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A MOLECULAR PHYLOGENETIC STUDY OF *GRAPTOPETALUM* (CRASSULACEAE) BASED ON ETS, ITS, *RPL*16, AND *TRNL-F* NUCLEOTIDE SEQUENCES[1]

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Nuclear ETS and ITS, as well as plastid *rpl16* and *trnL-F* DNA sequences were used to determine relationships among species of *Graptopetalum* (Crassulaceae) and closely related genera. *Graptopetalum* is member of a group of taxa restricted to North America, one of the centers of diversity of Crassulaceae; however, their phylogenetic relationships are not yet understood. Nineteen species of *Graptopetalum* and 24 species from nine other genera of Crassulaceae were sampled for use in three separate parsimony analyses: ITS alone, ETS alone, and a combined nuclear + plastid DNA analysis using all four gene regions. The ETS data set had the highest number of parsimony-informative sites, about 30% more than in ITS, but the most fully resolved tree resulted when the four DNA regions were combined. Only four subclades of the tree received moderate to strong bootstrap support, one of which includes all species of *Graptopetalum* having a single whorl of stamens. However, *Graptopetalum* is not monophyletic. Instead, *Tacitus bellus* and select species of *Cremnophila*, *Sedum*, and *Echeveria* are interspersed among species of *Graptopetalum* and show evidence of grouping according to geographical range of distribution more so than habit or floral morphology.

Key words: Crassulaceae; ETS; *Graptopetalum*; ITS; molecular; *rpl16*; succulents; *trnL-F*.

DNA sequences from several different genes and gene regions, as well as chloroplast DNA restriction-site data, have been employed to determine relationships at both higher and lower taxonomic levels in Crassulaceae (van Ham et al., 1994; van Ham and 't Hart, 1998; 't Hart et al., 1999; Gehrig et al., 2001; Mort et al., 2001, 2002; Jorgensen and Frydenberg, 1999). However, sequences from the nuclear ribosomal external transcribed spacer such as the ETS region have not yet been used in this group. It has been demonstrated that the proportion of variable and potentially informative sites is about 30% higher in ETS compared with ITS (Baldwin and Markos, 1998). Sequences of ETS analyzed in combination with other DNA regions have been valuable for resolving phylogenetic relationships within several different groups of angiosperms (Clevinger and Panero, 2000; Beardsley and Olmstead, 2002; Andreasen and Baldwin, 2003). We investigated the utility of sequences of the ETS region, among others, to infer relationships among species of *Graptopetalum* Rose, a genus of Crassulaceae of the New World.

Crassulaceae is a family of approximately 35 genera that is divided into six subfamilies based on a variety of morphological characters (Berger, 1930). However, according to recent molecular phylogenetic studies, there are only two major lineages in the family. One is the ''*Crassula* lineage'' that includes genera from three of the traditional subfamilies, Crassuloideae, Cotyledonoideae, and Kalanchoideae, which are found predominantly in southern Africa. The second is the ''*Sedum* lineage'' that includes genera from the other three subfamilies: Echeverioideae, Sedoideae, and Sempervivoideae. These are found predominantly in the Northern Hemisphere ('t Hart and Eggli, 1995). One of the clades in the ''*Sedum* lineage'' has been informally named the ''*Acre* clade'' (van Ham and 't Hart, 1998) and contains a group of genera from Echeverioideae as well as some species from the large genus *Sedum* L. (Sedoideae). According to Mort et al. (2001), the ''*Acre* clade'' comprises one-third of the species in Crassulaceae, but is plagued by a number of unresolved relationships.

Graptopetalum, a genus of about 19 species, is a member of the Acre clade (Mort et al., 2001). The clade includes species representative of genera such as *Cremnophila* Rose, *Echeveria* DC., *Pachyphytum* Link, Klotzsch & Otto, *Sedum*, *Tacitus* Moran and Meyrán, and *Thompsonella* Britton & Rose (Mort et al., 2001). With the exception of *Sedum*, which is a genus widely distributed, the rest of the taxa are restricted to North America. By focusing in *Graptopetalum*, in its phylogenetic postion as well as in its circumscription, a better understanding of the relationships of this American group will be gained. Two former species of *Sedum* were transferred to *Graptopetalum* (*G. craigii* and *G. suaveolens*). The study of this group will help evaluating the notoriously difficult ''*Sedum* sensu lato'' group.

Species of *Graptopetalum* are mostly found in semiarid vegetation from Arizona in the United States to Oaxaca in Mexico (Moran and Uhl, 1968; Uhl, 1970). The genus is traditionally

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TABLE 1. DNA site variation and tree statistics for the three data sets used in the cladistic analyses presented in this study. CI = consistency index; HI = homoplasy index; RI = retention index; RC = rescaled consistency index.

DNA region	No. taxa	No. characters	No. variable sites	No. informative sites	Percent informative sites	No. trees	Tree length	CI	HI	RI	RC
ETS	41	614	123	211	34.3	792	774	0.51	0.48	0.62	0.37
ITS	43	666	135	169	25.3	296	691	0.53	0.47	0.60	0.39
ETS + ITS + rpl16 + trnL-F	31	3641	—	417	11.4	33	1200	0.54	0.46	0.57	0.41

divided into two sections based on stem characters. Section *Byrnesia* includes caulescent species, whereas section *Graptopetalum* includes acaulescent species with sessile leaf rosettes. This latter group occurs mainly in northwestern Mexico (Moran, 1984).

A recent phylogenetic analysis of *Graptopetalum* using morphological characters (Acevedo-Rosas et al., 2004) does not support the monophyly of the genus unless certain species of *Sedum* are transferred into *Graptopetalum*. Among a number of clades, two well-supported groups were recovered, one including the acaulescent species and another containing all the haplostemonous taxa. However, the question as to how many species should be recognized in *Graptopetalum* still remains.

The objective of this paper is to determine phylogenetic relationships among species of *Graptopetalum* using DNA sequence data from the nuclear *ETS*, *ITS*1, and ITS2 regions, as well as from the chloroplast genome in the form of the *rpl*16 intron and flanking regions, plus the *trnL* intron and *trnL-F* intergenic spacer. The resulting phylogenies will assist in determining if *Graptopetalum* is indeed monophyletic and to gain understanding in the relationships of the American group of Crassulaceae taxa part of the ''*Acre* clade.'' Furthermore, we explore the utility of the ETS region as a potential source of variable characters to help resolve relationships within Crassulaceae.

MATERIALS AND METHODS

Taxon sampling—Nineteen species of *Graptopetalum* and 24 species from nine closely related genera in Crassulaceae were sampled. Appendix 1 (see Supplemental Data accompanying the online version of this article) includes voucher information and GenBank accession numbers. Members of *Cremnophila*, *Dudleya* Britton & Rose, *Echeveria*, *Lenophyllum* Rose, *Pachyphytum*, *Sedum*, *Tacitus*, *Thompsonella*, and *Villadia* Rose were designated as outgroups based on molecular phylogenies of the family published by Mort et al. (2001). Most of the material of *Graptopetalum* was field collected, whereas cultivated plants provided material for many of the other genera.

DNA extraction, amplification, and sequencing—DNA was extracted from fresh or silica-gel-dried leaves using the modified 2× hexadecyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1990) or the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) for a few species. Leaf tissue was ground in liquid nitrogen before using CTAB. For *ETS*, the amplification and sequencing primers were 18S-ETS (Baldwin and Markos, 1998) and a new primer designed specifically for Crassulaceae (ETS-IGSf). Baldwin and Markos (1998) primers were used to obtain ETS sequences in one direction. Sequences were aligned and an internal ETS primer was then designed (ETS-IGSf: AGTTCACGTACGGCGGCCTTTTA). The primers used to amplify and sequence ITS were the universal primers ITS1 and ITS4 (White et al., 1990); for *rpl16*, *rpl16*-1216F and *rps*3-42R (Asmussen, 1999); and for *trnL-F*, the universal primers c–f (Taberlet et al., 1991) (listed in Appendix 2; see supplemental data accompanying online version of this article). Polymerase chain reaction (PCR) fragments were purified using QIAquick silica columns (PCR purification kit, Qiagen) according to the manufacturer's protocols. These purified PCR products were sequenced in both directions using the BigDye Terminator Mix and an ABI 377 automated sequencer (Applied Biosystems, Foster City, California, USA) in the molecular systematics laboratories of The New York Botanical Garden.

Sequence alignment and phylogenetic analysis—Contigs were assembled using Sequencher 4.1 (Gene Codes, Ann Arbor, Michigan, USA). Sequence alignments for the four DNA regions were mostly unambiguous and were performed manually. Parsimony analyses were performed with PAUP* 4.0b10 (Swofford, 2002). Three parsimony analyses were performed, ITS alone, ETS alone, and a combined analysis with all four DNA regions (ETS + ITS + *rpl16* + *trnL-F*). A plastid alone analysis was not performed, because only a few informative characters were obtained for *rpl16* and *trnL-F*. The number of taxa considered in each analysis varied. There were 43 taxa included for *ITS*, 41 taxa for ETS, and 31 taxa for the combined analysis. Heuristic searches were performed with 1000 random addition sequence replicates using tree bisection reconnection (TBR) branch swapping, MulTrees in effect, and with Fitch parsimony (Fitch, 1971). Parsimony analyses weighted all characters and character-state transformations equally; gaps were treated as missing data. Support was evaluated through bootstrapping (Felsenstein, 1985) with 550 replicates using TBR branch swapping for the combined matrix, and 50 000 replicates of fast stepwise-addition for the separate ETS and ITS data sets. Incongruence among data partitions (*rpl16* + *trnL-F* and ETS + ITS) was evaluated with the partition—homogeneity test of Farris et al. (1994) implemented in PAUP* 4.0b10 (Swofford, 2002). The partition homogeneity test used 1000 resamplings under the parsimony criterion with only variable characters included, all characters equally weighted.

RESULTS

Sequence comparisons—DNA site variation for the three data sets (*ETS*, *ITS*, and the combined ETS + ITS + *rpl*16 + *trn*L-F) of this study as well as tree statistics are shown in Table 1. The ETS matrix contained 614 aligned characters, whereas the ITS matrix contained 666. The combined four-gene matrix contained 3641 characters. The percentage of parsimony-informative sites in the ITS region is 25.3% (169), but the ETS data matrix contains proportionally more informative sites (34.3%) than the other data sets. The plastid sequences were not analyzed alone because of the small number of informative sites for each gene: *rpl16* had only 25 parsimony-informative sites from 1424 characters, and *trnL-F* had only 12 parsimony-informative sites in 937 characters.

The ETS data set contained a higher number of indels (Table 1) compared with the ITS data. For 10 taxa, the *rpl16* intron was completely missing, resulting in a 1000-bp deletion for these taxa (the matrix is in Appendix 3; see the supplemental data accompanying the online version of this article).

Phylogenetic analyses—The partition homogeneity test ($P < 0.18$) identified incongruence among the two data sets (ETS + ITS and *rpl16* + *trnL-F*). However, because none of the apparent conflict was between well-supported clades, the data were combined. The resulting cladogram from the ETS analysis had the greatest number of well supported-clades. Con-

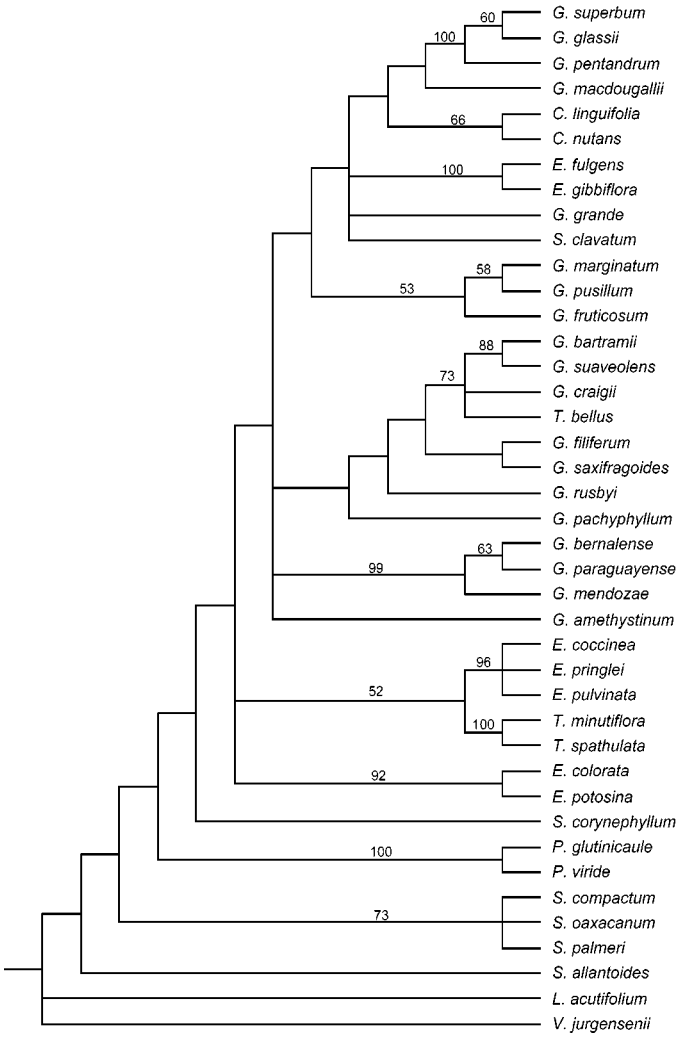

Fig. 1. Consensus tree recovered from the 792 most parsimonious trees inferred from ETS sequences for 41 taxa. Numbers below branches indicate bootstrap support (>50%).

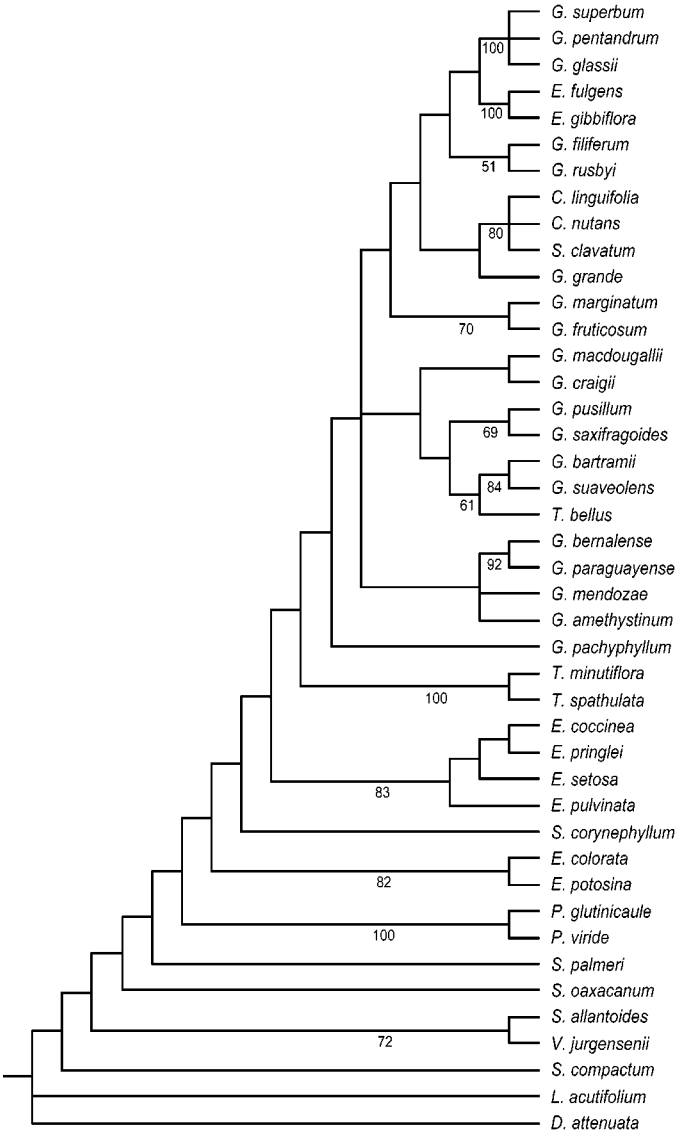

Fig. 2. Consensus tree recovered from the 296 most parsimonious trees inferred from ITS sequences for 43 taxa in Crassulaceae. Numbers below branches indicate bootstrap support (>50%).

sensus of the 792 equally parsimonious trees is shown in Fig. 1. *Graptopetalum* is not monophyletic because some species are forming different groups with outgroup taxa such as *Tacitus bellus*, *Cremnophila lingufolia*, *C. nutans*, *Echeveria fulgens*, *E. gibbiflora*, and *Sedum clavatum*. Within this clade, the two species of *Cremnophila*, *C. linguifolia* and *C. nutans*, form a subclade with a bootstrap value of 66% and *Echeveria fulgens* is sister to *Echeveria gibbiflora* (100%). Some *Graptopetalum* species form well-supported subclades such as (1) *G. glassi*, *G. superbum*, and *G. pentandrum*; (2) *G. bartramii* and *G. suaveolens*; and (3) *G. bernalense*, *G. paraguayense*, and *G. mendozae*. *Tacitus bellus* is closely related to three *Graptopetalum* species: *G. bartramii*, *G. suaveolens*, and *G. craigii* (Fig. 1). Only trees 28 steps longer find *Graptopetalum* monophyletic.

The most parsimonious trees obtained with ITS have nearly the same relationship as recovered with *ETS*. Consensus from the 296 trees is shown in Fig. 2 with bootstrap support. In this case, however, only the terminal branches receive strong bootstrap support, and there is less resolution.

Analysis of the combined data resulted in 33 equally parsimonious trees. The strict consensus of these is presented as Fig. 3. The same two smaller groups of *Graptopetalum* found in the separate analyses were recovered. *Graptopetalum glassii*, *G. superbum*, and *G. pentandrum* form a subclade, whereas *G. bernalense*, *G. paraguayense*, and *G. mendozae* form another subclade.

DISCUSSION

Several studies have shown that greater resolution and support for phylogenetic estimations is achieved by increasing characters and/or taxon representation (Graybeal, 1998; Hillis, 1998; Soltis et al., 1998; Bremer et al., 1999). Our study confirms the importance of adding characters, because relationships among the species of *Graptopetalum* and allied genera are better resolved when the four DNA regions sequenced are analyzed together. Our results also corroborate the results of previous studies (Baldwin and Markos, 1998; Clevinger and

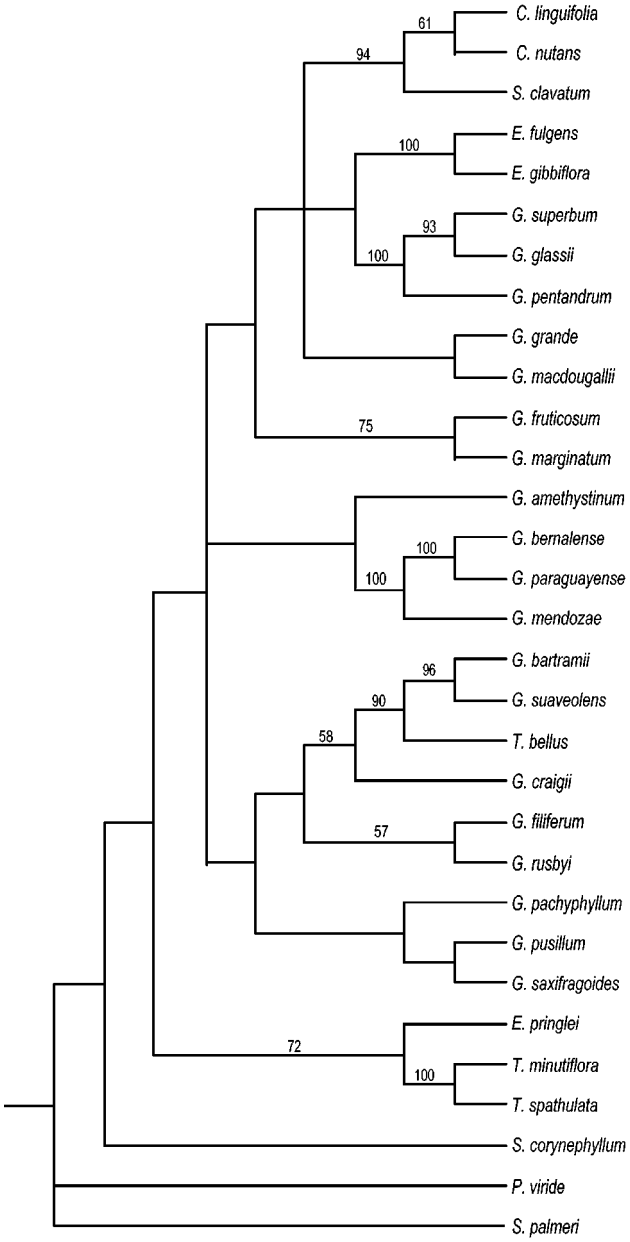

Fig. 3. Consensus tree recovered from 33 most parsimonious trees inferred from the combined data of the four DNA regions (ETS + ITS + *rpl16* + *trnL-F*) for 31 taxa. Numbers below branches indicate bootstrap support (>50%).

Panero, 2000; Beardsley and Olmstead, 2002; Andreasen and Baldwin, 2003) by confirming that the ETS region is an excellent choice for studies at the interspecific level because it has a higher number of parsimony-informative sites than the ITS or other sequences.

This study is also the first to employ *rpl*16 sequence data for Crassulaceae. The presence of a large deletion in this gene region (missing data equal to the complete intron) may have had a negative effect on the analysis, but it is present in taxa that appear to belong to different subclades according to the *ITS, ETS,* and combined trees. It seems to be a homoplasious character. In fact, loss of the intron was not even observed for closely related species of the same genus (e.g., *Cremnophila nutans* is missing the intron, but its sister species, *C. liguli-folia*, is not). This large deletion was found in eight species of *Graptopetalum* belonging to different subclades. In a preliminary analysis, we coded this deletion and other gaps as separate characters, as suggested by Simmons and Ochoterena (2000). However, this strategy had no effect on the overall tree topology.

Despite using the combination of four data sets, the number of parsimony-informative characters was still not sufficient to resolve all relationships among the taxa studied, and only a few subclades received bootstrap support (>50%). Mort et al. (2001) suggested two possible reasons for finding so many unresolved polytomies in their phylogenetic study of the ''*Acre* clade'' of Crassulaceae. Their cladistic analysis used the chloroplast *matK* gene, and they mentioned the possibility that intergeneric hybridization could make for frequent chloroplast exchange among taxa, thus severely affecting chloroplast phylogenies. Two of the gene regions in our study, *rpl*16 and *trnL-F*, are from the chloroplast genome. Furthermore, a variable chromosome number has been reported for 11 *Graptopetalum* species, ranging from $n = 30$ to 270 (Uhl, 1970). This large variation in chromosome numbers is indicative of polyploidy and hybridization. However, polyploidy has never been evaluated in a scientific manner in *Graptopetalum.* Crassulaceae is easily hybridized in cultivation, but there is no empirical evidence for the hybrid origin of any of the species, and nearly all *Graptopetalum* species were wild collected. One reason that both plastid and nuclear genes were chosen was to look for obvious evidence for such a phenomenon in the resulting trees, but this was not found. Moreover, the current geographic distribution and isolation of the species (with the exception of one widespread species) tend to favor against hybrid origins.

Mort et al. (2001) also considered that the ''*Acre* clade'' is a group of relatively recent origin, and this would account for the low levels of variation as seen in our results as well. In our study, incongruence between data partitions was restricted to the positions of only three species of *Graptopetalum*: *G. grande*, *G. pusillum*, and *G. saxifragoides*. However, their alternative positions did not receive strong bootstrap support, suggesting soft rather than hard conflicting phylogenetic signal.

In the combined DNA analysis, we recovered a topology in which at least three major clades of *Graptopetalum* are evident. Within these clades, only four smaller subgroups of *Graptopetalum* species receive bootstrap support (>50%), but these largely correlate with the geographic distribution of the species. *Graptopetalum glassii*, *G. superbum*, and *G. pentandrum* form a well-supported clade that is also present in all most parsimonious trees obtained with ETS and ITS data sets alone. Morphological analyses (Acevedo-Rosas and Cházaro, 2003; Acevedo-Rosas et al., 2004) also recover this monophyletic group, which is characterized by flowers with only one whorl of stamens. These three species are confined to the states of Colima, Jalisco, and Michoacán in west-central Mexico. They appear to be closely related to species of *Cremnophila*, *Echeveria*, and *Sedum*, as well as other *Graptopetalum* species such as *G. fruticosum* and *G. marginatum*, which are also from the west-central Mexican states of Jalisco and Nayarit. The second supported subclade consists of *G. bernalense*, *G. paraguayense*, and *G. mendozae*. These three species are geographically restricted to the states of Tamaulipas and northern Veracruz in east-central Mexico. The third subclade contains *Tacitus bellus*, *Graptopetalum bartramii*, *G. suaveolens*,

and *G. craigii*. These species are restricted to Sonora, Chihuahua, and Durango in northwestern Mexico and are sister to the pair of *G. filiferum* and *G. rusbyi*, which are also restricted in their range to the extreme northwest of Mexico and Arizona. This clade of six species is sister to another group of species, *G. pusillum*, *G. saxifragoides* (both from Durango as well), and the widespread *G. pachyphyllum*.

These subclades do not correspond to the two sections of *Graptopetalum* defined by Moran (1984). Species placed in section *Byrnesia* (caulescent species) as well as those classified in section *Graptopetalum* (acaulescent species) are distributed throughout the cladogram. For example, the acaulescent species *G. marginatum* is sister to *G. fruticosum*, one of the caulescent species. If we are to accept these gene tree topologies, then the separate lineages of *Graptopetalum* species cannot be characterized by those morphological characters that Acevedo-Rosas et al. (2004) found to be synapomorphic. Among these characters are habit, flower fragrance, color of petals, and position of petal maculae. *Tacitus bellus* has large flowers with colorful, dark pink petals that lack spots. It is also aromatic and grouped with *G. bartramii*, *G. suaveolens*, and *G. craigii*, all of which have whitish petals. It seems, therefore, that geography rather than habit or flower morphology may be a better indicator of phylogenetic relationships within this group. The acaulescent and caulescent habits appear to have evolved independently from ancestors native to different geographic areas, perhaps to fill vacant ecological niches within each of these isolated environments. Most *Graptopetalum* species are found in semiarid vegetation, and populations are usually isolated on rocky hills of ravines in these habitats (Acevedo-Rosas et al., 2004).

Our results clearly indicate that *Graptopetalum* is not monophyletic as currently circumscribed (trees 28 steps longer find the genus monophyletic). However, they do not conclusively indicate the exact composition of the genus. The type species of the genus is *G. pusillum*, but this species is in a group that did not receive bootstrap support (>50%). *Cremnophila* and apparently some species of *Sedum* and *Echeveria* are embedded within *Graptopetalum*, but we have not sampled those large genera well enough to know which ones and how many. The monotypic genus *Tacitus* (*T. bellus*) is also embedded within *Graptopetalum* and probably should not be considered a separate genus. As mentioned, *T. bellus* has been difficult to classify because of its unique and horticulturally prized flowers, which are almost certainly not fly-pollinated as are most *Graptopetalum* species. *Tacitus bellus* has been considered a species of *Graptopetalum* by some authors (Hunt, 1979), and its close relationship to *G. suaveolens* in our trees is supported by their similar aromatic floral fragrance.

Shifts in pollinator syndromes (e.g., from fly to bee) leading to convergent flower morphologies have been documented in many other groups of flowering plants (e.g., Hapeman and Inoue, 1997; Borba et al., 2002). These shifts may help to explain the patterns observed in *Graptopetalum* as well. Variation in color and distribution of bands over petals and dissimilar fragrances in same groups of *Graptopetalum* suggest that different pollinator syndromes exist. However, due to the remote places in which most *Graptopetalum* species grow, no evidence on pollination biology has been gathered.

More data from additional gene regions with high levels of variation are needed ultimately to address the question of which species should be included in *Graptopetalum*. Greater taxon sampling is needed as well, especially from within the large and problematic genera *Echeveria* and *Sedum* within the "*Acre*" clade. Nevertheless, this study sheds new light on interpretations of systematic relationships within Crassulaceae and the role that geography, habitat, pollinators, and other ecological factors may play in driving the evolution of these succulents.

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