–a clear water-saving adaptation. Additional analysis of morphological trends in Cylindropuntieae is provided in Majure et al. (2019).

Past efforts to interpret infrageneric morphological pattern among the species of *Cylindropuntia* have shown limited success. The taxonomic series proposed by Benson (1982) and Hunt (2006), identify morphological profiles that do not correlate well with the clade composition revealed in the molecular analyses presented here and elsewhere (Griffith and Porter 2009; Barcenas 2016; Majure et al. 2019, etc.). The phylogenetic patterns revealed in the present study are largely congruent with other recent studies involving chloroplast sequences (Barcenas 2016) and genomic skimming (Majure et al. 2019) in the chollas, but provoke new hypotheses of evolutionary pattern and process. Welcomed future investigations would focus on the possible synergistic adaptive value of polyploidy and vegetative propagation in the history of the chollas, and the parentage of the many hybrid diploid and polyploid taxa.

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