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PHYLOGENETIC STUDIES OF *MAMMILLARIA* (CACTACEAE)—INSIGHTS FROM CHLOROPLAST SEQUENCE VARIATION AND HYPOTHESIS TESTING USING THE PARAMETRIC BOOTSTRAP[1]

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The genus *Mammillaria* is likely the most species-rich and morphologically variable genus in the Cactaceae. There is doubt as to whether the genus is monophyletic, and past infrageneric treatments differ regarding generic circumscription. Phylogenetic questions about *Mammillaria* were addressed using chloroplast DNA sequence data from the *rpl16* intron and the *psbA-trnH* intergenic spacer for 125 taxa (113 *Mammillaria*, 10 *Coryphantha*, *Escobaria*, *Neolloydia*, *Pelecyphora*, *Ortegocactus*, and two outgroup taxa from *Ferocactus* and *Stenocactus*). Parsimony analyses were conducted using various heuristic search strategies. Bayesian analyses were conducted using the F81 and F81 + I + G models of sequence evolution. Tree topologies from the parsimony and Bayesian analyses were largely congruent. Hypothesis testing was undertaken using the parametric bootstrap to test the monophyly of the genus and the taxonomic status of *Mammillaria candida*. Phylogenies derived from the parsimony and Bayesian analyses indicate that *Mammillaria* is not monophyletic and that the genus *Mammilloydia* (synonym *Mammillaria*) is embedded within a ''core'' group of *Mammillaria* species. Both these results were corroborated by the parametric bootstrap tests. The entire *rpl16* intron was deleted from species in the *Mammillaria crinita* group.

Key words: Cactaceae; *Mammillaria*; phylogeny; *psbA-trnH* intergenic spacer; *rpl16* intron.

Following reorganization of the genus *Opuntia* by Wallace and Dickie (2002) into a number of segregate genera, the genus *Mammillaria* has taken precedence as the most species-rich genus in the cactus family. Modern estimates of species numbers vary greatly depending upon circumscription at both the generic and specific levels. Of 181 species recognized by Pilbeam (1999), Hunt (1999) accepts 145 species.

Members of the genus *Mammillaria* are low-growing, globular cacti with distinctly tuberculate stem morphology. Plants may either be solitary or form massive mounds. These traits are shared with other members of the ''Mammilloid clade'' (Butterworth et al., 2002), which also share the presence of dimorphic areoles—the vegetative (spine-bearing) areole is borne on the tubercle apex, while the flowering areoles are located in the axils of the tubercles. *Mammillaria* is distinct from these other genera (*Coryphantha*, *Escobaria*, *Pelecyphora*, *Neolloydia*, and *Ortegocactus*) in lacking an adaxial groove running from the vegetative areole, in some cases, along the entire length of the tubercle. Distribution of the genus ranges from Venezuela and Colombia to the Southwestern United States, with maximal diversity and species richness in Mexico.

Although used by Linnaeus (1753) as type species for the genus *Cactus*, *C. mammillaris* L. was transferred to and designated type species (as *M. simplex*) by Haworth (1812). The name *Mammillaria* as described by Haworth is a later homonym; the name was first used to describe a genus of algae by Stackhouse (1809). The name *Mammillaria* is conserved for the cactus genus (Greuter et al., 2000).

Pfeiffer (1837) introduced the first infrageneric division of *Mammillaria*. This classification divided the genus into two groups based upon spine characteristics and was followed in 1845 by a more complex classification by Salm-Dyck (1845), who recognized eight groups just below the rank of genus. Both these early classifications of *Mammillaria* were broadly circumscribed, and in 1856, George Engelmann, a St. Louis physician, laid the groundwork for future splitting of the genus into segregate genera. Engelmann (1856) explicitly recognized and described two subgenera in *Mammillaria*. Members of subgenus *Coryphantha* Engelmann included species with grooved tubercles and flowers produced from the current year's growth, whereas the species in subgenus *Eumammillaria* Engelmann had ungrooved tubercles, and flowers produced from tubercles of the previous year.

Schumann (1898) published a comprehensive work on the cactus family. Although he included within *Mammillaria* members of the genus *Coryphantha* (as subgenus *Coryphantha*), he recognized three other subgenera—*Dolichothele* Schumann, *Cochemiea* Brandegee, and *Eumamillaria* Schumann. Even though previous authors (Pfeiffer, 1837) had described infrageneric taxa above the level of species in *Mammillaria*, Schumann explicitly named the infrageneric ranks of section and series. Both subgenera *Dolichothele* and *Cochemiea* in-

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cluded a single series each; however, subgenus *Eumamillaria* was further divided into sections *Hydrochylus* Schumann and *Galactochylus* Schumann, depending upon whether the members had watery or milky sap, respectively. Section *Hydrochylus* was further split into six series and section *Galactochylus* into five series.

Since Schumann's work on *Mammillaria*, a number of subsequent authors have held differing opinions regarding generic delimitations in *Mammillaria*. Britton and Rose (1923) recognized only a narrow circumscription of *Mammillaria*, splitting Schumann's view of the genus into nine genera. Contrary to Britton and Rose, Berger (1929) took a slightly broader view of *Mammillaria* and recognized many of the infrageneric taxa of Schumann.

Buxbaum (1951b) believed that *Mammillaria* was not monophyletic, stating that there was a ''*Mammillaria* stage'' in the evolution of North American barrel cacti (tribe Cacteae) in which plants had the appearance of members of *Mammillaria*. Furthermore, the ''*Mammillaria* stage'' had been reached in a number of independent lineages. During the following years, Buxbaum (1951a, 1954, 1956a, b) modified his infrageneric and generic delimitations of *Mammillaria* and closely related taxa into a narrow circumscription of *Mammillaria* and recognized a number of segregate genera. However, when Moran (1953) proposed reunifying Buxbaum's segregate genera with *Mammillaria* for Hortus Third, Buxbaum relented, accepting a much broader circumscription of the genus *Mammillaria* (Buxbaum, 1956a, b).

Two later authors attempted to produce up-to-date classifications of *Mammillaria*. David Hunt, working in the 1960s and 1970s, attempted to combine the work of Schumann (1898) and Buxbaum (1951a, b, 1954, 1956a, b) into a simple infrageneric classification. Hunt (1971, 1977a, b, c, 1981) did not hesitate in recognizing the genus *Coryphantha* as being clearly separate from *Mammillaria*. Within the genus *Mammillaria*, Hunt recognized five subgenera—*Mammilloydia* (Buxb.) Moran, *Oehmea* (Buxb.) Hunt, *Dolichothele*, *Cochemiea*, *Mamillopsis* Morren ex B. & R., and *Mammillaria*. Of these subgenera, only subgenus *Mammillaria* was divided further, being split into three sections, which were modified from Schumann's (1898) sections *Hydrochylus* (divided into *Hydrochylus* and *Subhydrochylus* Backeberg ex Hunt) and *Galactochylus* (as section *Mammillaria*). Hunt further recognized a number of series within the sections of subgenus *Mammillaria*.

Lüthy (1995) took a phenetic approach to the classification of *Mammillaria* and undertook a detailed morphological analysis of the genus. These data, supplemented with biochemical and ecological data, were used to infer relationships in the genus and produce a classification that was independent of past taxonomic treatments of the genus. Lüthy recognized a fairly narrow circumscription of *Mammillaria*, preferring to treat *Coryphantha* and *Mammilloydia* as distinct from *Mammillaria*. The classification produced by Lüthy (1995, 2001) includes five subgenera, six sections, and 22 series.

The infrageneric classifications of Hunt (1981) and Lüthy (1995) have a number of significant differences (see Fig. 1) and represent the endpoints of different approaches in taxonomic inference. In the last two decades, the use of molecular sequence data in cladistic studies has had a significant impact on the world of taxonomy and systematics. Such methods provide a unique way of investigating taxonomic problems such as the differences of judgment between Hunt and Lüthy. The aim of the study presented in this paper was to use molecular phylogenetic techniques (namely, sequence data from the *rpl16* intron and *psbA-trnH* intergenic spacer regions of the chloroplast) to investigate cladistic relationships and to assess and resolve the differences in past infrageneric classifications of the genus.

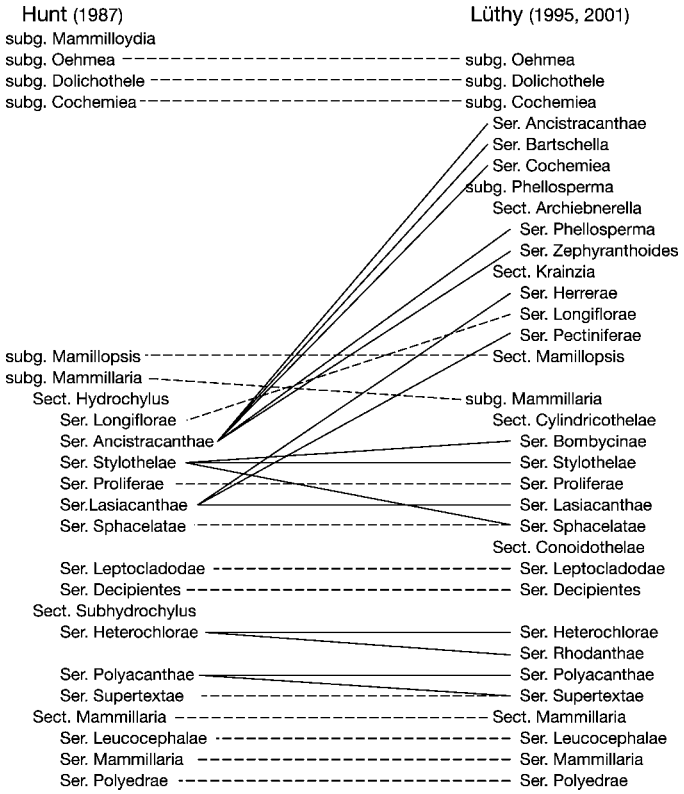

Fig. 1. Comparison of Hunt's (1987) infrageneric classification of *Mammillaria* with that of Lüthy (1995, 2001). subg. = subgenus; Sect. = section; Ser. = series. The dashed lines indicate infrageneric groupings with similar circumscriptions between the two classifications; solid lines show circumscriptional differences between the two classifications.

MATERIALS AND METHODS

Taxonomic sampling—A total of 125 taxa were sampled (Appendix 1; see Supplemental Data accompanying the online version of this article) including 113 representative taxa from *Mammillaria*. Other members from the ''Mammilloid clade'' (Butterworth et al., 2002) included individual taxa from *Ortegocactus*, *Pelecyphora*, and *Neolloydia*, four taxa from *Escobaria*, and three taxa from *Coryphantha*. Selected outgroup taxa for the study were *Ferocactus robustus* and *Stenocactus multicostatus*.

DNA extraction and purification—Total genomic DNA of representative taxa was extracted using one of three methods. (1) In a modified organelle pellet method suitable for mucilaginous material, DNA was extracted from despined, green plant material according to previously published methods (Wallace, 1995; Wallace and Cota, 1996; Butterworth et al., 2002), and the DNA pellet was resuspended in 1 mL of Tris-EDTA and stored at −20°C. (2) In the Nucleon Phytopure plant and fungal kit for 1 g samples (Amersham Biosciences, Little Chalfont, UK), extracted DNA was resuspended in 1 mL Tris-EDTA and stored at −20°C. (3) Using the DNEasy Plant Mini kit (Qiagen, Valencia, California, USA), approximately 90 mg of green plant material was used for each extraction, and the manufacturer's protocol was followed with the exception that the DNA was eluted in 50 μL of sterile distilled water and stored at −20°C.

Amplification and sequencing—Double-stranded amplification of the target sequences was done using the polymerase chain reaction (PCR) in an MJ Research (Waltham, Massachusetts, USA) PTC-100 thermal cycler. Primer sequences of amplification and sequencing primers are shown in Appendix 2 (see Supplemental Data accompanying the online version of this article).

The rpl16 intron—The *rpl16* intron was amplified in 100-μL reaction volumes that included 10 μL of 10× buffer, 5 μL of 25 mmol/L magnesium chloride solution, 8 μL of 25 mmol of an equimolar dNTP solution, 20 pmol of each primer (F71 and R1661), 0.5 μL of *Taq* polymerase, and 2 μL (<10 ng) of DNA template. The following temperature cycles gave sufficient amplification of the *rpl16* intron: an initial melting at 95°C for 5 min followed by 24 cycles of the following protocol: 95°C melt for 2 min; 50°C annealing for 1 min; ramp temperature increase of 15°C at 0.125°C /s; 65°C extension for 4 min. A final extension step at 65°C for 10 min completed the PCR amplification.

In 17 of the *Mammillaria* species sampled for this study, the *rpl16* intron was not amplified with any combination of forward and reverse primers. To check for the presence of the intron, PCR amplifications were conducted for the entire *rpl16* gene using primers RPL16F (Campagna and Downie, 1998) and R1661. Amplicons and subsequent sequences clearly demonstrated that in these species, the entire *rpl16* intron has been deleted (C. A. Butterworth, unpublished data).

The psbA-trnH intergenic spacer—The *psbA-trnH* intergenic spacer was amplified in 50-μL reaction volumes that included 5 μL of 10× buffer, 2.5 μL of 25 mmol/L magnesium chloride solution, 4 μL of 25 mmol of an equimolar dNTP solution, 10 pmol of each primer (PSBAF and TRNHR), 0.25 μL of *Taq* polymerase, and 1 μL of unquantified DNA template. The following temperature cycling parameters gave sufficient amplification of the *psbA-trnH* IGS: an initial melting at 94°C for 2 min followed by 31 cycles of the following protocol: 94°C melt for 1 min; 50°C annealing for 1 min; ramp temperature increase of 15°C at 0.125°C /s; 65°C extension for 2 min. A final extension step at 65°C for 10 min completed the PCR amplification.

Purification and sequencing of PCR products—The PCR products were spun in a vacuum centrifuge to reduce solution volumes to approximately 10 μL, then separated on a 1.5% TAE agarose gel. The amplicon bands were excised from the gel and cleaned using one of the following two methods. (1) Using the Geneclean II kit (Qbiogene, Carlsbad, California, USA) according to the manufacturer's instructions, elution from the glassmilk pellet was achieved in 10 μL of sterile distilled water followed by a second elution in 5 μL of sterile distilled water. (2) Using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions, elution was in 30 μL sterile distilled water followed by a second elution in 20 μL of sterile distilled water. The purified product was further concentrated in a vacuum centrifuge to a final volume of approximately 10 μL. Purified PCR products from both protocols were quantified using agarose electrophoresis using a 1% gel in TAE buffer. Concentrated, purified PCR product (1 μL) was run on a gel with two lanes of a standard, either 5 or 10 μL of ϕX174-*HAEIII* (Invitrogen, Carlsbad, California, USA) at 25 μg/mL.

Sequence data were obtained in chain-termination reactions using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Foster City, California, USA). Approximately 200 ng and 100 ng of purified PCR products were used to sequence the *rpl16* intron and *psbA-trnH* IGS, respectively. Sequencing primers for the *rpl16* intron were F543, R637, and R1516, and for the *psbA-trnH* IGS, the amplification primers were used for sequencing. Only partial sequences for the *rpl16* intron were obtained with approximately 200 nucleotides from the beginning of the intron being omitted. Kelchner and Clark (1997) demonstrated very limited levels of sequence divergence in this region. For most of the sequencing reactions, 1 : 4 dilutions of the BigDye solution gave acceptable reads; however for some amplicons, dilutions of 1 : 1 BigDye solution were required to yield acceptable DNA sequences. Electrophoresis and automated sequence readings were undertaken at the Iowa State University Protein Facility using Perkin Elmer/Applied Biosystems automatic sequencing units (ABI Prism 377).

TABLE 1. Relative positions and lengths of binary encoded indels and excluded regions (for the *rpl16* intron) of unalignable sequence.

Sequence	Position	Length
rpl16 intron	29–32	4
	104–106	3
	203–212	10
	230–233	4
	317	1
	531–534	4
	617–639	23
	640–644	5
	645–685	41
	735–754	20
	727–838	112
	770–775	6
	875–880	6
	881–887	7
	888–892	5
	995–997	3
rpl16 intron, unalignable regions	588–615	28
	686–713	28
psbA-trnH intergenic spacer	28–41	14
	63–67	5
	68–74	7
	104–109	6
	110–115	6
	148–151	4
	225–312	88
	240–246	7
	294–297	4
	356–367	12

Sequence alignment—Sequences were aligned using AutoAssembler (Applied Biosystems, 1995) and Se-Al (Rambaut, 1995). Sequence alignment was carried out manually, following the principles of Kelchner and Clark (1997) for the alignment of noncoding DNA. Insertion/deletion events (indels) considered to be phylogenetically informative were coded in binary (presence/absence) following the treatment of Graham et al. (2000) and added to the end of the data matrix (summarized in Table 1). There were two regions of doubtful homology in the *rpl16* intron, which totaled 56 nucleotides. These nucleotides were excluded from all subsequent analyses.

To test the robustness of our manual alignment, we also performed alignments using ClustalX (Thompson et al., 1997). The raw sequence data was aligned using three different gap penalties—10, 15, and 100. The cost for extending gaps was kept at 1 for all three alignments. Following alignment, the aligned data matrices were saved and compared for number of informative characters.

Congruence testing—Both markers sampled for this study are located in the chloroplast and thus are inherited as a single unit such that phylogenies based upon these markers should yield congruent topologies. Although such congruence has been demonstrated by numerous authors, including Cronn et al. (2002) who clearly showed congruence for four chloroplast markers in cotton and by Nyffeler (2002) for two chloroplast markers in the Cactaceae, we felt that congruence testing should still be a fundamental part of analysis when dealing with multiple data sets. For this reason, congruence between the *rpl16* intron and *psbA-trnH IGS* data sets was tested using the incongruence length difference (ILD) test (Farris et al., 1995) as implemented by the partition homogeneity test in PAUP* for 25 replicates, each saving a maximum of 1000 most parsimonious trees per replicate.

Parsimony analyses—Parsimony analyses were undertaken using PAUP* 4.0b10 (Swofford, 2002). Both the *rpl16* and *psbA-trnH* IGS were tested for phylogenetic signal by calculation of the *G* statistic (Hillis and Huelsenbeck, 1992) for 10 000 random trees. All substitutions and indels were equally weighted. Because of the large number of taxa in the data set, a number of

TABLE 2. A comparison between different sequence alignment methods. For the manually aligned sequences, the aligned length in excluding binary-encoded indels and the numbers inside the brackets are character counts including unalignable regions.

Method	Aligned length	Constant characters	Variable (uninformative)	Variable (informative)
rpl16 manual	1036 (1092)	723 (744)	151 (167)	162 (181)
rpl16 ClustalX default	1058	569	157	332
rpl16 ClustalX 10:1	1305	845	176	284
rpl16 ClustalX 100:1	1076	503	143	430
PsbA-trnH manual	367	247	41	79
PsbA-trnH ClustalX default	354	199	55	100
PsbA-trnH ClustalX 10:1	356	224	49	83
PsbA-trnH ClustalX 100:1	342	154	56	132

heuristic search strategies were employed to maximize the likelihood of finding the most parsimonious tree(s) for the data set. Heuristic searches were performed on separate and combined data sets. An initial heuristic search employed tree bisection-reconnection (TBR) branch swapping on a starting tree obtained by stepwise addition, saving multiple parsimonious trees with MAXTREES set to autoincrement as necessary. Further heuristic searches limited the number of saved parsimonious trees to 1000 (MAXTREES = 1000). Additional random-addition searches of 50 replications, with each replicate limited to saving a maximum of 1000 parsimonious trees (NCHUCK = 1000, CHUCKSCORE = 1), were performed in an attempt to find islands of shorter trees. Parsimony ratchet (Nixon, 1999) searches were also performed on the combined manually aligned data matrix using the software PaupRat (Sikes and Lewis, 2001) under the following conditions: 2000 iterations with 25% of informative characters being perturbed. Bootstrap values for the combined data sets were calculated for 45 replicates each saving a maximum of 1000 trees. For the individual data sets, bootstrap values were calculated using the ''fast'' option for 10 000 replicates. Decay estimates (Bremer, 1988) were calculated using the converse constraint method as implemented using AutoDecay (Eriksson, 1998).

Bayesian analyses—Phylogenetic reconstruction of discrete data (such as molecular sequences) using a Bayesian approach has become increasingly popular as an alternative to maximum likelihood approaches, mainly because Bayesian methods are much less computationally intensive. Given the large number of taxa in our data set, we opted for Bayesian rather than maximum likelihood analyses. Prior to running the Bayesian analyses, two methods were utilized to estimate the most appropriate model of sequence evolution—Modeltest 3.06 (Posada and Crandall, 1998) and DT-ModSel (Minin et al., 2003). Both programs recommended using the F81 model (Felsenstein, 1981) with Modeltest adding parameters for invariable sites and a gamma distribution (F81 + I + G). For each of the two recommended models, five independent Bayesian analyses were performed on the combined data set using the software ''MrBayes'' (Huelsenbeck and Ronquist, 2001, 2002). Each analysis was initiated from a random tree and run in a Markov chain for 1×10^6 cycles with tree sampling every 100th cycle in the chain. Four chains were run simultaneously for each analysis. Following the analyses, the posterior probabilities were graphed to allow an estimate of the number of trees that should be discarded as ''burn-in.'' After the ''burn-in'' trees were removed from the data set, trees from the five analyses were combined and used to produce a majority-rule consensus in which the percentage support is equivalent to Bayesian posterior probabilities.

Hypothesis testing—A number of hypotheses were tested using the parametric bootstrap (Huelsenbeck et al., 1996). Constraint trees that represented the hypothesis under investigation were constructed using MacClade (Maddison and Maddison, 2000). The constraint tree was then used to construct a constraint neighbor-joining tree using maximum-likelihood distances derived from the DT-ModSel analysis. For each hypothesis, 100 data sets were simulated using the computer program Seq-Gen (Rambaut and Grassly, 1997), and the F81 model of sequence evolution. For each of the simulated data sets and the empirical data set, parsimony searches (saving only 1000 most parsimonious [MP] trees) were undertaken in PAUP*, with and without the topological constraint. The distribution of length differences between the constrained and unconstrained MP trees for the 100 simulated data sets was then plotted and compared with the length differences observed for the empirical data set.

RESULTS

Sequence alignment—A comparison of Clustal and manual alignments was undertaken in PAUP* by comparing the number of phylogenetically informative sites in each data matrix (Table 2). The data matrix that presented the smallest number of informative sites should represent the most parsimonious and hence, the most conservative alignment of the sequences. In all cases, the manually aligned sequences had the smallest number of parsimony-informative sites.

Sequence length of the *rpl16* intron varied considerably among those taxa in which it was present. The shortest sequences of the *rpl16* intron were observed in *M. blossfeldiana* and *M. goodridgei* (589 base pairs [bp]) and *M. mammillaris* (615 bp). The longest sequences were observed in *Escobaria hesteri* (964 bp) and *M. wrightii* (949 bp). Sequence length variation in the *psbA-trnH* IGS was much more uniform than in the *rpl16* intron and ranged from 206 bp in *Mammillaria candida* to 307 bp in *Stenocactus multicostatus*. Length characteristics of the aligned sequences are summarized in Table 3. The aligned sequence length of the full data set (including binary-coded indels) totaled 1428 bp. Including the binary-encoded indels, the data set contained 266 parsimony informative sites. There appears to be considerable phylogenetic signal in the *psbA-trnH* IGS, *rpl16* intron, and combined data

TABLE 3. Summary of sequences of the *rpl16* intron, *psbA-trnH* intergenic spacer (IGS), and combined data sets.

Sequence characteristics	*rpl16* intron	*psbA-trnH* IGS	Combined data
Length of aligned matrix (sites)	1036[a]	367	1403
Number of informative gaps	16[b]	9	25
Number of informative sites (% of total sites)	162 (16%)	79 (22%)	266 (17%)

[a] The number of sites after the exclusion of unalignable regions.
[b] The number of informative indels for the *rpl16* intron includes the presence/absence of the entire intron.

TABLE 4. Summary of parsimony analyses of the *rpl16* intron, *psbA-trnH* intergenic spacer (IGS), and combined data sets. Tree length reported is for heuristic searches with MAXTREES to autoincrement/MAXTREES = 1000/Parsimony Ratchet. Other statistics are reported from a single tree drawn from the pool of shortest trees.

Analysis data	*rpl16* intron[a]	*psbA-trnH* IGS	Combined data
Tree length	625/624/624	277/276/276	918/916/916
Consistency index	0.646	0.601	0.622
Consistency index (excluding uninformative characters)	0.522	0.524	0.512
Homoplasy index	0.354	0.399	0.377
Homoplasy index (excluding uninformative characters)	0.478	0.476	0.488
Retention index	0.826	0.809	0.814
Number of resolved clades[b]	94	100	108
Resolution index[c]	0.90	0.82	0.89

[a] Taxa lacking the *rpl16* intron were pruned from the data set.

[b] The number of resolved clades is for strict consensus trees recovered from heuristic searches.

[c] Resolution index is the percentage of clades recovered vs. the maximum number of possible clades in a bifurcating tree that has *n* taxa in the ingroup (for data sets with all taxa included, the number of bifurcating clades is $n - 1 = 122$ clades and for those data sets with taxa lacking the *rpl16* intron excluded, $n - 1 = 105$).

matrices with *G* statistics of −0.32, −0.38, and −0.45 respectively. All of these fall within the 95 and 99% confidence limits for 25 taxa and 500 characters (Hillis and Huelsenbeck, 1992) The aligned data matrix and consensus trees are available from TreeBase (http://www.treebase.org).

Parsimony analyses—The results of the heuristic searches are summarized in Table 4. Heuristic searches on the individual data sets did not find the most parsimonious trees when MAXTREES was set to autoincrement. The trees found and saved by these searches exceeded 150 000 in number and the MacIntosh G4 computer with 990 Mb of memory ran out of memory. For this reason, subsequent heuristic searches were limited to saving a maximum of 1000 trees (MAXTREES = 1000), and under this option shorter trees were actually found (see Table 4). Random addition searches failed to find islands of shorter trees. Strict consensus trees for the *rpl16* intron and *psbA-trnH* IGS are shown in Figs. 2 and 3, respectively. Heuristic searches using the ''parsimony ratchet'' (Nixon, 1999) also recovered trees with lengths equivalent to those of the shortest trees from the random addition searches.

The *rpl16* and *psbA-trnH* IGS data sets have significantly high degrees of congruence. The ILD tests gave a *p* value of 0.82, which suggests that the null hypothesis (tree lengths from random partitions being statistically similar to those from the original partitions) should not be rejected (see Johnson and Soltis, 1998). This result indicates that the data sets can be combined. The strict consensus tree from the combined *rpl16* intron and *psbA-trnH* IGS is shown in Fig. 2.

Making assessments regarding the utility of the different data sets for producing robust phylogenies is not simple. With the use of standard measures, it would appear that the *rpl16* intron with 162 informative sites and 16 scored indels should produce a better resolved phylogeny than the *psbA-trnH* IGS, which only has 79 informative sites and nine scored indels. Indeed, with 22% of sites being parsimony informative, we could reason that the *psbA-trnH* IGS should include more multiple hits than the *rpl16* intron, which only has 16% informative sites. A visual comparison of trees produced from heuristic searches may be a suitable indicator of the resolving powers of particular markers. However, to compare numerically the ''resolving power'' of the two data sets in this study, we opted to create a ''resolution index'' for the individual markers and the combined data set for both the strict and majority rule consensus trees. A fully resolved, bifurcating rooted-tree contains $n - 1$ clades, where *n* is the number of taxa. The ''resolution index'' is simply the proportion of the clades recovered in parsimony analysis to the maximum number of possible clades (from the previous equation). This index gives a very clear and easily interpretable indication of how well different data sets produce resolved trees, either as a comparison between markers for a single set of taxa (as in this study) or between different taxa or taxonomic ranks for a single marker. Using the ''resolution index,'' we conclude that in this study, the *rpl16* intron sequence data provide slightly better resolution than the *psbA-trnH* intergenic spacer region (0.90 vs. 0.82, respectively).

The strict consensus (Fig. 2) reveals a major basal dichotomy that distinguishes two major clades within the ingroup taxa. Twenty-seven of the sampled taxa of *Mammillaria* form a clade (clade A) that is sister to sampled species of *Coryphantha*, *Escobaria*, and *Pelecyphora*. Within clade A, there are two non-*Mammillaria* taxa—*Neolloydia conoidea* and *Ortegocactus macdougallii*. The second group of the major basal dichotomy contains the remaining *Mammillaria* taxa sampled in this study. Within this group of mammillarias, there are a number of resolved clades: (1) clade B consists of five species—*M. beneckei*, *M. oteroi*, *M sphacelata*, *M. tonalensis*, and *M. zephyranthoides* (bootstrap <50%, decay 3); (2) clade C—members of series *Stylothelae* (sensu Hunt) including *M. pottsii* (bootstrap <50%, decay 2); (3) clade D—*M. carmenae*, *M. glassii*, *M. pectinifera*, *M. picta*, *M. plumosa*, and *M. prolifera* (bootstrap <50%, decay 2); (4) *M. vetula* subsp. *gracilis*, which forms the sister group to a large clade that forms a dichotomy of the two remaining clades; (5) clade E—remaining members of series *Stylothelae* (Pfeiffer) Schumann and *M. hernandezii*, *M. longimamma*, *M. herrerae*, *M. humboldtii*, *M. candida*, *M. decipiens*, *M. elongata*, and *M. microhelia* (bootstrap <50%, decay 1); (6) clade F—a large clade containing the remaining 31 sampled taxa of *Mammillaria* (bootstrap 50%, decay 4).

Bayesian analyses—The five individual Bayesian analyses using the F81 and F81 + I + G produced trees that are topologically congruent. Trees from the different models of sequence evolution only differed in the number of clades recovered. The majority-rule tree from the F81 analyses has a resolution index of 0.69, compared with 0.65 for F81 + I + G. The majority-rule tree from the combined F81 Bayesian anal-

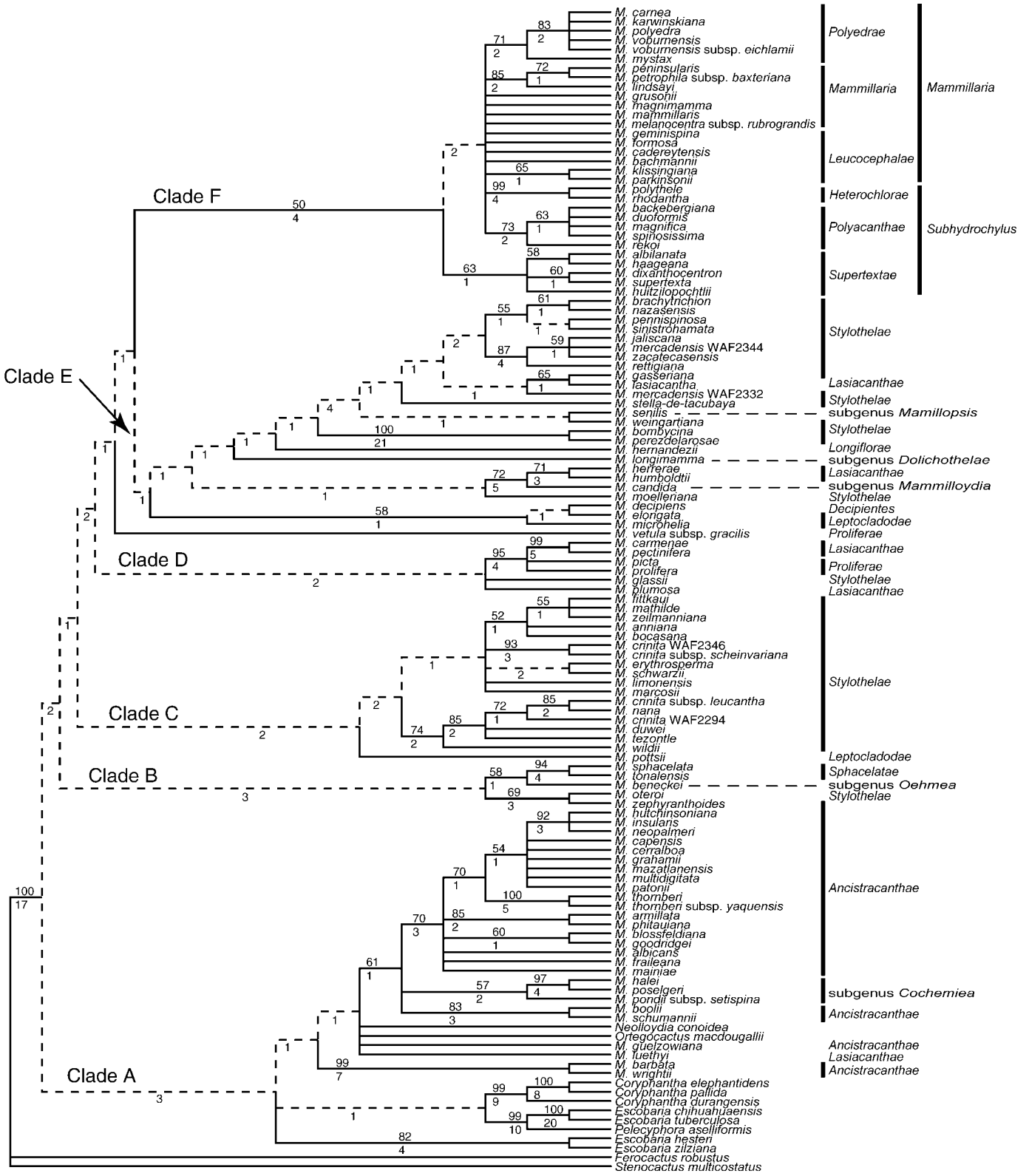

Fig. 2. Strict consensus of 1000 most parsimonious trees for combined *rpl16* intron and *psbA-trnH* IGS sequence data for sampled species of *Mammillaria* and closely related taxa. Percentage support for majority rule is shown above the branches. Bootstrap values greater than 50% are shown below the branches. Decay values are shown below the branches following the bootstrap values. The WAF collection numbers are shown for multiple accessions of *M. mercadensis* and *M. crinita*. The placement of taxa within the infrageneric classification of Hunt (1987) is indicated to the right of the taxon names: column 1 = series, column 2 = section. *Mammillaria* taxa are in subgenus *Mammillaria* section *Hydrochylus* unless indicated otherwise.

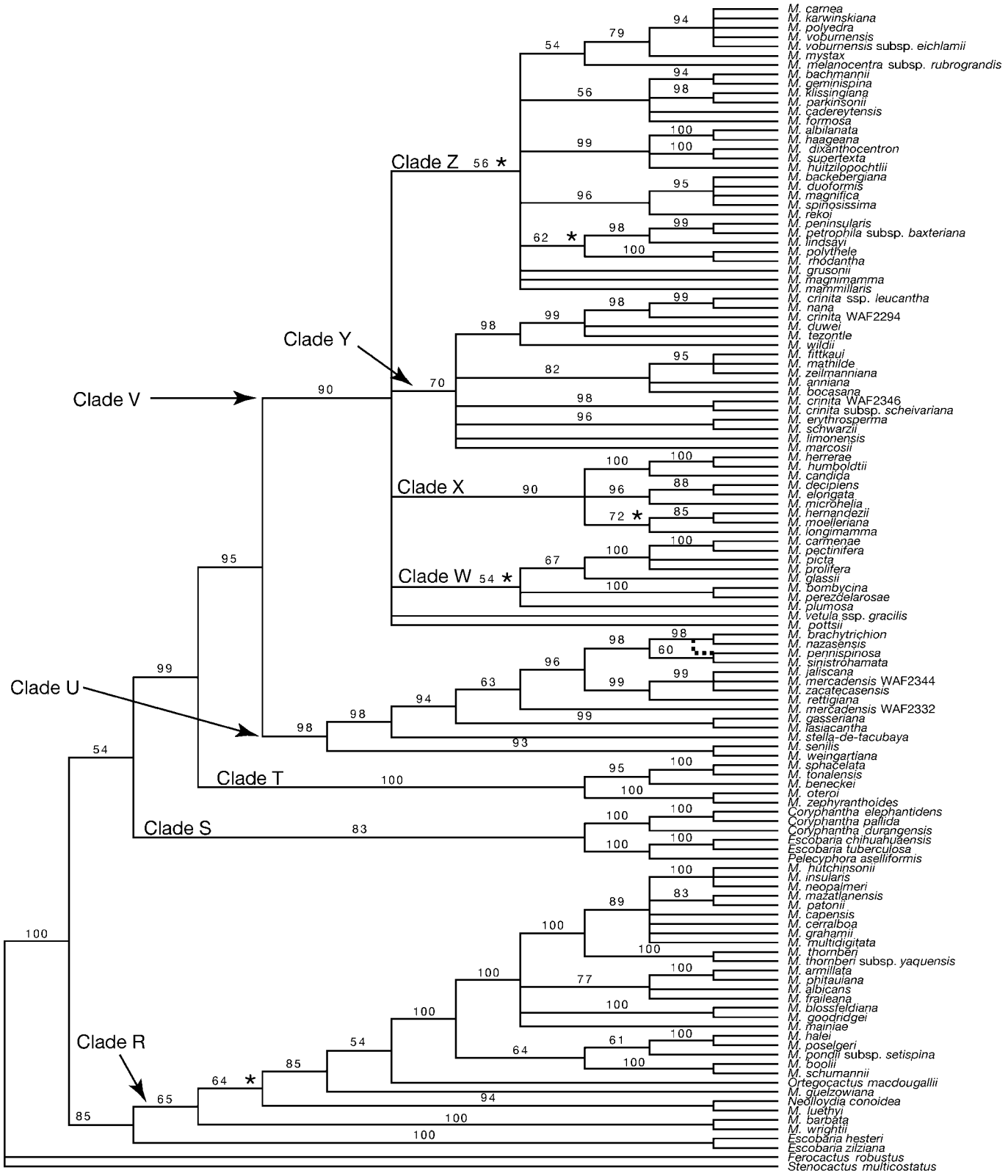

Fig. 3. Majority-rule consensus trees from combined F81 Bayesian analyses for *rpl16* intron and *psbA-trnH* IGS sequence data for *Mammillaria* and closely related taxa. Clades marked by an asterisk are collapsed in the F81 + I + G combined majority-rule consensus. The dashed branch leading to *M. pennispinosa* indicates an alternative topology in the F81 + I + G Bayesian analyses.

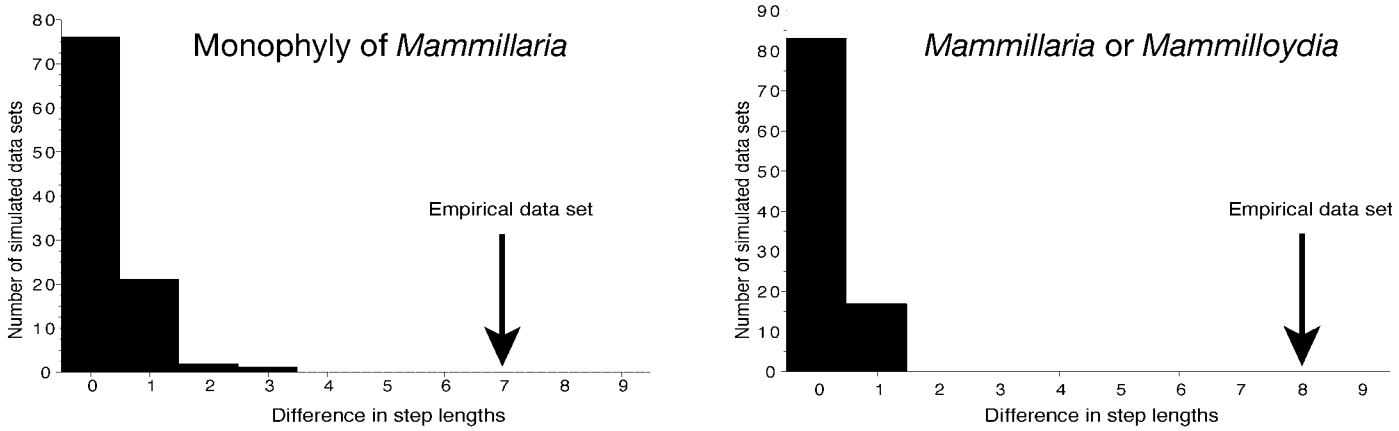

Fig. 4. Results from the parametric bootstrap analyses for testing the hypotheses of monophyly of *Mammillaria* and separate generic status for *Mammillaria*/*Mammilloydia candida*. The plots show the differences in tree length between trees constrained and not constrained for the hypothesis under test. The arrow indicates the differences in tree length observed for the empirical data set. Both hypotheses were rejected at the 5% probability level.

yses is shown in Fig. 3, with alternative/collapsed clades in the F81 + I + G shown by asterisks and dashed lines.

The Bayesian analyses reveal a major basal dichotomy within the ingroup taxa. Clade R includes *Escobaria hesteri* and *E. zilziana*, which form a sister clade to a clade containing 27 of the *Mammillaria* taxa studied, *Neolloydia conoidea*, and *Ortegocactus macdougallii*. The second clade of the major basal dichotomy also resolves a number of distinct clades: (1) clade S—the sampled members of genus *Coryphantha* reside in this clade along with *Escobaria chihuahuaensis* and *E. tuberculosa*; (2) clade T—*M. beneckei*, *M. oteroi*, *M. sphacelata*, *M. tonalensis*, and *M. zephyranthoides*; (3) clade U—members of series *Stylothelae* (sensu Hunt) plus *M. senilis*; (4) clade V—sister group to Clade Y and contains the remaining 67 taxa of *Mammillaria*, which are further divided among a number of clades (W, X, Y, and Z) with relatively low Bayesian posterior probabilities.

A comparison of parsimony and Bayesian trees—The majority-rule consensus tree from the F81 Bayesian analyses (Fig. 3) resolved fewer clades (resolution index = 0.69) than the majority-rule consensus tree from the parsimony analysis (Fig. 2), which had a resolution index of 0.89. In spite of the differences in resolution index, both methods of phylogenetic reconstruction produced trees that were not dramatically dissimilar. Both methods of phylogeny reconstruction show a fairly nested arrangement of clades within the ingroup: (1) clade A in the parsimony tree is equivalent to clade R from the Bayesian analysis, with the exception that members of clade S are included in clade A; (2) clade B and clade T are identical in their membership and placement of the clade as sister to the remaining sampled taxa of *Mammillaria*; (3) clade C of the parsimony analysis is equivalent to clade Y of the Bayesian analysis with the exception that *M. pottsii* is excluded from clade Y and the position of the clades in the two analyses differ—in the Bayesian tree, clade Y is placed within clade V; (4) clades D and W share a similar membership, with the exception of *M. bombycina* and *M. perezdelarosae*, which are included in clade W but not clade D; (5) clade X in the Bayesian tree is not supported in the parsimony analyses, although subclade groupings are fairly congruent between both analyses.

Hypothesis testing—*Monophyly of Mammillaria*—Differences in tree length between constrained and unconstrained trees for the simulated datasets ranged from zero to three steps (see Fig 4). For the empirical data, the branch lengths in constrained and unconstrained trees differed by seven steps, which clearly rejects the null hypothesis of monophyly of *Mammillaria* at greater than the 95% probability level.

The taxonomic status of Mammilloydia—Constrained and unconstrained parsimony searches for the 100 simulated data sets resulted in tree length differences of either zero or one step. The difference in tree length between constrained and unconstrained parsimony searches for the empirical yielded trees with a length difference of eight steps (see Fig. 4). Thus, the null hypothesis of a phylogeny in which *Mammillaria candida* is distinct from other members of *Mammillaria* must be rejected at greater than the 95% level.

DISCUSSION

Phylogenetic relationships in Mammillaria—Based upon the phylogeny produced from the parsimony analyses (Fig. 2), a number of conclusions can be drawn regarding phylogenetic relationships in *Mammillaria*.

Clade A—With the exclusion of the non-*Mammillaria* taxa, clade A corresponds favorably with Hunt's (1981) circumscription of series *Ancistracanthae* Schumann and Lüthy's (1995) circumscription of subgenus *Cochemiea*. Members of series *Ancistracanthae* are often slender, cylindric, and densely clustering with stout, firm tubercles. Central spines of the spine-bearing areoles are typically hooked, although some species have straight spines. Flowers of series *Ancistracanthae* tend to be large (relative to other species in *Mammillaria*) and funnelform, and color ranges from purplish-pink to creamy-yellow to white. Their distribution is predominantly in northwestern Mexico and southwestern United States. However, embedded within series *Ancistracanthae* (sensu Hunt) is subgenus *Cochemiea* (sensu Hunt), whose species (represented in the study by *M. poselgeri*, *M. halei*, and *M. pondii* subsp. *setispina*) are very distinct in *Mammillaria* for their elongated cylindrical stems that may be either upright or prostrate and flowers that are unique in *Mammillaria* for their narrowly tu-

bular shape with bilateral symmetry and hummingbird pollination. Britton and Rose (1923) recognized *Cochemiea* at the level of genus. The phylogeny presented in this study suggests that in spite of unique gross morphology, the recognition of *Cochemiea* at a rank equal to or higher than series would render paraphyletic Hunt's circumscription of series *Ancistracanthae*. The other non-*Ancistracanthae* species of *Mammillaria* included within clade A is *M. luethyi*. With morphology and distribution somewhat different from the typical *Ancistracanthae*, *M. luethyi* is probably one of the most recognizable species of the genus, in having minute spines that branch repeatedly near their apex. Originally discovered by Norman Boke in Coahuila, Mexico, in 1952 as a cultivated specimen, the species went undescribed, and all cultivated material was eventually lost. George Hinton and Jonas Lüthy subsequently rediscovered the plant in habitat in 1996, and it was later described by George Hinton (1996). Hunt (1997) placed *M. luethyi* in series *Lasiacanthae* Hunt with other species that possess mainly undifferentiated numerous diminutive spines.

Clade A as circumscribed in Fig. 2 includes sampled members of the genera *Coryphantha*, *Escobaria*, and *Pelecyphora*, which form sister lineages to sampled taxa of Hunt's and Lüthy's series *Ancistracanthae* and subgenus *Cochemiea*, respectively, thus clearly demonstrating paraphyly within *Mammillaria*. Furthermore, within the core group of series *Ancistracanthae* sensu Hunt and subgenus *Cochemiea* sensu Lüthy, our phylogeny places *Ortegocactus macdougallii* and *Neolloydia conoidea*. Discovered by MacDougall in the early 1950s and described by Alexander (1961), *Ortegocactus macdougallii* has been contentious in its placement in relation to other members of tribe Cacteae. Bravo-Hollis and Sánchez-Mejorada (1991) sank this genus into *Neobesseya*, members of which are now commonly accepted as species of *Escobaria* (Hunt, 1992, 1999; Barthlott and Hunt, 1993). Hunt and Taylor (1986, 1990) suggested that *Ortegocactus* may be referable to the genus *Mammillaria*, although an official transfer to *Mammillaria* was not made. Barthlott and Hunt (1993) also commented on the similarities of *Ortegocactus* and *Mammillaria*, going so far as to suggest that *Ortegocactus* is reminiscent of *M. schumannii*. Butterworth et al. (2002) also suggested that *Ortegocactus* shared a greater affinity with members of *Mammillaria* than with *Escobaria* or *Coryphantha*. The data presented in this paper do indeed show that *O. macdougallii* is embedded within members of *Mammillaria*, its closest *Mammillaria* relatives including *M. schumannii*. However, at present the transfer of *Ortegocactus* to *Mammillaria* would be inappropriate because of the polyphyletic nature of *Mammillaria* as seen in our analyses.

Past circumscriptions of *Neolloydia*, such as those of Hunt and Taylor (1986, 1990), have included the genera *Gymnocactus* and *Turbinicarpus*. Barthlott and Hunt (1993) noted that there were significant differences in the morphology between *N. conoidea* (type species) and other members of the genus and suggested that a separate genus *Turbinicarpus* (presumably including *Gymnocactus*) may be preferable. Hunt (1999) and Anderson (2001) accepted a more narrow circumscription of *Neolloydia* by excluding from the genus those species that lack a tubercular groove and do not have axillary flowering areoles. Butterworth et al. (2002) supported the exclusion of members of *Turbinicarpus* from *Neolloydia*, clearly demonstrating that *Neolloydia conoidea* is phylogenetically positioned within their ''Mammilloid clade,'' whose members have flowers arising from an axillary position between the tubercles. The phylogeny presented here further suggests that *Neolloydia conoidea* has a closer relationship to *Mammillaria* species in Hunt's series *Ancistracanthae* and Lüthy's subgenus *Cochemiea* than to other species of *Mammillaria*.

Clade B—Clade B and clade T of the parsimony and Bayesian analyses, respectively, are identical in their inclusivity and position (as a sister lineage to remaining members of *Mammillaria*). Hunt's (1981) treatment of *Mammillaria* distributed members of clade B among series *Sphacelatae* Hunt (*M. sphacelata* and *M. tonalensis*), *Ancistracanthae* (*M. zephyranthoides*), and *Stylothelae* (*M. oteroi*) all within subgenus *Mammillaria*, and subgenus *Oehmea* (*M. beneckei*). Lüthy's (1995) treatment of the genus placed these species into three groups—*Sphacelatae* (*M. sphacelata*, *M. tonalensis*, and *M. oteroi*) in subgenus *Mammillaria*, series *Zephyranthoides* Kuhn & Hoffmann (*M. zephyranthoides*) in subgenus *Phellosperma* (Britton & Rose) Lüthy, and subgenus *Oehmea* (*M. beneckei*).

Mammillaria beneckei was recognized as a separate genus (*Oehmea*) by Buxbaum (1951c) based on the highly rugose nature of the seeds, which allied the genus to his *Thelocactus* lineage. Hunt (1971) reunited *Oehmea* with *Mammillaria*, sinking it within subgenus *Dolichothele* of *Mammillaria*. Hunt later separated it from subgenus *Dolichothele* (Hunt, 1977a, 1981), but kept it as a subgenus in its own right because of various morphological differences from subgenus *Mammillaria*. The same stance on subgeneric recognition was also taken by Lüthy (1995), who accepted *M. beneckei* in subgenus *Oehmea*. Butterworth et al. (2002) noted that generic status for subgenus *Oehmea* is unwarranted, that Buxbaum's phylogenetic hypothesis of a close relationship between *Oehmea* and *Thelocactus* is incorrect, and that *Oehmea* should be retained within *Mammillaria*. The phylogeny presented here affirms Butterworth et al. (2002) and suggests that the inclusion of *Oehmea* within *Mammillaria* is justified.

When Buxbaum (1951b) described the genus *Ebnerella*, he also described the subgenus *Archiebnerella* Buxbaum, whose type species (*M. zephyranthoides*) formed the connecting (intermediate) group between *Neobesseya* and *Ebnerella*. Hunt (1977a, 1981) subsequently sank *M. zephyranthoides* within his circumscription of series *Ancistracanthae*. Lüthy (1995) recognized *M. zephyranthoides* as being distinct from members of series *Ancistracanthae* and placed the species together with *M. heidiae* Krainz in series *Zephyranthoides*, which is itself placed alongside series *Phellosperma* in section *Archiebnerella*. Our phylogeny suggests that Hunt's placement of *M. zephyranthoides* into series *Ancistracanthae* is incorrect, although our sampling is insufficient to allow us to draw any firm conclusions regarding section *Archiebnerella*.

Clade C—Hunt's (1977b, 1981) circumscription of series *Stylothelae* included species possessing slender, soft-textured tubercles. The series was split into two groups by Hunt (1977b)—those species from the northwestern range of the series, with firm, blunt tubercles and acicular radial spines (*M. bombycina* group) and those with a more southeastern distribution (*M. wildii* group). Lüthy (1995) had a narrower circumscription of series *Stylothelae* than Hunt—a circumscription similar to Hunt's *M. wildii* group. The other species were placed in series *Bombycinae* Lüthy. With the exclusion of *M. pottsii*, members of clade C correspond to Lüthy's circumscription of series *Stylothelae*. The inclusion of *M. pottsii* within this clade warrants further investigation. Hunt (1977b,

1986) and Pilbeam (1999) both allude to distinctive characteristics of this species, which both Hunt and Lüthy placed within series *Leptocladodae* (Lemaire) Schumann. The phylogeny presented in this paper suggests that *M. pottsii* is likely misplaced by both Hunt and Lüthy in series *Leptocladodae*.

Clade D—With the exception of *M. glassii*, members of clade D were treated by Hunt as members of series *Lasiacanthae* and *Proliferae* Hunt. In his description of series *Proliferae*, Hunt (1977b) stated that this group is distinct from members of series *Stylothelae* for having straight central spines that intergrade with the radial spines rather than having two distinct series of spines. Hunt (1977b) further stated that this series is linked to series *Lasiacanthae*, which lack central spines altogether. *Mammillaria prolifera* and *M. picta* of clade D were included by Hunt (1981) in series *Proliferae*; and *M. carmenae*, *M. pectinifera*, and *M. plumosa* were included in series *Lasiacanthae*. Lüthy (1995) accepted Hunt's placements of these species with the exception of *M. pectinifera*, which, along with *M. solisioides*, Backeberg, he believed deserved the recognition given them by Kuhn and Hoffmann (1979) as series *Pectiniferae* Kuhn & Hoffmann.

Mammillaria glassii, placed by Hunt (1984) and Lüthy (1995) into series *Stylothelae* and *Bombycinae*, respectively, is distinguishable within series *Stylothelae* and *Bombycinae* by its spination with a single central spine that may be hooked or straight, and 6–8 subcentral spines that may be difficult to distinguish from the radial spines. For this reason, Hunt (1984) further suggested that *M. glassii* may form a link between series *Stylothelae* and *Proliferae*. Indeed, the phylogeny presented here suggests that *M. glassii* has a greater affinity with members of series *Proliferae* and *Lasiacanthae* than it does to members of series *Bombycinae* and *Stylothelae*. Furthermore, our data suggest that series *Proliferae*, *Lasiacanthae*, and *Pectiniferae* are very closely related.

Clade E—The topology of clade E forms a nested series of small clades, many of which lack strong statistical support. *Mammillaria decipiens*, *M. elongata*, and *M. microhelia* seem to form a well-supported clade that forms a sister lineage to remaining members of clade E. These species were placed within series *Decipientes* Hunt and *Leptocladodae* by both Hunt (1981) and Lüthy (1995). *Mammillaria decipiens* was used as the type species for Hunt's (1979) series *Decipientes*, which he placed in subgenus *Dolichothele* based on its long tubercles, few spines, and green colored fruits. Subsequently, Hunt (1981) removed series *Decipientes* from subgenus *Dolichothele* and allied it with members of series *Leptocladodae* in subgenus *Hydrochylus*. Hunt further noted that the only known interseries hybrid in *Mammillaria* occurred between series *Decipientes* and *Leptocladodae* in the cross between *M. decipiens* and *M. elongata*. Our phylogeny places members of series *Decipientes* and *Leptocladodae* in a single clade, confirming Hunt's (1981) placement of these series alongside each other.

The clade containing *M. herrerae*, *M. humboldtii*, *M. candida*, and *M. moelleriana* is supported by a bootstrap value of only one. Hunt (1981) grouped *M. herrerae* and *M. humboldtii* in series *Lasiacanthae*, based mainly on the lack of central spines, numerous radial spines, and globose, clustering habit. Lüthy (1995) separated these species from series *Lasiacanthae*, placing them in series *Herrerae* Lüthy within section *Krainzia* (Backeberg) Buxbaum because of their seed and fruit morphology. The phylogeny presented in this paper supports the separation, by Lüthy, of these two species from series *Lasiacanthae*.

The treatment of *Mammillaria candida* has been a source of debate since Buxbaum (1951a) elevated the species to genus level (*Mammilloydia*), based upon the verrucose nature of the seed testa. Hunt (1971) accepted that the seed of *M. candida* was unique among *Mammillaria*, because it lacked intracellular pits. However, he felt little else separated it from *Mammillaria* and adopted the treatment of Moran (1953) in accepting the subgenus *Mammilloydia* (Buxbaum) Moran. Riha and Riha (1975) examined seeds of *M. candida* from various sources and found that seeds had a smooth testa rather than a verrucose testa, as reported by Buxbaum (1951a). They concluded that Buxbaum's observations of the seed of *M. candida* were inaccurate, even postulating that his material might have been contaminated. Furthermore, Riha and Riha (1975) concluded that the lack of a pitted testa was not sufficient to warrant recognition of *M. candida* in its own genus, subgenus or series and suggested that the species would be better placed with members of Hunt's (1971) series *Lasiacanthae*. Hunt (1977a) contested the conclusions of Riha and Riha (1975) as superfluous and continued to recognize the placement of *Mammillaria candida* within subgenus *Mammilloydia*. In 1986 and 1990, the working party of the International Organization for Succulent Plant Study (IOS) published preliminary findings on their search for a consensus classification for the cactus family (Hunt and Taylor, 1986, 1990), in which *Mammillaria candida* was provisionally accepted within the genus *Mammillaria* in spite of unspecified differences that possibly warranted recognition as genus *Mammilloydia*. The International Cactaceae Systematics Group (formerly the IOS working party) finally concluded that generic-level recognition for *Mammilloydia candida* was justified (Hunt, 1999). Butterworth et al. (2002) concluded that recognition of the genus *Mammilloydia* would render *Mammillaria* paraphyletic. The phylogeny and results from the parametric bootstrap presented here further support this conclusion. Furthermore, our phylogeny groups *Mammillaria candida* with *M. herrerae* and *M. humboldtii* (series *Lasiacanthae* sensu Hunt). Pilbeam (1999) comments on the resemblance of some forms of *M. humboldtii* to *M. herrerae*. More significantly, however, past circumscriptions of *Mammillaria candida*, such as those by Schumann (1898), Britton and Rose (1923), and Berger (1929), sank *Mammillaria humboldtii* within *Mammillaria candida*, whereas recent authorities such as Hunt (1984) and Pilbeam (1999) dismissed similarities between these two species as misleading. The phylogeny presented here suggests that *Mammillaria candida* should not be recognized at genus level (as *Mammilloydia*) and that this species is closely related to *Mammillaria humboldtii* and *M. herrerae*.

Also included within clade E is *Mammillaria longimamma*. Schumann (1898) viewed the elongate, soft tubercles of this species as sufficiently important to warrant its own subgenus—*Dolichothele* within *Mammillaria*. Britton and Rose (1923) elevated subgenus *Dolichothele* to genus level, and it remained that way until Hunt (1971) sank it back into *Mammillaria*, arguing that acceptance of *Dolichothele* at genus level based only on one character or character group was unjustified. Lüthy (1995) also accepted the sinking of *Dolichothele* into *Mammillaria* and (like Hunt) recognized subgenus *Dolichothele*. Butterworth et al. (2002) concluded that Hunt and Lüthy were correct in treating *Mammillaria longimamma* as a

member of *Mammillaria* and that this species was clearly not a separate genus. Our phylogeny further supports this view, placing *M. longimamma* within the core group of *Mammillaria* species. However, the phylogeny does not support recognition of *Dolichothele*, even at subgeneric level.

The sister group to *Mammillaria longimamma* includes *M. hernandezii*, which is the only representative taxon from series *Longiflorae* Hunt. Members of this series are typically low-growing, cespitose plants with large flowers and black seeds. Hunt (1971) suggested that this group has affinities with members of series *Ancistracanthae*, placing them alongside each other in his classification, and that the disjunct distributions may be relictual or indicative that the two groups are not closely related. Lüthy (1995, 2001) also recognized series *Longiflorae*, but differed from Hunt in the placement of the group in section *Krainzia* and not closely allied with series *Ancistracanthae*.

With the exception of *M. lasiacantha* and *M. senilis*, the large clade that forms the sister group to *Mammillaria hernandezii* contains members treated within the *M. bombycina* group of series *Stylothelae* by Hunt (1977b, 1981). Lüthy (1995) formally named this group as series *Bombycinae* Lüthy. The *M. bombycina* group, which includes the northern and western species of series *Stylothelae*, tend to have larger, firmer, and blunter tubercles than the other members of series *Stylothelae* sensu Hunt, and the radial spines are acicular and form a single series.

Included within this clade is *M. senilis*, whose distinct long-tubed, slightly zygomorphic flowers are bird-pollinated. This species had been considered distinct within the genus *Mammillaria*. Britton and Rose (1923) believed that morphological differences warranted treatment of this species in its own genus—*Mamillopsis*. However, Hunt (1971) believed that *M. senilis* was not sufficiently different from other members of *Mammillaria* to justify its segregation as a genus and preferred to retain *Mamillopsis* at the rank of subgenus, a stance also taken by Lüthy (1995). The phylogeny presented in Fig. 2 clearly indicates that recognition of *M. senilis* at subgenus level would render subgenus *Mammillaria* polyphyletic. The placement of this species with *M. weingartiana* appears unusual and warrants further investigation. However, it must be noted that the distribution of *M. senilis* in northern Mexico (Chihuahua, Durango, and Jalisco) is sympatric with the distribution of clade E members of series *Stylothelae* sensu Hunt.

Clade F—Schumann (1898) divided Engelmann's subgenus *Eumamillaria* (Engelmann, 1856) into two sections—*Hydrochylus* and *Galactochylus* for those species that have watery and milky sap, respectively. Backeberg (1938) described section *Subhydrochylus* as containing those species that possess watery sap in the tubercles but milky sap in the stem core. Members of clade F correspond to sections *Mammillaria* (*Galactochylus*) and *Subhydrochylus* as recognized by Hunt (1971, 1977b, c, 1981, 1987). However, according to our phylogeny, section *Subhydrochylus* as currently circumscribed by Hunt is paraphyletic.

Within clade F, the clade containing *M. dixanthocentron*, *M. supertexta*, *M. huitzilopochtlii*, *M. albilanata*, and *M. haageana* is supported with 63% bootstrap and a decay value of 1 step. This clade corresponds with series *Supertextae* Hunt. Members of this series typically are shortly cylindrical to stoutly columnar, often clustering plants with small tubercles; have small to very small flowers; central spines that are absent or, if present, are straight or curved; and have numerous fine radial spines that obscure the stem. These morphological attributes are striking, and the series was also recognized by Lüthy (1995).

The well-supported clade containing *M. backebergiana*, *M. duoformis*, *M. magnifica*, *M. spinosissima*, and *M. rekoi* was placed within series *Polyacanthae* (Salm-Dyck) Schumann by both Hunt (1977b, 1981) and Lüthy (1995). Members of series *Polyacanthae* possess very small flowers. Spines are numerous and differentiated into central spines (which may be hooked), and numerous radial spines that rarely obscure the plant stem as they do in series *Supertextae*.

The clade consisting of *Mammillaria carnea*, *M. karwinskiana*, *M. polyedra*, *M. voburnensis*, *M. voburnensis* subsp. *eichlamii*, and *M. mystax* were recognized by Hunt (1977c, 1981) and by Lüthy (1995) within series *Polyedrae* (Pfeiffer) Schumann and are characterized by their medium-sized flowers, few spines, with little or no distinction between central and radial spines, and more-or-less conspicuous axillary bristles (absent in *M. carnea*).

Mammillaria peninsularis, *M. petrophila* subsp. *baxteriana*, and *M. lindsayi* form a well-supported clade within Clade F (bootstrap 85%, decay 2 steps). The first two species of this clade are found in southern Baja California, while the *M. lindsayi* is found across the Sea of Cortez in adjacent regions of Sinaloa and Chihuahua. The only other members of *Mammillaria* that occur in Baja California are from series *Ancistracanthae* sensu Hunt, clearly indicating independent migrations from mainland Mexico.

Generic circumscription of Mammillaria—The phylogenies presented in Butterworth et al. (2002) and in this paper clearly show that, as currently circumscribed, the genus *Mammillaria* is likely polyphyletic. Species within the genus *Coryphantha* and *Escobaria* are morphologically distinct from members of *Mammillaria*, which lack a tubercular groove. The number of species in *Coryphantha* and *Escobaria* is 55 and 23, respectively (Hunt, 1999). For this reason, firm conclusions regarding the polyphyly of *Mammillaria* because of the inclusion of members of these genera must be viewed with caution until more species are sampled. If increased sampling of species from *Coryphantha* and *Escobaria* reveals a monophyletic origin for these genera, then the obvious solution indicated by the phylogeny in Fig. 2 is to restrict the genus *Mammillaria* to clades B through F.

Even if the genera *Coryphantha* and *Escobaria* form a clade separate from the remaining members of clade A, the membership of clade A is still problematic. *Neolloydia conoidea* and *Ortegocactus macdougallii* would need to be transferred from their respective genera. *Mammillaria halei*, *M. poselgeri*, and *M. pondii* subsp. *setispina* are currently placed by both Hunt (1981, 1987) and Lüthy (1995) in subgenus *Cochemiea* Brandegee, which itself was validly elevated by Walton (1899) to the rank of genus. Thus the *Mammillaria* members of clade A, *Neolloydia conoidea* and *Ortegocactus macdougallii* could be transferable to the genus *Cochemiea* (Brandegee) Walton.

Methodological considerations—*The utility of the rpl16 intron and psbA-trnH IGS for phylogeny reconstruction in Mammillaria*—Considering the large number of recoverable most parsimonious trees and the relatively high homoplasy indices for the individual and combined data sets, it is reasonable to suggest that both of the chosen markers are highly variable in

the genus *Mammillaria*. This conclusion is also supported by the lack of bootstrap and decay-value support for the deeper, internal branches of the phylogeny illustrated in Fig. 2. For this reason, the addition of sequence data from more slowly evolving markers such as *ndhF* or the *trnL-F* spacer region may likely result in a more phylogenetically robust data set, in which the slower evolving markers provide resolution in the deeper nodes, while the faster evolving markers provide resolution towards the tips of the cladogram.

Heuristic search strategies for large data sets—The analysis of large data sets presents special problems for heuristic search strategies, especially when homoplasious characters form a large proportion of variable sites. The large number of most parsimonious trees exceeded the memory available to the computer used in this study. Setting an upper limit to the number of trees to save in the analysis, i.e., setting MAXTREES in PAUP* to a reasonable number (in our case, 1000 trees) allowed the heuristic search to store a maximum number of trees, then begin branch-swapping on the saved trees. This method of heuristic search quickly found shorter trees than if the heuristic search was allowed to save all most parsimonious trees.

Closing remarks—In summary, the phylogeny presented in this paper suggests that as currently circumscribed, the genus *Mammillaria* is likely polyphyletic on a number of levels. Within the core group of *Mammillaria*, past taxonomic classifications (chiefly Hunt and Lüthy) have had limited success in identifying ''natural,'' phylogenetic groups and to some extent, have been thwarted by morphological convergence in a genus that likely contains numerous ''micro'' taxa.

We are cautious with regard to a more detailed infrageneric classification of *Mammillaria* because of the amount of uncertainty caused by poorly supported clades within the core group of *Mammillaria*. Investigations are ongoing to increase the depth of sampling within the genera *Coryphantha* and *Escobaria*, as well as to fill in sampling gaps within *Mammillaria*. It is also imperative to add more molecular data, such as other ''fast'' evolving chloroplast and nuclear markers, to further add support at branch tips, and slower evolving markers to increase the statistical robustness of major branches towards the root of the phylogeny. Once a well-supported phylogeny has been produced, assessments of morphology can be utilized along with phylogenetic information to yield a reliable infrageneric classification within *Mammillaria*.

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