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MOLECULAR EVIDENCE FOR THE HYBRID ORIGIN OF *OPUNTIA PROLIFERA* (CACTACEAE)

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ABSTRACT

Opuntia prolifera Engelm., (Coastal Cholla) is common to the coastal sage scrub community extending from Ventura County, California to El Rosario, Baja California. On the basis of morphological intermediacy, *O. prolifera* is suspected to have originated through hybridization between *O. alcahes* and *O. cholla,* both species of coastal and inland deserts of Baja California and Baja California Sur. For an independent test of this hypothesis, we generated RAPD banding patterns from exemplars of different populations of *O. prolifera* and the putative parents. In order to exclude other potential parents and to distinguish species-specific RAPD bands we included *O. bigelovii* Engelm., *O. ganderi, O. tesajo,* and *O. wolfii* (L. Benson) M. Baker in the screening. The results provide support for the hybridization hypothesis as well as some insight into the speciation process. Twenty-nine primers revealed 44 bands shared only between *O. prolifera* and one or the other putative parent. No other species included in the screening proved to be comparable alternatives to *O. alcahes* or *O. cholla* as the parents of *O. prolifera.* Unique bands are rare (=2) in *O. prolifera* compared with *O. alcahes* (=19) and *O. cholla* (=23). Trends in the degree of band sharing between *O. prolifera* and representatives of *O. alcahes* and *O. cholla* suggest a central Baja California origin of the species.

The dynamic geologic and climatic history of Baja California has fostered a diverse and highly endemic flora on the peninsula, and one of the most speciose genera is *Opuntia* (Cactaceae). The speciation routes taken by *Opuntia* have also been diverse: many species are proven hybrids and many more exhibit multiple ploidal levels (D. Pinkava pers. comm.). One suspected hybrid, *Opuntia prolifera* Engelm., was until recently considered a species derived through cladogenesis.

Opuntia prolifera (Coastal Cholla) occurs in the coastal sage scrub community adjacent to the Pacific Ocean between Cedros Island, Baja California and Ventura County, California. This taxon is triploid (Pinkava et al. 1992) and reproduces almost exclusively asexually, usually through dispersal of detached stem segments. Morphological intermediacy of *O. prolifera* between *O. alcahes* F. A. C. Weber and *O. cholla* F. A. C. Weber in several characteristics (Table 1) has prompted speculation that *O. prolifera* may have arisen through hybridization of these species (Rebman 1995). *Opuntia alcahes* and *O. cholla* are desert taxa of Baja California and typically diploid (Pinkava et al. 1992; Rebman 1995). The two species commonly grow sympatrically without hybridizing (Fig. 1), however a hybrid swarm involving the two species exists near El Rosario (Rebman 1995)—which is in the southern part of the range of *O. prolifera,* an area of overlap between the Sonoran Desert and the California Floristic Province (Fig. 1). Despite the general intermediacy of *O. prolifera,* phenotypic plasticity of the putative hybrid and parent species prevents a strong case for hybridization to rest on morphological data alone.

To subject the hybridization hypothesis to further scrutiny, we surveyed patterns of Random Amplified Polymorphic DNA (RAPD) markers obtained from *O. prolifera* and its putative parents. The RAPD technique allows relatively quick assessment of a large number of highly polymorphic loci, largely of the nuclear genome (Welsh and McClelland 1990; Williams et al. 1990). Recent studies have successfully applied the RAPD approach to questions of interspecific hybridization (Pham and Smith 1995; Barker et al. 1996; Daehler and Strong 1997) and hybrid speciation (Smith et al. 1995; Lifante and Aguinagalde 1996; Allan et al. 1997; Padgett et al. 1998).

We used RAPD data to test if *Opuntia prolifera* exhibits the classic genetic expectations of hybrid taxa. If the putative parent species, *O. alcahes* and *O. cholla,* were sufficiently divergent genetically prior to a hybridization event, then the hybrid, i.e., *O. prolifera,* should exhibit additivity of genetic markers specific to the parent species as well as a lack of unique markers (Gallez and Gottlieb 1982). Additionally, the sterile triploid nature of *O. prolifera* suggests the possibility that "fixed" heterozygosity (***sensu*** Roose and Gottlieb 1976) in *O.*

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TABLE 1. SELECTED MORPHOLOGICAL CHARACTERISTICS OF *OPUNTIA PROLIFERA* AND ITS HYPOTHESIZED PARENTS, *O. ALCAHES* AND *O. CHOLLA*, IN REGIONS OF SYMPATRY (FROM REBMAN 1995).

Characteristic	*O. alcahes*	*O. prolifera*	*O. cholla*
Inner tepal color	yellow, green, or red-magenta	magenta to deep red	light to dark pink
Stem segment shape	long and narrow (3.5–13 × 1.5–4.5 cm)	intermediate (7.5–12.6 × 3.0–4.1 cm)	short and wide (6–11.5 × 3–5.5 cm)
Tubercule length	4–22 mm	12–24 mm	20–35 mm
Tubercule height	2–9 mm	5–9 mm	10–20 mm
Spine shape	short and thin (4–20 × 0.3–0.5 mm)	intermediate (14–18 × 0.7–0.9 mm)	long and thick (20–35 × 0.8–1.3 mm)
Areole size	3–5 × 2–4 mm	5–8 × 3–5 mm	6–11 × 3–5 mm
Proliferating fruit	rare	yes	yes

prolifera could endow it with a higher overall number of RAPD markers relative to its putative parents. Finally, patterns in the degree of band sharing between hybrid and parents can also be used to make preliminary inferences regarding the geographic region in which the hybrid taxon arose, as well as the possibility that this event occurred multiple times.

METHODS

Field collection and DNA extraction. Stem segments were gathered from a single plant (exemplar) at each location (Table 2). DNA was extracted from fresh or frozen stem tissue following a modification (Doyle and Doyle 1987) of the hot CTAB method of Saghai-Maroof et al. (1984).

Initial RAPD screening. DNA extracts from *Opuntia alcahes, O. prolifera,* and *O. cholla* were subjected to DNA amplification via the polymerase chain reaction (PCR) using the 100 10-mer primers of RAPD Oligo Set 3 (Nucleic Acid-Protein Service Unit of the University of British Columbia). Each 25 μL reaction contained 1 unit of Promega (Madison, WI) *Taq* polymerase, 1× reaction buffer,

TABLE 2. COLLECTIONS OF *OPUNTIA* FROM CALIFORNIA AND MEXICO ANALYZED IN THE PRESENT STUDY; PRECISE LAT./LONG. DATA ARE AVAILABLE UPON REQUEST. Exemplars are given abbreviated names for reference in text, tables, and figures; B.C. = Baja California, B.C.S. = Baja California Sur; asterisk denotes collections used in initial screening only.

Species	Collection	Location
O. alcahes F. A. C. Weber		
	Rebman s.n.*	CA., San Diego Co., Quail Botanical Gardens
alc 1	Voss 1174	B.C.S., Cape Region
alc 2	Rebman 4157	B.C., southwest of Cataviña
alc 3	Rebman 4835	B.C.S. near Rt. 1 and rd. to Punta Abreojos
alc 4	Rebman 5183	B.C. Sur, Sierra Guadalupe
O. bigelovii Engelmann var. *Bigelovii*	Rebman 4956	CA., San Diego Co., Hwy S-2 at Canebrake
O. cholla F. A. C. Weber		
cho 1	Rebman 4158	B.C., southwest of Cataviña
cho 2	Rebman 4501	B.C.S., Sierra San Francisco
cho 3	Rebman 4827	B.C.S., Isla Margarita
cho 4	Rebman 5184	B.C.S., Sierra Guadalupe
O. ganderi (C. B. Wolf) J. Rebman & D. J. Pinkava	Rebman 4973	B.C., San Felipe Desert, n. of Laguna Diablo
O. prolifera Engelmann		
pro 1	Mayer 591	CA., San Diego Co., U.S.D. campus, West Point
pro 2	Rebman 3951	B.C., between La Bocana and Puerto Santo Tomás
pro 3	Rebman 3977	B.C., s. of Punto Canoas
pro 4	Rebman 5119	B.C., near La Mision
O. tesajo Engelmann	Rebman 4972	B.C., San Felipe Desert, n. of Laguna Diablo
O. wolfii (L. D. Benson) M. A. Baker	Rebman 3820	CA., Imperial Co., along I-8 at Mountain Springs Grade

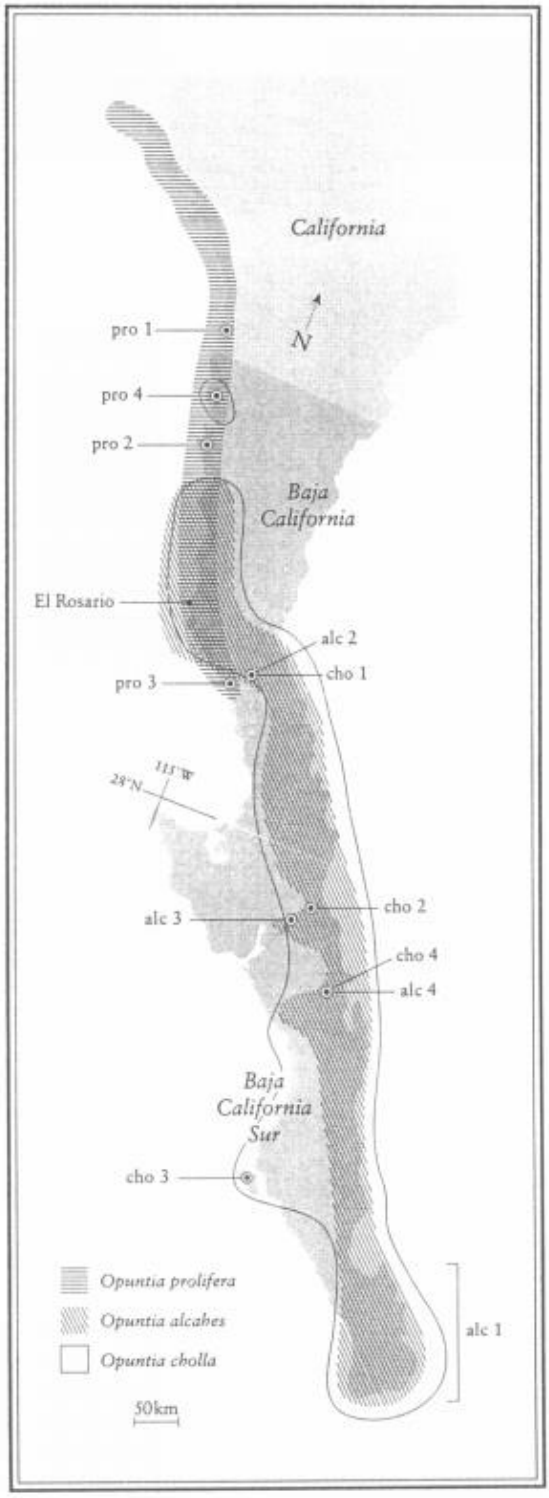

FIG. 1. Ranges of *Opuntia prolifera, O. alcahes,* and *O. cholla*; locations of collections used for population-level comparisons are noted by abbreviated names listed in Table 2.

1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.2 μM of one primer, and 1 μl dilute DNA extract. After 2 min at 94°C, the following cycle was repeated 40 times: denaturing at 94°C for 15 s, annealing at 36°C for 1 min, and elongation at 72°C for 1 min. A final elongation segment was held at 72°C for an additional 6 min. The PCR products were separated electrophoretically in 2% agarose gels and banding patterns were visualized by staining with ethidium bromide and inspection under ultraviolet light. Of the 100 primers, twenty-one showed banding polymorphism and the sharing of bands between exemplars of *O. prolifera* and either *O. alcahes* or *O. cholla*; therefore these primers were used in subsequent screening experiments.

To replicate the patterns observed in the first round of screening and to identify bands shared between *O. prolifera* and only *O. alcahes* or *O. cholla,* we included other related species (*O. bigelovii, O. ganderi, O. tesajo*) in new screening experiments using the primers identified in the first round. We assumed that any marker that was also present in one of these additional species was a symplesiomorphic characteristic and not helpful in a rigorous test of the hybridization hypothesis. *Opuntia prolifera* exhibited a total of five bands that it shared only with both the putative parents, six bands that it shared only with *O. alcahes,* and eight bands that it shared only with *O. cholla.* When *O. prolifera* was compared in the same way with *O. bigelovii, O. ganderi,* and *O. tesajo,* the numbers of exclusively-shared markers were zero, two, and one, respectively.

Primary RAPD screening. The results of the initial rounds of screening increased our confidence that *Opuntia prolifera* was a hybrid derivative of *O. alcahes* and *O. cholla.* We then examined the distribution of RAPD markers among populations within these species. We employed the same primers that had proven useful in previous rounds of screening and increased our sample sizes of *O. prolifera, O. alcahes,* and *O. cholla* to include an exemplar from four populations of each taxon (Table 2). In addition, one exemplar each was included from *O. bigelovii, O. ganderi, O. tesajo,* and *O. wolfii.* This allowed us to (1) replicate previously observed patterns and identify additional bands shared only between the putative hybrid and its parents, (2) get a cursory look at intraspecific RAPD polymorphism, and (3) assess the degree of band sharing on a pairwise population level and, subsequently, compare these data to the geographic distribution of the populations represented.

RESULTS

Banding patterns derived from screening 16 exemplars using 29 RAPD primers revealed a greater number of markers in support of the hybridization hypothesis than did the initial comparisons (Table 3), presumably because more of the total variation

TABLE 3. PRIMERS THAT RESOLVE MARKERS SHARED EXCLUSIVELY BETWEEN *OPUNTIA PROLIFERA* AND ITS PUTATIVE PARENTS. A = *O. alcahes*, C = *O. cholla*, A+C = both species.

Primer	Sequence	Markers shared with *O. prolifera*		
		A	C	A+C
UBC 202	GAGCACTTAC	2	0	0
UBC 204	TTCGGGCCGT	1	0	0
UBC 218	CTCAGCCCAG	1	0	0
UBC 219	GTGACCTCAG	1	1	2
UBC 220	GTCGATGTCG	3	1	0
UBC 225	CGACTCACAG	1	2	1
UBC 226	GGGCCTCTAT	1	2	0
UBC 227	CTAGAGGTCC	0	1	0
UBC 228	GCTGGGCCGA	1	1	0
UBC 238	CTGTCCAGCA	0	1	0
UBC 245	CGCGTGCCAG	1	0	0
UBC 246	TATGGTCCGG	1	1	0
UBC 247	TACCGACGGA	0	2	0
UBC 250	CGACAGTCCC	0	1	1
UBC 253	CCGTGCAGTA	1	2	0
UBC 259	GGTACGTACT	2	1	0
UBC 260	TCTCAGCTAC	1	0	0
UBC 269	CCAGTTCGCC	1	2	0
UBC 270	TGCGCGCGGG	1	2	0
UBC 275	CCGGGCAAGC	0	1	0
UBC 281	GAGAGTGGAA	3	0	1
UBC 283	CGGCCACCGT	1	0	0

within each species was assessed and more primers were successful. Pairwise comparisons between exemplars of *O. prolifera* and *O. alcahes*, or *O. prolifera* and *O. cholla* revealed 23 and 21 bands, respectively, present in at least one population of the two species compared, and found in no other species (Tables 3, 4). Of these 44 marker loci, the group of *O. prolifera* exemplars is polymorphic for at least 31 (>70%). A comparison between *O. alcahes* and *O. cholla* detected just one shared band, which was unique to just one exemplar of each species. A comparison of *O. prolifera* exemplars against representatives of *O. bigelovii, O. ganderi, O. tesajo,* and *O. wolfii* revealed one, one, zero, and zero bands, respectively, that were exclusively shared. Of the aforementioned markers of hybridization, only a small number are fixed in all exemplars of *O. prolifera* and *O. alcahes* (=3) or *O. prolifera* and *O. cholla* (=5) (Table 4). Five additional bands were shared exclusively among *O. prolifera* and both putative parents.

Opuntia prolifera did not possess a significantly greater number of bands (P = 0.97) compared with its putative parents: 167 bands total versus 164 in both *O. alcahes* and *O. cholla* (Table 4). Comparison of *O. prolifera* with its putative parents also revealed significantly (P < 0.01) fewer unique bands in *O. prolifera* (n = 2) than in either *O. alcahes* (n = 19) or *O. cholla* (n = 23) (Table 4). A factor analysis (Statview 5.0, SAS Institute, Inc. 1998) of

TABLE 4. SUMMARY DATA FROM PRIMARY SCREENING OF RAPD PATTERNS IN *OPUNTIA PROLIFERA* (P) AND ITS PUTATIVE PARENTS *O. ALCAHES* (A) AND *O. CHOLLA* (C).

Characteristic	P	A	C
Total bands examined	167	164	164
Unique bands	2	19	23
Bands shared only with P	—	23	21
Bands shared only with P, fixed for both taxa	—	3	5
Bands shared only between A and C	—	1	
Bands shared only among A, C, and P		5	

the RAPD data provided the means to assess overall similarity among the exemplars included in the study. The first two factors account for 41.5% and 16.8% of the variance in the data set. Plotting the exemplars by their scores along factors one and two places *O. prolifera* clearly intermediate between *O. alcahes* and *O. cholla* (Fig. 2).

Estimates of banding pattern similarity between pairs of populations of *O. prolifera* and *O. alcahes* or *O. cholla* were made in two ways: using the Simple Matching Coefficient (Sokal and Michener 1958) and the Coefficient of Jaccard (Sneath 1957). We tallied presence or absence of marker bands for all pairwise comparisons of exemplars of *O. prolifera* vs. *O. alcahes* or *O. cholla*. We ignored bands that were fixed for all exemplars of the two taxa being compared in an effort to minimize the effect of symplesiomorphies on the coefficient. The Simple Matching Coefficient (SMC) was calculated by adding the matches (shared absences plus shared presences of markers) and dividing by the total number of matches and mismatches. The Coefficient of Jaccard (CJ) omits shared absences from the numerator and denominator. We were concerned that the SMC would be biased by artefacts

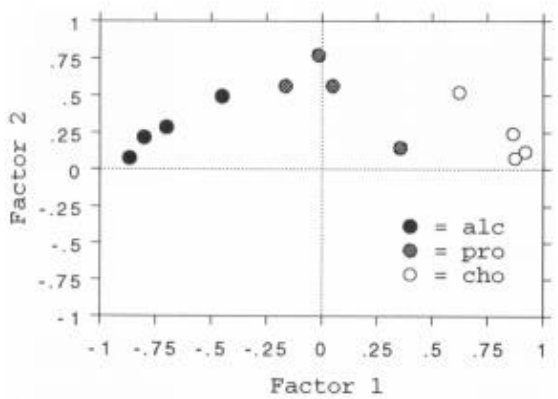

FIG. 2. Unrotated factor plot showing position of exemplars along factors one and two; refer to Table 2 for key to abbreviations.

TABLE 5. PAIRWISE SIMILARITY COEFFICIENTS BETWEEN EXEMPLARS MEASURING *OPUNTIA PROLIFERA* (PRO) × *O. ALCAHES* (ALC) AND *O. PROLIFERA* × *O. CHOLLA* (CHO). Simple Matching Coefficients before slash, Coefficient of Jaccard after; see Table 2 for key to abbreviations.

	pro 1	pro 2	pro 3	pro 4
alc 1	0.539/0.143	0.524/0.231	0.359/0.242	0.225/0.184
alc 2	0.583/0.000	0.568/0.111	0.250/0.069	0.189/0.063
alc 3	0.475/0.160	0.512/0.259	0.525/0.424	0.342/0.308
alc 4	0.436/0.154	0.475/0.250	0.539/0.455	0.400/0.368
cho 1	0.528/0.150	0.421/0.120	0.297/0.212	0.447/0.364
cho 2	0.650/0.263	0.600/0.200	0.263/0.125	0.250/0.167
cho 3	0.528/0.105	0.526/0.182	0.243/0.152	0.368/0.273
cho 4	0.514/0.182	0.462/0.192	0.324/0.242	0.487/0.412

arising from poor amplification, thereby inflating estimates of similarity between two populations. As expected, the SMC values were uniformly greater than the CJ values (Table 5), but in some cases the two approaches yield different patterns of relationships among the populations. For example, the two sets of coefficients comparing pro 2 with the four populations of *O. alcahes* display almost opposite rankings by magnitude (Table 5), perhaps indicating that the inclusion of shared absences does indeed bias the SMC in this application.

Considering, therefore, only the CJ values we see that among all the exemplars of *O. alcahes* and *O. cholla,* two exemplars of *O. prolifera* (pro 1 and 2) exhibited greater similarity, albeit by narrow margins, to alc 3 and cho 2 (Table 5). In contrast, pro 3 and pro 4 exhibited greater similarity to alc 4 and cho 4. These relationships also had geographic significance: exemplars alc 4 and cho 4 were collected from the same vicinity in northern Baja California Sur, and alc 3 and cho 2 were collected just 40 km apart, also in northern Baja California Sur (Fig. 1).

DISCUSSION

Molecular evidence supports the proposition that hybridization between *Opuntia alcahes* and *O. cholla* gave rise to *O. prolifera.* Forty-four RAPD markers are shared only between *O. prolifera* and one or the other parent species; no other candidates emerge as comparable alternatives to *O. alcahes* or *O. cholla* as the parents of *O. prolifera.* As expected for a hybrid, *O. prolifera* exhibits significantly fewer unique RAPD markers than its parent species. Moreover, multivariate analysis of marker distribution places exemplars of *O. prolifera* intermediate between those of *O. alcahes* and *O. cholla.*

Some results of this study, however, were contrary to early expectations. First, *O. prolifera* banding patterns did not exhibit the greater numbers of loci predicted for a sterile hybrid or allopolyploid (Table 4). This observation may indicate a relatively low degree of divergence between *O. alcahes* and *O. cholla,* or a low amount of variation derived from the actual hybridization event, or it may expose a limitation of RAPD markers in this application: RAPDs are dominant, diallelic markers and thus may not show the same patterns of additivity as codominant markers. Another surprising outcome was the RAPD polymorphism evident among exemplars of *O. prolifera,* indicating interpopulational genetic diversity. Because *O. prolifera* is only known to reproduce asexually, this variation may signify one or more of the following: (1) multiple independent hybrid origins of *O. prolifera,* (2) undetected sexual reproduction, or (3) genetic divergence via somatic mutations. We introduce these alternative processes briefly below, but leave a critical analysis to future studies specifically targeted to discriminating among these phenomena.

First, recurrent origin of a triploid *O. prolifera* would require either that multiple diploid-level hybridizations must each have been followed by the production of triploid offspring, or that a pairing of a diploid parent with a tetraploid parent must have occurred multiple times. Because both *Opuntia alcahes* and *O. cholla* are diploid with rare exception (Rebman 1995), the latter scenario seems unlikely. If the former scenario occurred, diploid hybrids should be common and widespread in the zone of sympatry. However, only one diploid count has been documented for *O. prolifera* (Pinkava and Parfitt 1982). Despite the apparent obstacles to recurring origins of *O. prolifera,* RAPD-based relationships among exemplars employed in the present study provide some evidence in its support. Two exemplars of *O. prolifera* (pro 1 and 2) are more closely related to alc 3 and cho 2 than to the other representatives of *O. alcahes* and *O. cholla.* In contrast, the other two exemplars of *O. prolifera* (pro 3 and 4) are more closely related to alc 4 and cho 4. Furthermore, specimens alc 4 and cho 4 were collected from the same vicinity, and the locations of alc 3 and cho 2 were separated by just 40 km.

Next, for sexual reproduction to be the source of interpopulational variation, triploid *O. prolifera* plants must give rise to triploid offspring. If meiosis could occasionally generate viable gametes of varying ploidy in *O. prolifera,* we should expect more ploidal levels than just triploid in these populations. Currently, only a hybrid swarm of the El Rosario

area (Fig. 1) has yielded counts in *O. prolifera* that exceed triploidy, including a hexaploid—presumably an autopolyploid that formed through the fusion of two unreduced gametes (Rebman 1995).

Lastly, reproduction in *O. prolifera* relies perhaps exclusively on establishment of detached stem segments (Rebman 1995). Long-term clonal growth allows for the possibility that somatic mutations in branch primordia could generate RAPD variation among populations of *O. prolifera.* The importance of somatic mutations in clonal species has long been suspected and is gaining more experimental support (Ellstrand and Roose 1987, reviewed in de Kroon and van Groenendael 1997).

Origin of Opuntia prolifera. Although it is almost uniformly triploid across its range, *O. prolifera* could have originated as a diploid, through hybridization of diploid *O. alcahes* and *O. cholla.* Meiotic irregularities in this diploid hybrid allowed the production and subsequent fusion of a reduced and unreduced gamete, generating a triploid offspring. This route from diploidy to triploidy has been seen repeatedly among cactus species (D. Pinkava pers. comm.). A notable example is *O. bigelovii,* a close relative of *O. prolifera,* which apparently arose as a diploid but is now predominantly triploid (D. Pinkava pers. comm.). If indeed *O. prolifera* originated in this way, some set of factors then allowed the triploid to surpass its diploid progenitor and thrive in the coastal sage scrub of the Californias, a habitat to which few other chollas are well-adapted.

All exemplars of *O. prolifera* showed the greatest similarity to representatives of *O. alcahes* (alc 3 and 4) and *O. cholla* (cho 2 and 4) collected from the northern end of Baja California Sur, indicating a possible region of origin of *O. prolifera.* Surprisingly, this region is greatly disjunct from the present range of *O. prolifera* (Fig. 1). However, the repeated shifts in climate and vegetation in the history of Baja California cautions us from excluding this proposition prior to further investigation.

Establishment of *Opuntia alcahes* and *O. cholla* as the parents of *O. prolifera* now sets the stage for further population genetic studies, which should be aimed towards testing for recurrent origins of *O. prolifera* and the route by which it attained triploidy.

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