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Phylogenetic evaluation of chloroplast *trn*L–*trn*F DNA sequence variation in the genus *Mammillaria* (Cactaceae)

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Abstract: HARPKE, D., PETERSON, A., HOFFMANN, M.H. & RÖSER, M. 2006: Phylogenetic evaluation of chloroplast *trn*L–*trn*F DNA sequence variation in the genus *Mammillaria* (Cactaceae). Schlechtendalia **14**: 7–16.

Phylogenetic relationships among 21 species of the large Cactaceae genus *Mammillaria* were investigated using DNA sequence data from the chloroplast intergenic region *trn*L–*trn*F. The study contains representatives of all subgenera (except subg. *Cochemiea*) of *Mammillaria* as well as the sections and series of *M.* subg. *Mammillaria*. Sequences were aligned and a neighbour-joining (NJ) tree was constructed. Although variation of the *trn*L–*trn*F intergenic region was low, the NJ tree showed that *Mammillaria candida* (subg. *Mammilloydia*) clustered within the genus *Mammillaria* which supports to include the segregate genus *Mammilloydia* Buxbaum under synonymy of *Mammillaria*. *Mammillaria mazatlanensis* was revealed as sister to the other *Mammillaria* species investigated.

Zusammenfassung: HARPKE, D., PETERSON, A., HOFFMANN, M.H. & RÖSER, M. 2006: Phylogenetic evaluation of chloroplast *trn*L–*trn*F DNA sequence variation in the genus *Mammillaria* (Cactaceae). Schlechtendalia **14**: 7–16.

Die phylogenetischen Beziehungen zwischen 21 Vertretern der Cactaceae-Gattung *Mammillaria* wurden mittels DNA-Sequenzdaten der *trn*L–*trn*F Chloroplastenregion untersucht. Die Auswahl der Arten umfasste Vertreter aller Untergattungen (außer subg. *Cochemiea*) sowie aller Sektionen und Serien der großen Untergattung *Mammillaria*. Zur Auswertung der erhaltenen Daten wurde ein Sequenz-Alignment mit anschließender Stammbaum-Berechnung nach der neighbour-joining-Methode durchgeführt. Trotz geringer Unterschiede in der untersuchten *trn*L–*trn*F Region ließen sich Rückschlüsse auf die phylogenetischen Verhältnisse innerhalb der Gattung *Mammillaria* ziehen. Der NJ-Baum zeigt, dass *Mammillaria candida* (subg. *Mammilloydia*) innerhalb der Gattung *Mammillaria* gruppiert, was die Einbeziehung der Gattung *Mammilloydia* unter Synonymie von *Mammillaria* unterstützt. *Mammillaria mazatlanensis* erwies sich als Schwester aller übrigen hier untersuchten Arten von *Mammillaria*.

Key words: Molecular phylogenetics, *Mammilloydia.*

Introduction

With an estimated age of about 30 million years (HERSHKOVITZ & ZIMMER 1997) the Cactaceae represent a comparatively recent but species-rich family which includes about 100 genera and 1500 species (BARTHLOTT & HUNT 1993). Within this family, the genus *Mammillaria* Haw. is the most species-rich genus with about 180 species

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(PILBEAM 1999), since *Opuntia* Mill., the formerly largest genus of Cactaceae, became disassembled into several segregate genera recently (WALLACE & DICKIE 2002).

Molecular studies and null hypothesis testing (BUTTERWORTH and WALLACE 2004) revealed the genus *Mammillaria* as a polyphyletic group including species of, for example, *Coryphantha* (Engelm.) Lem. and *Escorbaria* Britton & Rose. The genus in its traditional circumscription is found chiefly in Mexico, extending northwards into the south-western United States and southwards into Central America, Venezuela, and northern Colombia (PILBEAM 1999).

At the beginning of this study there was a lack of molecular data for the genus *Mammillaria*. Therefore, we wanted to study the phylogeny of *Mammillaria* with the *trn*L–*trn*F region of the chloroplast DNA. The selection of species was based on the classification of HUNT (1971; 1977a, b, c; 1981). HUNT recognised six subgenera: *Oehmea*, *Mamillopsis*, *Dolichothele*, *Mammilloydia*, *Cochemiea* K. Brandegee and *Mammillaria*. Only subgenus *Mammillaria* was divided further into sections: *Hydrochylus*, *Subhydrochylus* and *Mammillaria* and each section became subdivided into series. LÜTHY (1995, 2001) considered subg. *Mammilloydia* as a genus separate from *Mammillaria* and recognised it as genus *Mammilloydia* Buxb. LÜTHY's classification of *Mammillaria* contained the subgenera *Oehmea*, *Dolichothele*, *Cochemiea* (three series), *Mammillaria* (three sections with 14 series), and subg. *Phellosperma* (Britton & Rose) Lüthy (three sections with five series) which was newly erected for parts of the subg. *Mammillaria* sect. *Hydrochylus* in HUNT's circumscription. The subg. *Mamillopsis* became subsumed by LÜTHY as a section under an expanded subg. *Cochemiea*.

Our research deals with *trn*L–*trn*F sequences of 21 out of the c. 180 *Mammillaria* species representing plants from all subgenera (except subg. *Cochemiea*) as well as the sections and series of the large subgenus *Mammillaria* (Table 1).

The intergenic spacer between *trn*L (Leu) and *trn*F (Phe) has often been used for phylogenetic studies (SANG et al. 1997, MCDADE & MOODY 1999, RICHARDSON et al. 2000, NYFFELER 2002, PETERSON et al. 2004).

Material and Methods

Plant material

Fresh plant material (21 *Mammillaria* species, Table 1) was provided by the Botanical Garden Halle, where the reference collection of the 'Arbeitskreis für Mammillarienfreunde' e.V. (AfM) is cultivated . This collection contains about 80 % of all *Mammillaria* species. The cultivated individuals can be traced back in most cases to old wild collections or to seeds collected in the natural habitats. Thus, the study of unintended hybrids due to open pollination in the greenhouses can be excluded.

DNA isolation

10–50 mg of fresh plant material was macerated in liquid nitrogen and used for DNA isolation with the DNeasy Plant Mini Kit (Qiagen), following the manufacturer's protocol. DNA concentration was determined by spectrophotometry (Genequant, Pharmacia).

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Tab. 1: Overview of the *Mammillaria* species investigated with classification (HUNT 1971; 1977 a, b, c; 1981), accession numbers/cultivation code of plants of the Botanical Garden Halle and EMBL accession numbers of the *trn*L-*trn*F sequences.

Species	Subgenus	Section	Series	Cultivation code	trnL-trnF accession numbers (EMBL)
M. beneckei Ehrenb.	*Oehmea* (Buxb.) D.R. Hunt			999240	AJ583216
M. candida Scheidw.	*Mammilloydia* (Buxb.) Moran			910574	AJ583218
M. longimamma DC.	*Dolichothele* K. Schum.			910662	AJ583225
M. senilis Lodd. ex Salm-Dyck	*Mamillopsis* (E. Morren ex Britton & Rose) D.R. Hunt			-	AJ583212
M. camptotricha Dams	*Mammillaria*	*Hydrochylus* K. Schum.	*Decipientes* D.R. Hunt	009455	AJ583215
M. deherdtiana Farwig	*Mammillaria*	*Hydrochylus* K. Schum.	*Longiflorae* D.R. Hunt	971522	AJ583221
M. elongata DC.	*Mammillaria*	*Hydrochylus* K. Schum.	*Leptocladodae* Lem.	951037	AJ583224
M. mazatlanensis K. Schum. ex Gürke	*Mammillaria*	*Hydrochylus* K. Schum.	*Ancistracanthae* K. Schum.	882035	AJ583226
M. multiceps Salm-Dyck	*Mammillaria*	*Hydrochylus* K. Schum.	*Proliferae* D.R. Hunt	971607	AJ583228
M. pectinifera F.A.C. Weber	*Mammillaria*	*Hydrochylus* K. Schum.	*Pectiniferae* E. Kuhn & B. Hofm.	-	AJ583229
M. plumosa F.A.C. Weber	*Mammillaria*	*Hydrochylus* K. Schum.	*Lasiacanthae* D.R. Hunt	-	AJ583230
M. rettigiana Boed.	*Mammillaria*	*Hydrochylus* K. Schum.	*Stylothelae* Pfeiff.	971537	AJ583231
M. viperina J.A. Purpus	*Mammillaria*	*Hydrochylus* K. Schum.	*Sphacelatae* D.R. Hunt	889906	AJ583232
M. carnea Zucc. ex Pfeiff.	*Mammillaria*	*Mammillaria*	*Polyedrae* (Pfeiff.) K. Schum.	928010	AJ583217
M. centralifera Repp.	*Mammillaria*	*Mammillaria*	*Mammillaria*	951035	AJ583220
M. gigantea Hildm. ex K.Schum.	*Mammillaria*	*Mammillaria*	*Mammillaria*	900376	AJ583223
M. lloydii Orcutt	*Mammillaria*	*Mammillaria*	*Leucocephalae* (Lem.) Salm-Dyck in Walp.	900447	AJ583222
M. melanocentra Poselg.	*Mammillaria*	*Mammillaria*	*Mammillaria*	961144	AJ583227
M. backebergiana Buchenau	*Mammillaria*	*Subhydrochylus* Backeb. ex D.R. Hunt	*Polyacanthae* (Salm-Dyck) K. Schum.	951031	AJ583213
M. conspicua J.A. Purpus	*Mammillaria*	*Subhydrochylus* Backeb. ex D.R. Hunt	*Elegantes* K. Schum.	950906	AJ583219
M. wiesingeri Boed.	*Mammillaria*	*Subhydrochylus* Backeb. ex D.R. Hunt	*Heterochlorae* (Salm-Dyck) K Schum.	951045	AJ583214

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Amplification of the *trn*L–*trn*F region

The *trn*L–*trn*F region was amplified by polymerase chain reaction (PCR) using the *trn*L–*trn*F forward and reverse primers (SANG et al. 1997; Table 2). PCR was performed with 50 ng genomic DNA in 20 µl reactions (Ready To Go PCR Beads, Amersham Bioscience) in a GeneAmp PCR System 9700 (Perkin Elmer) with a primer concentration of 50 µM. The PCR program is described in Table 3. PCR products were purified after gel separation on 1.8 % agarose gels using the Mini Elute Gel Extraction Kit (Qiagen) following the manufacturer's protocol, eluted in 10 µl H_2O and stored at -20 °C. PCR products were quantified in an agarose gel.

Sequencing

PCR fragments were sequenced directly following the cycle sequencing procedure with BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kits (Applied Biosystems) in a volume of 20 µl containing 100 ng DNA and 5 µM primer by using the same primer as for PCR amplification. In Table 4 the cycling parameters are shown. Cleaning of sequencing products by ethanol precipitation was followed by seperation and analysis on an automated analyser (ABI 310, Applied Biosystems). All sequences have been deposited into the EMBL database under the accession numbers listed in Table 1.

Alignment and phylogenetic analysis

Sequences were aligned with the software program ClustalW (THOMPSON et al. 1994) and corrected manually. Transitions (A ↔ G or C ↔ T) were weighted with a value of 0.5, mismatches with 0 and matches with 1. For deletions affine gap penalty was used. This function charges an initial penalty of 15 for opening a gap and then a lesser penalty of 5 per space for each additional space in the gap [d(g) = 15 + 5 g, g = number of spaces, d(g) = penalty of a deletion of the lenght g].

Phylogenetic analyses were performed by the neighbour-joining (NJ) method using the molecular evolutionary genetic analysis program TREECON (Version 1.3b, VAN DE PEER & DE WACHTER 1994). NJ analyses were conducted by calculating KIMURA's (1980) two-parameter distance (insertion/deletion in account). Sequences of the two outgroup taxa *Blossfeldia liliputana* Werderm. and *Eriosyce napina* (Phil.) Katt. were taken from the EMBL database (AY064324, AY015384). Bootstrap analyses with 1000 replicates were done by TREECON. The topology of the NJ tree was interpreted by the following categories of bootstrap support (ZOMLEFER et al. 2001): unsupported (<50 %), weak (50–74 %), moderate (75–84 %) and strong (85–100 %).

Results

The *trn*L–*trn*F intergenic spacer varied in length between 273 bp (*Mammillari rettigiana*) and 392 bp (*M. plumosa*). Sequences with the identical lenghts were found in different species of *Mammillaria*. For example, in *M. centralifera, M. lloydii, M. gigantea, M. longimamma* and *M. melanocentra* the spacers were 381 bp, in *M. conspicua*, *M. deherdtiana*, and *M. elongata* 385 bp long.

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Among the taxa of *Mammillaria* investigated, the sequence divergence was up to 8 %, found e.g. between *M. senilis* and *M. conspicua* or between *M. carnea* and *M. senilis*. Identical *trn*L–*trn*F sequences were found in *M. centralifera* and *M. melanocentra* which belong to same series within *M*. subg. *Mammillaria* sect. *Mammillaria* (Table 1). Similar sequences differing only by indels occurred in *M. plumosa* (subg. *Mammillaria* sect. *Hydrochylus*), *M. centralifera* (subg. *Mammillaria* sect. *Mammillaria*), *M. melanocentra* (subg. *Mammillaria* sect. *Mammillaria*), *M. pectinifera* (subg. *Mammillaria* section *Hydrochylus*) and *M. wiesingeri* (subg. *Mammillaria* sect. *Subhydrochylus*).

Among the investigated Mammillarias altogether eight indels (indel 1: 61–69 bp, indel 2: 82–88 bp, indel 3: 82–99 bp, indel 4: 85–89 bp, indel 5: 87–89 bp, indel 6: 125–134 bp, indel 7: 184–293 bp, indel 8: 263–272 bp), two substitution sites and three point mutations were found in the spacers.

A total of 402 nucleotide sites from *Mammillaria* and outgroup taxa were aligned. The NJ tree showed *M. mazatlanensis* (*subg. Mammillaria* sect. *Hydrochylus*) to be sister to all other species of *Mammillaria* investigated with strong bootstrap support of 87 % (Fig. 1). *Mammillaria viperina*, *M. senilis* and *M. rettigiana* formed a weakly supported clade in which *M. senilis* and *M. rettigiana* clustered with a bootstrap value of 89 %. A weakly supported branch contained *M. multiceps, M. longimamma*, *M. camptotricha*, *M. candida*, *M. beneckei*, *M. deherdtiana*, *M. conspicua*, and *M. elongata*. *Mammillaria beneckei* was opposed as sister to the other species, although bootstrap support was weak (64 %). Within this assemblage, a minor clade consisting of *M. deherdtiana*, *M. conspicua* and *M. elongata* received strong bootstrap support of 98 %.

Mammillaria melanocentra, *M. centralifera*, *M. lloydii*, *M. gigantea* and *M. wiesingeri* formed a weakly supported branch (62 % bootstrap value), but with *M. lloydii* and *M. gigantea* forming a strongly supported separate group (88 % bootstrap value).

Discussion

Main lineages within *Mammillaria*

According to our *trn*L–*trn*F sequence data, we found two weakly supported *Mammillaria* clades which comprised (1) most species of subgenus *Mammillaria* (sections *Mammillaria*, *Subhydrochylus*, *Hydrochylus*) and (2) the species of the other four subgenera (i.e., *Dolichothele*, *Mammilloydia*, *Oehmea*, *Mamillopsis*) including some species of subgenus *Mammillaria* (from the sections *Subhydrochylus* and *Hydrochylus*), respectively. Representatives of the subgenera *Dolichothele*, *Mammilloydia* and *Oehmea* are nested within species belonging to the subgenus *Mammillaria*, which corresponds to the findings of BUTTERWORTH & WALLACE (2004). In the latter study using *rpl*16 and *psb*A–*trn*H cpDNA sequence data, *M. longimamma* (subg. *Dolichothele*), *M. beneckei* (subg. *Oehmea*) and *M. candida* (subg. *Mammilloydia*), however, clustered with species of subg. *Mammillaria* sect. *Hydrochylus*, whereas subg. *Mammillaria* sections *Mammillaria* and *Subhydrochylus* were unified in a separate clade (termed clades F and Z in the parsimony and Bayesian trees, respectively).

The NJ tree (Fig. 1) shows that *M. candida* (subg. *Mammilloydia*) clusters within the genus *Mammillaria*, together with species of subgg. *Mammillaria* and *Dolichothele*.

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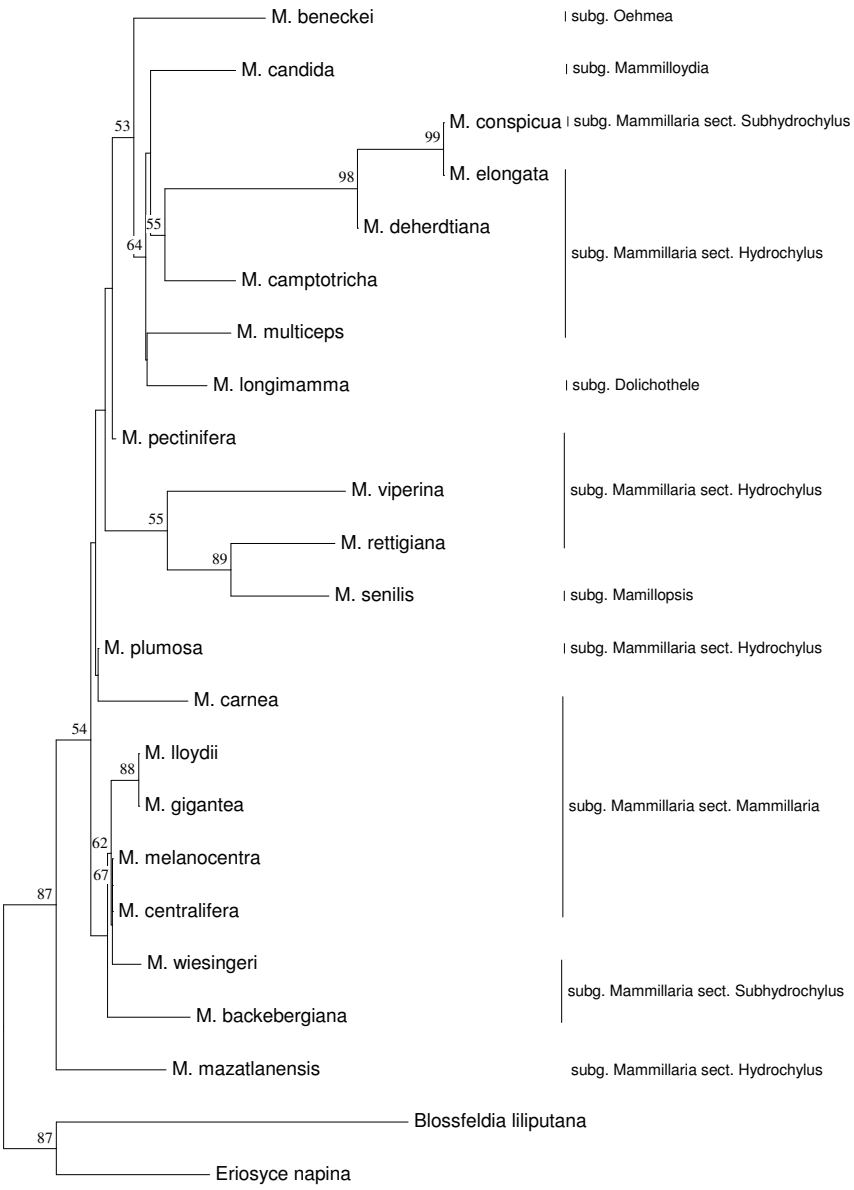

Fig. 1: Neighbour-joining tree of 21 *Mammillaria* species investigated and outgroups. Bootstrap analyses were conducted by TREECON version 1.3b (VAN DE PEER & DE WACHTER 1994) running 1000 replicates. Bootstrap values are given above the branches.

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This accords to the results of BUTTERWORTH & WALLACE (2004) and supports the inclusion of the segregate genus *Mammilloydia* Buxbaum under synonymy in the genus *Mammillaria*.

Mammillaria mazatlanensis (subg. *Mammillaria* sect. *Subhydrochylus*) is sister to the other species of *Mammillaria* studied. This topology has the highest bootstrap support in our analyses and corresponds well to the topology recovered by the broader sampling of BUTTERWORTH & WALLACE (2004) where *M. mazatlanensis* was nested within a basal clade of Mammillarias including the subg. *Cochemiea* and presumably related genera of *Mammillaria* (*Coryphantha*, *Escobaria*, *Ortegocactus* Alexander).

Mammillaria senilis (subg. *Mamillopsis*) and *M. rettigiana* (subg. *Mammillaria* series *Stylothelae*) are grouped together in the NJ tree with strong support. This corresponds basically to the topology recovered by BUTTERWORTH & WALLACE (2004) where *M. senilis* also grouped with members of the subg. *Mammillaria* ser. *Stylothelae* although species different from *M. rettigiana* had been investigated

The placement of all studied representatives of subg. *Mammillaria* sections *Mammillaria* and *Subhydrochylus* (except *M. conspicua*) within the same clade (Fig. 1) suggests a close relationship of both sections as apparent also from the *rpl*16 and *psb*A–*trn*H chloroplast DNA sequence data (BUTTERWORTH & WALLACE 2004). BUTTERWORTH & WALLACE (2004) argued for the additional application of the *trn*L–*trn*F spacer to resolve the deeper branches of *Mammillaria* in the phylogenetic tree. This marker appears to evolve more slowly, but the resolution in our data set is not high. Most sequence variation arises from indels. Nucleotide substitutions that may most unambiguously fortify the phylogenetic hypothesis are rather rare.

Intersectional hybridisation

The strongly supported placement of *M. conspicua* (subg. *Mammillaria* sect. *Subhydrochylus*) together with two representatives of subg. *Mammillaria* sect. *Hydrochylus*, viz. *M. elongata* und *M. deherdtiana* (Fig. 1), conflicts with the present classifications and probably with the results of BUTTERWORTH & WALLACE (2004). In the latter study, *M. haageana* Pfeiff. was sampled instead of *M. conspicua* and it grouped with the other members of subg. *Mammillaria* sect. *Subhydrochylus* as expected. *Mammillaria conspicua* und *M. haageana*, however, are highly similar and sometimes treated as subspecies under *M. haageana*. Perhaps, *M. conspicua* may be a hybrid between *M. haageana* s.str. or a related species from sect. *Subhydrochylus* and most likely *M. elongata* from sect. *Hydrochylus*. In this supposed hybridisation event *M. elongata* may have been the maternal parent. According to the usual maternal inheritance of chloroplasts in angiosperms this would explain the corresponding *trn*L–*trn*F sequences found in *M. conspicua* and *M. elongata* (Fig. 1). An alternative explanation, i.e. that the comparatively small taxon sampling in our study caused this arrangement of species in the tree, can be dismissed, because some taxa clustering between theses species in the cladograms of BUTTERWORTH & WALLACE (2004) were included also in the present study.

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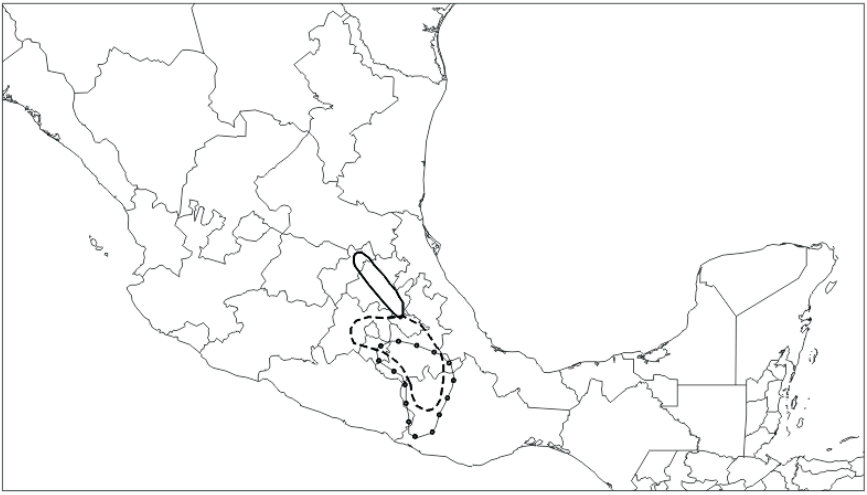

Fig. 2: Distribution of *Mammillaria elongata* (continuous line), *M. haageana* s.str. (dashed line), and *M. conspicua* (knotted line). Redrawn from PILBEAM (1999).

Biogeography of hybrids

This hypothesis, however, is not fully corroborated by biogeographical data. The three taxa in question are restricted to southern Mexico and occur at similar altitudes reported as 450–2550 m for *M. haageana* s.str., 600–2600 m for *M. conspicua* and 1350–2400 m for *M. elongata* (PILBEAM 1999). The horizontal distributions, however, are only partially overlapping, because *M. elongata* and *M. haageana* s.str. are clearly parapatric whereas the distributions of *M. conspicua* and *M. haageana* s.str. are almost congruent (Fig. 2). This is diametrically opposed to the assumption derived from our cpDNA results in which *M. elongata* was suspected as maternal parent of *M. conspicua*, because then *M. elongata* and *M. conspicua* would be expected to have similar ranges, instead of *M. haageana* s.str. and *M. conspicua*.

Conclusions

The chloroplast DNA *trn*L–*trn*F intergenic spacer tested for phylogenetic utility reveals only low level of variation within the genus *Mammillaria*. Thus, phylogenetic relationships among the 21 species of this genus studied are only poorly resolved. A possible reason might be the comparatively recent age of this genus, which on biogeographical reasons is dated after the collision of the north and south American continents some 7 mio years ago (COATES et al. 2004). Therefore, insufficient time might have elapsed to accumulate chloroplast DNA sequence variation suitable to identify different lineages.

Our parsimony-based analysis of *trn*L–*trn*F sequeces, however, yielded only partial support to current classifications of the genus *Mammillaria*, since the previously cir-

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cumscribed large subg. *Mammillaria* appears to be polyphyletic, the former genus *Mammilloydia* submerges within the genus *Mammillaria*, and the other subgenera held within the genus *Mammillaria* (i.e. subgg. *Dolichothele*, *Oehmea*) appear as derivatives of subg. *Mammillaria.* Therefore, we suggest to increase the sample size and to include more variable chloroplast and nuclear markers to get a well supported phylogeny of this large genus.

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