Molecular Phylogenetics and Evolution 65 (2012) 668–681

Contents lists available at SciVerse ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Molecular phylogeny and character evolution in terete-stemmed Andean opuntias (Cactaceae–Opuntioideae)

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ARTICLE INFO

Article history:
Received 22 November 2011
Revised 23 April 2012
Accepted 23 July 2012
Available online 2 August 2012

Keywords:
South America
Andes
Topological conflict
Nuclear ribosomal DNA
Secondary structure
Seed anatomy

ABSTRACT

The cacti of tribe Tephrocacteae (Cactaceae–Opuntioideae) are adapted to diverse climatic conditions over a wide area of the southern Andes and adjacent lowlands. They exhibit a range of life forms from geophytes and cushion-plants to dwarf shrubs, shrubs or small trees. To confirm or challenge previous morphology-based classifications and molecular phylogenies, we sampled DNA sequences from the chloroplast *trnK/matK* region and the nuclear low copy gene *phyC* and compared the resulting phylogenies with previous data gathered from nuclear ribosomal DNA sequences. The here presented chloroplast and nuclear low copy gene phylogenies were mutually congruent and broadly coincident with the classification based on gross morphology and seed micro-morphology and anatomy. Reconstruction of hypothetical ancestral character states suggested that geophytes and cushion-forming species probably evolved several times from dwarf shrubby precursors. We also traced an increase of embryo size at the expense of the nucellus-derived storage tissue during the evolution of the Tephrocacteae, which is thought to be an evolutionary advantage because nutrients are then more rapidly accessible for the germinating embryo. In contrast to these highly concordant phylogenies, nuclear ribosomal DNA data sampled by a previous study yielded conflicting phylogenetic signals. Secondary structure predictions of ribosomal transcribed spacers suggested that this phylogeny is strongly influenced by the inclusion of paralogous sequence probably arisen by genome duplication during the evolution of this plant group.

1. Introduction

Cacti have fascinated botanists since the discovery of the Americas in the 15th century. Botanists and gardeners have paid much attention to their remarkable evolution of succulence and their large colourful flowers. But whereas cactus enthusiasts have generally focused their interest on the smaller-growing members of the very diverse subfamily Cactoideae (containing approximately 80% of cactus species), the subfamily Opuntioideae has not enjoyed the same attention. The subfamily Opuntioideae is widespread throughout the Americas from Canada to southern Patagonia. It has traditionally been recognised as a monophyletic taxonomic entity (Anderson, 2001; Backeberg, 1966; Britton and Rose, 1919; Hunt et al., 2006; Schumann, 1897–1898; Stuppy, 2002). It is characterised by a number of synapomorphies: (1) presence of glochids: small, deciduous barbed spines (Robinson, 1974); (2) woody funicular tissue surrounding the seed ('funicular envelope', Stuppy, 2002); (3) high amounts of calcium oxalate monohydrate druses and monoclinic cluster crystals in the outer hypodermis of stems (Gibson and Nobel, 1986; Hartl et al., 2007), and (4) polyporate pollen grains with peculiar exine structures (Leuenberger, 1976).

Molecular phylogenetic investigations support the monophyly of the Opuntioideae (Bárcenas et al., 2011; Edwards et al., 2005; Griffith and Porter, 2009; Hernández-Hernández et al., 2011; Nyffeler, 2002; Wallace and Dickie, 2002) but the sister group relationship to one of the other subfamilies of the Cactaceae remains unclear (Bárcenas et al., 2011; Hernández-Hernández et al., 2011; Nyffeler, 2002). Traditional classifications of the Opuntioideae based on gross morphology have treated *Opuntia* (L.) Mill. itself as a large genus of up to 200 species, subdivided into infrageneric units (Barthlott and Hunt, 1993; Britton and Rose, 1919; Endler and Buxbaum, 1974; Rowley, 1958; Schumann, 1897–1898), or independent genera (Backeberg, 1958–1962). Current classifications recognise about 15 genera (Anderson, 2001, 2011; Hunt

Abbreviation: nrITS, nuclear ribosomal transcribed spacer.

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http://dx.doi.org/10.1016/j.ympev.2012.07.027

et al., 2006; Nyffeler and Eggli, 2010; Stuppy, 2002) arranged in up to five tribes (Doweld, 1999; Wallace and Dickie, 2002).

In the present study we focus on the South American spherical to terete-stemmed Opuntioideae of the tribe Tephrocacteae sensu Hunt (2011), which consists of the genera *Austrocylindropuntia* Backeb., *Cumulopuntia* F.Ritter, *Maihueniopsis* Speg., *Punotia* D.R.Hunt, *Pterocactus* K.Schum. and *Tephrocactus* Lem. (Fig. 1). Thus, Tephrocacteae in its broader circumscription sensu Hunt (2011) include the tribes Austrocylindropuntieae Wallace & Dickie and Pterocacteae Doweld. These genera develop many different life forms ranging from small geophytes, hemispherical cushion-plants, dwarf shrubs, shrubs and columnar cacti consisting of either indeterminate branches (in *Austrocylindropuntia*) or determinate terete or spherical segments. Although some of the genera are closely similar morphologically, seed anatomical structures provide diagnostic characters to differentiate them (Stuppy, 2002).

The phylogenetic relationships within the Tephrocacteae and its position within the Opuntiodeae are not yet firmly understood. In the molecular phylogenetic studies of Bárcenas et al. (2011) and Hernández-Hernández et al. (2011), the Tephrocacteae formed a polytomy with the two major clades of Opuntioideae: the terete stemmed Opuntioideae (Cylindropuntieae sensu Hunt et al.,

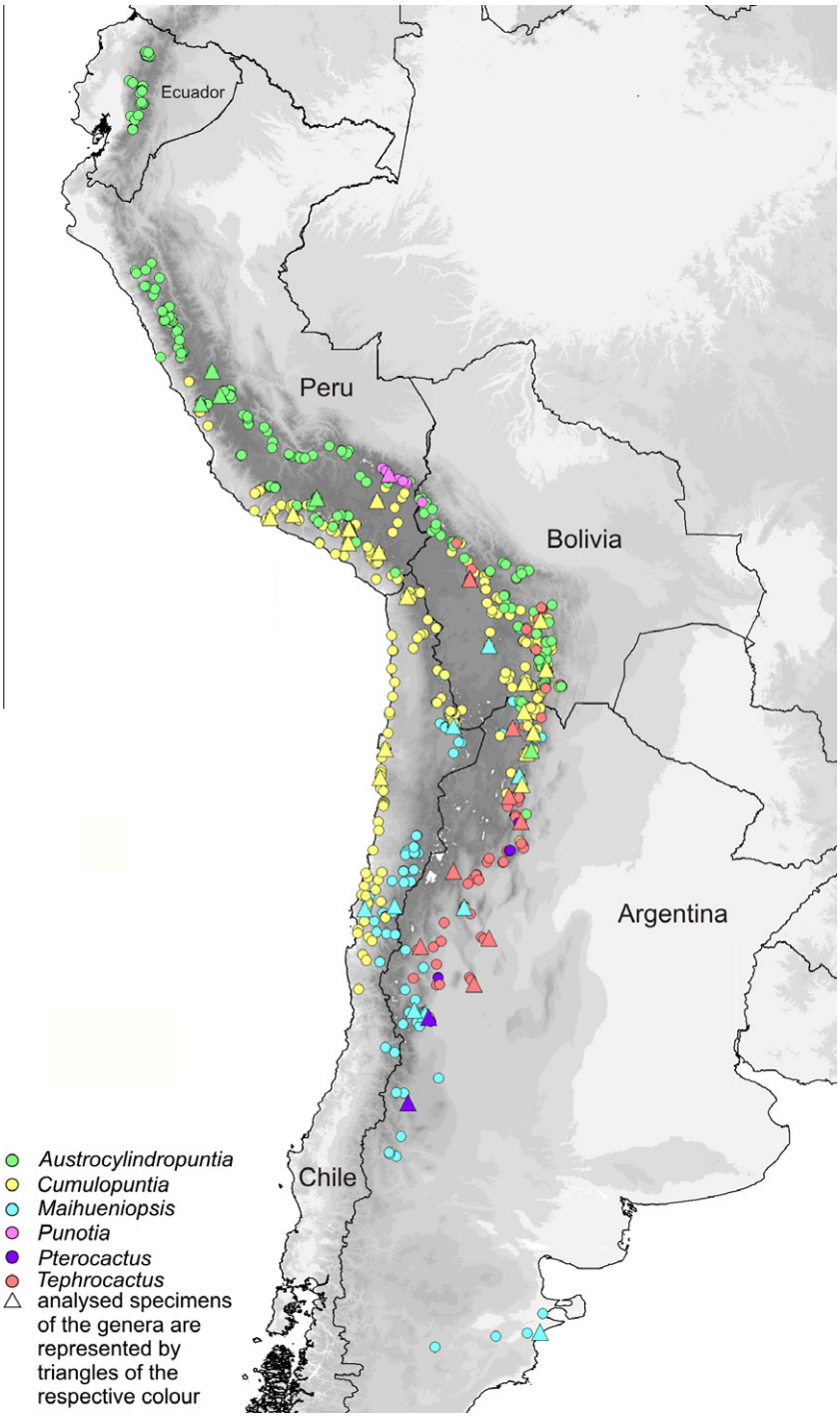

Fig. 1. Distribution area of the genera of the tribe Tephrocacteae in Southern South America. Localities known to the authors are presented as dots, localities of specimens analysed during this study are presented as triangles.

2006) and the mainly flat-stemmed Opuntioideae (Opuntieae sensu Hunt et al., 2006). However, the phylogeny presented by Griffith and Porter (2009) indicated extensive polyphyly of the genus *Maihueniopsis* because some species clustered with the flat- or terete-stemmed Opuntioideae or were sister to all other Opuntiodeae.

The phylogeny from Griffith and Porter (2009) was based on a combined data set of the chloroplast *trnL-trnF* intergenic spacer and the nuclear ribosomal internal transcribed spacer sequences (*nrITS*). In this study the chloroplast DNA sequences were much less variable than the *nrITS* sequences (72% of variable sites in the combined alignment). Thus, the *nrITS* data obviously strongly influenced the topology of the resulting tree. Nuclear *rITS* data have been widely used for phylogenetic reconstructions, especially at infrageneric level e.g. (Baldwin et al., 1995; Bruyns et al., 2006; Martins, 2006; von Hagen and Kadereit, 2001), because the high copy numbers of the tandemly repeated ribosomal DNA arrays in the nucleolar organiser region (NOR) facilitates its amplification. Variation between individual *nrITS* copies is usually rapidly homogenised by unequal cross overs and gene conversion described as concerted evolution (Bailey et al., 2003; Buckler and Holtsford, 1996; Eickbush and Eickbush, 2007). However, several studies, including some within Cactaceae, have demonstrated that intra-individual *nrITS* polymorphisms originating by gene or genome duplication (paralogous genes) persist because mechanisms of sequence homogenisation are retarded or lacking (Harpke and Peterson, 2006, 2007, 2008b; Hartmann et al., 2001, 2002). Some of the paralogous copies might become non-functional (pseudogenes) and often evolve at higher mutation rates, which can be reflected by a lower GC-content of the sequences and less stable RNA secondary structures (Harpke and Peterson, 2006, 2008b). The evolution and persistence of paralogous loci may thus result in erroneous species trees, if incompletely sampled, but may also provide the opportunity to identify ancient paralogs or to unravel the hybridogenic origin of a taxon when homeologs can be traced in parental species (Alvarez and Wendel, 2003; Bailey et al., 2003).

The aim of our study was to disentangle the phylogenetic relationships between the Tephrocacteae sensu Hunt (2011). We therefore sequenced the chloroplast *trnK/matK* region and the nuclear low copy gene *phyC* to reconstruct molecular phylogenies. Using ancestral character state reconstructions of morphological traits, we traced the evolution of different life-forms and anatomical structures of seeds within the Tephrocacteae. We closely examined *nrITS* sequences published by Griffith and Porter (2009) by analysing their GC-content and their secondary structure to assess whether the *nrITS* based phylogeny represents or fails to represent a species tree because of paralogous *nrITS* sequences.

2. Material and methods

2.1. Plant material

We analysed 45 taxa of the Tephrocacteae sensu Hunt (2011): we sampled five species out of six species of *Austrocylindropuntia*, 10 species out of 10 species of *Cumulopuntia*, 11 species out of 12 species of *Maihueniopsis*, three species out of nine species of *Pterocactus*, the monotypic genus *Punotia* and nine species out of nine species of *Tephrocactus*. We followed the nomenclature of Hunt et al. (2006) and Hunt (2011). Taxonomic adjustments resulting from this study have separately published by Hunt (2011). The plant material used was taken from vegetatively propagated specimens in documented collections held in Europe including the Royal Botanic Gardens Kew (Table S1). Specimens of investigated plants were deposited in the spirit collection of the Royal Botanic Gardens Kew (K). In accordance with previous studies (Bárcenas et al., 2011; Griffith, 2002; Hernández-Hernández et al., 2011; Nyffeler, 2002) we used sequences of *Portulaca oleracea* L., *Maihuenia patagonica* Britton & Rose, *Pereskia grandifolia* Pfeiff., *Pereskiopsis* spp. Britton & Rose, *Opuntia quimilo* K.Schum., *O. sulphurea* G.Don., *Brasiliopuntia brasiliensis* (Willd.) A.Berger and *Tunilla* spp. D.R.Hunt & Iliff as outgroups. Sampling details and GenBank accession numbers are presented in Table S1 in the Supplementary material.

2.2. DNA Extraction, sequence isolation

DNA was extracted using Qiagen Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions but all centrifugation steps were performed at 14,000 rpm.

The *trnK* intron including the *matK* gene (*trnK/matK* region) was amplified in 25 µl reactions using the primers trnK-3914F and trnK-2R (Johnson and Soltis, 1994). The reaction mixture contained: 2.5 µl 10-fold concentrated polymerase buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, and 0.8% (v/v) Nonidet (P40), 2.5 µl $MgCl_2$ (25 mM), 2.5 µl dNTPs (2 mM), 1 µl of each primer (10 µM), 1 unit of *Taq* polymerase (MBI Fermentas, St. Leon-Rot, Germany), and 1 µl DNA template from diluted extracts (app. 50 ng DNA). The amplification protocol started with an initial denaturation of 120 s at 94 °C, after which 35 cycles were performed each consisting of 30 s of denaturation at 94 °C, 45 s of annealing of 49.6 °C, and 180 s of extension at 72 °C and ended with a final extension of 180 s at 72 °C.

Amplification of the exon 1 region of *phyC* followed the same protocol as for the *trnK/matK* region but primers were taken from Helsen et al. (2009) and annealing temperature was set to 55 °C. Purified PCR products (Qiaquick Gel extraction Kit, Qiagen, Hilden, Germany) were cloned into vector pJet1 (CloneJet PCR cloning Kit, Thermo Fisher Scientific, Schwerte, Germany). Ligation products were electroporated into *E. coli* DH5α.

PCR products purified with ExoSAP-IT Kit (Affymetrix, Santa Clara, CA, USA) or plasmids of five positive clones per taxon were sequenced by the company Macrogen (South Korea, Seoul) using the same primers as for amplification and in case of the *trnK/matK* region with additional internal primers trnk-23F and trnK-71R (Nyffeler, 2002).

2.3. Phylogenetic analyses

Sequences of the *trnK/matK* region and *phyC* gene were aligned manually. Alignment of the *nrITS* data set (*nrITS-1*, *2* and 5.8S rDNA) was taken from Griffith and Porter (2009). The best fitting nucleotide substitution models for the *phyC* and the *trnK/matK* region (the *matK* gene within the *trnK* intron was analysed as separate partition) were estimated with MrModeltest v. 2.3 according to the corrected Akaike Information Criterion (Table 1, Nylander, 2004). The resulting model parameters were employed to reconstruct four phylogenies based on different data sets: (1) *trnK/matK* region, (2) *phyC*, (3) *nrITS* and (4) sequences combined *trnK/matK* region and *phyC* sequences.

The alignment of combined markers was pruned to taxa sequenced for both markers; we randomly chose one of the *phyC* sequences but excluded sequences which were apparently not orthologous (*C. boliviana* ssp. *ignescens* O-08/1-3, 5; *C. chichensis* O-13/2-3; *Maihueniopsis hickenii* O-22/2, 8 and *Tunilla orurenis* O-05/2). For *Pereskiopsis* we combined the chloroplast sequence of *P. diguetii* and *P. aquosa*. We controlled for combinability of *trnK* and *phyC* data sets using incongruence length difference (ILD) test (Farris et al., 1995) implemented in Paup 4.0b10 (Swofford, 2002) employing a heuristic search with TBR branch swapping, 1,000 partition replicates, each with 10 random sequence addition replicates and a maximum of 500 saved trees.

Table 1
Sequence information for the different sequence data sets.

Alignment characteristics	Genes			
	trnK/matK region	*phyC*	*nrITS* region	Combined *trnK/matK* and *phyC*
No. of taxa	54	40	29	40
No. of sequences	62	155	41	41
Range of sequence lengths	2374–2501	815–938	573–589	–
Alignment length	2530	957	622	3487
Informative positions within the ingroup	122	116	42	171
Constant positions within the ingroup	2326	674	518	3160
Nucleotide substitution model[a]	HKY + G (GTR + G)	HKY + G	GTR + G	

[a] Best-fit model according to Akaike information Criterion as implemented in MrModeltest (Nylander, 2004).

We ran all analyses with MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001) with two simultaneous runs over 10,000,000 generations, sampling every 100th generation and discarding the first 25,000 trees as burn-in resulting in a 50% majority rule consensus tree showing all compatible partitions supported by posterior probabilities (PP) for each node. Additionally, we calculated Maximum Likelihood Bootstrap percentages using RaxML (Stamatakis, 2006). We ran 1000 replicates of standard bootstrap using the model GTR + G.

2.4. Morphological traits and character state reconstruction

For this we used the phylogeny based on the alignment of combined sequences because statistical support was highest in this tree and the ILD test did not reject homogeneity between partitions ($P = 0.12$). The analyses were done using the Markov-k-state 1 parameter model (Maddison and Maddison, 2006) implemented in the Mesquite package (Maddison and Maddison, 2010). We reconstructed ancestral character states of seven morphological characters observed in living material or (seed characters) taken from Stuppy (2002). We assigned only unambiguous character states; in case several character states were observed in one species, we treated them as unknown. The following traits were scored: (1) roots (fibrous; tuberous (including taproots formed by root tissue only or from root and hypocotyls tissue); (2) growth mode (indeterminate, determinate); (3) life form (tree; columnar; shrub; dwarf shrub; cushion; geophytes); (4) leaf rudiments (persistent; caducous); (5) pericarp (juicy, indehiscent; dry dehiscent); (6) shape of embryo and amount of perisperm (circular embryo curved around large perisperm; hook-shaped embryo curved around reduced perisperm; hook-shaped embryo curved around very strongly reduced perisperm; spirally enrolled embryo bent around strongly reduced perisperm); (7) anatomy of the funicular envelope (all cells of the funicular envelope orientated parallel to the funicular girdle; cells subtending the funicular girdle transversely orientated and continuing on the insides of the flanks; cells subtending the funicular girdle transversely orientated and continuing on the outsides of the flanks; inner layers of the funicular envelope sclerenchymatous, outer layers and funicular girdle aerenchymatous; cells subtending the funicular girdle without distinct orientation). We analysed characters for species of the ingroup only, and for *Brasiliopuntia brasiliensis*, *Tunilla orurenis* and *Pereskiopsis* sp.

2.5. GC-content and secondary structure models of nrITS

In order to assess the presence of pseudogenes within the *nrITS* data set taken from Griffith and Porter (2009) we determined the GC-content of *nrITS* sequenced using Paup v. 4.0b10 (Swofford, 2002). Minimum free energy secondary structures of *nrITS-1* and *nrITS*2 were predicted with the program Mfold (Zuker, 2003) using default parameters. Resulting *nrITS-2* structures were compared to conserved structures within green algae and flowering plants (Mai and Coleman, 1997). Nuclear ribosomal ITS regions were checked for the occurrence of conserved sequence motifs: one motif within *nrITS*-1 (Liu and Schardl, 1994), six motifs in *nrITS-2* (Hershkovitz and Zimmer, 1996), the consensus sequence from (Harpke and Peterson, 2006), three motifs in 5.8S RNA (Harpke and Peterson, 2008a; Jobes and Thien, 1997).

3. Results

3.1. Phylogenetic reconstructions

Sequences of the *trnK/matK* region were 2374–2501 bp long, those of the *phyC* gene were 815–938 bp long and the variability of both markers was high within the ingroup (Table 1). Intraindividual sequence polymorphism among *phyC* copies obtained from one species ranged from 2 to 25 bp (mean = 10 bp). In four taxa we found apparently paralogous alleles, which differed by 56 bp in *Maihueniopsis hickenii* (O-22), 26 bp in *Cumulopuntia boliviana* ssp. *ignescens*, 18 bp in *C. chichensis* and 12 bp in *Tunilla orurensis*.

The phylogenies based on the *trnK/matK* region and the *phyC* gene indicated the same major clades (Figs. 2 and 3) but relationships between them were only supported by the combined analysis (Fig. 4). The topology of these three trees differed largely to that of the *nrITS* based tree (Fig. 5, see below). The ingroup was strongly supported by the *trnK/matK* region and the combined data set (1.00 PP, 96% BS; 1.00 PP, 96% BS, respectively) and weakly supported by *phyC* phylogeny (0.92 PP, 62% BS). The sister group relationship between *Maihueniopsis* and *Pterocactus* was only supported by the analyses based on combined sequences (1.00 PP, 92% BS; Fig. 4). *Maihueniopsis clavarioides*, which is the nomenclatural type of the small genus *Puna* R.Kiesling, was sister to the remaining species of *Maihueniopsis* in all phylogenies, except in the *nrITS* based one. Three species formerly assigned to different genera were nested within *Tephrocactus: Tephrocactus verschaffeltii*, formerly treated as *Austrocylindropuntia*; *T. recurvatus* formerly referred to *Cumulopuntia*, and *T. bonnieae* formerly assigned to *Puna*. (Figs. 2–4). The genera *Austrocylindropuntia* and *Cumulopuntia* including both subspecies of *C. subterranea* (formerly assigned to *Puna*), and *Punotia lagopus* (formerly treated as *Austrocylindropuntia lagopus*) were closely related (Figs. 2–4). In the *phyC* based tree these genera did not form monophyletic groups because some *phyC* copies of *Cumulopuntia boliviana* ssp. *ignescens* and *C. chichensis* appeared as unsupported sister group to the remaining species (Fig. 3). We also sequenced two *phyC* copies of *Maihueniopsis hickenii*, which did not cluster within the *Maihueniopsis* clade but with *Austrocylindropuntia shaferi* and *A. vestita* (O-22/4) and with *Tephrocactus alexanderi* (O-22/8). We also detected one *phyC* copy of *Tunilla orurensis* (O-05/2) within the *Maihueniopsis* clade (Fig. 3).

In accordance with the tree based on combined *nrITS* and *trnL-trnF* intergenic spacer sequences published by Griffith and Porter (2009) the phylogeny based on these *nrITS* sequences only

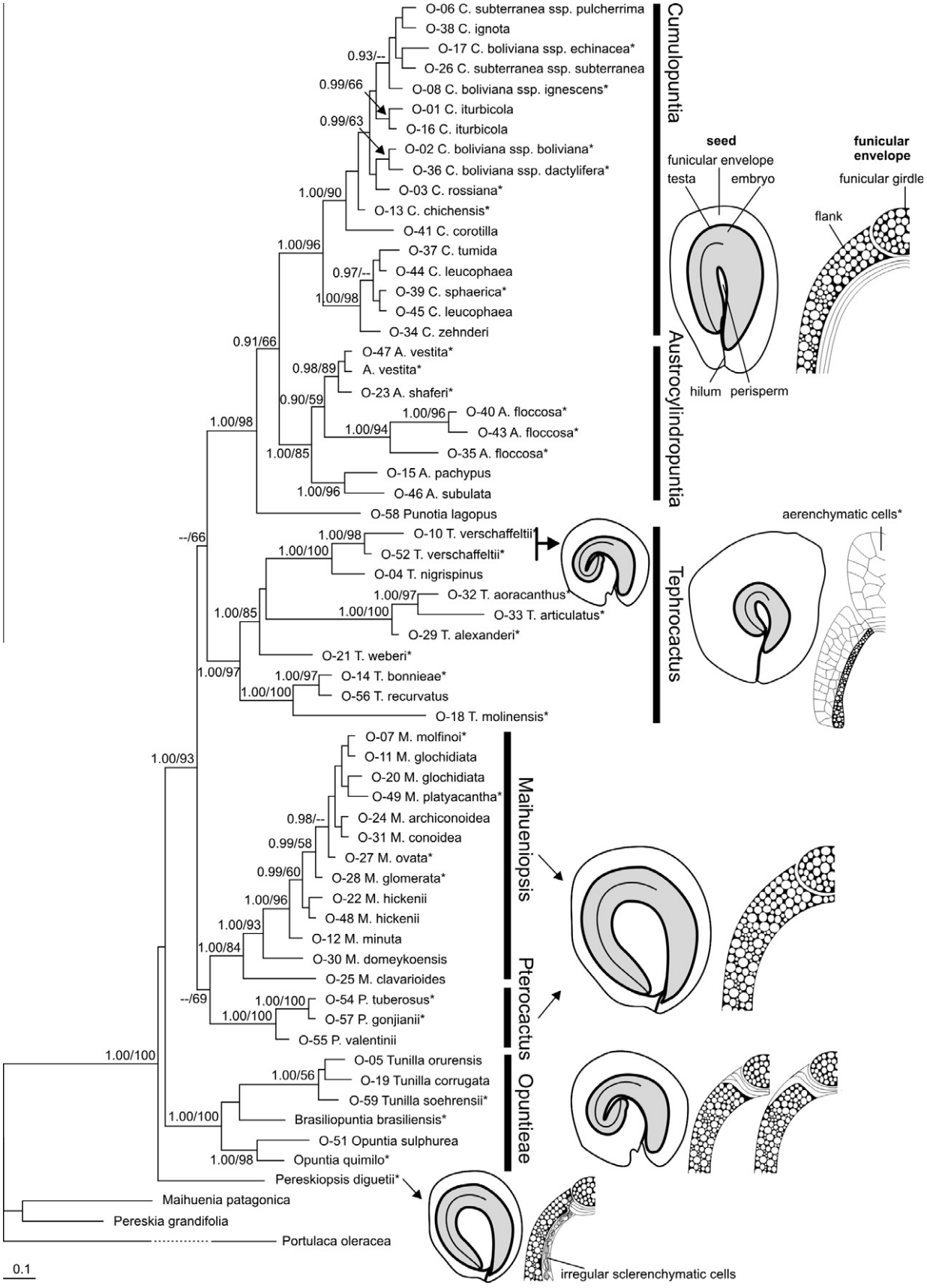

Fig. 2. Bayesian phylogeny based on the *trnK/matK* region of the chloroplast DNA. Posterior probabilities (PP) higher than 0.9 and Maximum Likelihood Bootstrap percentages (BS) higher than 70% are given above branches (PP/BS). The branch leading to *Portulaca oleracea* is not presented in the original scale of length. Seeds drawn after Stuppy (2002) are presented right to each clade. Seed structures were observed in species marked with an asterisk (Stuppy, 2002). The left scheme represents a longitudinal section of an opuntioid seed (principal structures are exemplified by the seed structures of *Cumulopuntia* and *Austrocylindropuntia*). The right scheme illustrates a cross section through the funicular envelope. Circles represent longitudinally elongated cells, lines represent crosswise elongated cells. Tissues marked with an asterisks are typical for *Tephrocactus* but were not observed in *T. nigrispinus*, *T. verschaffeltii* and *T. recurvatus* (Stuppy, 2002; Gilmer and Thomas, 2000; Stuppy pers. comm.).

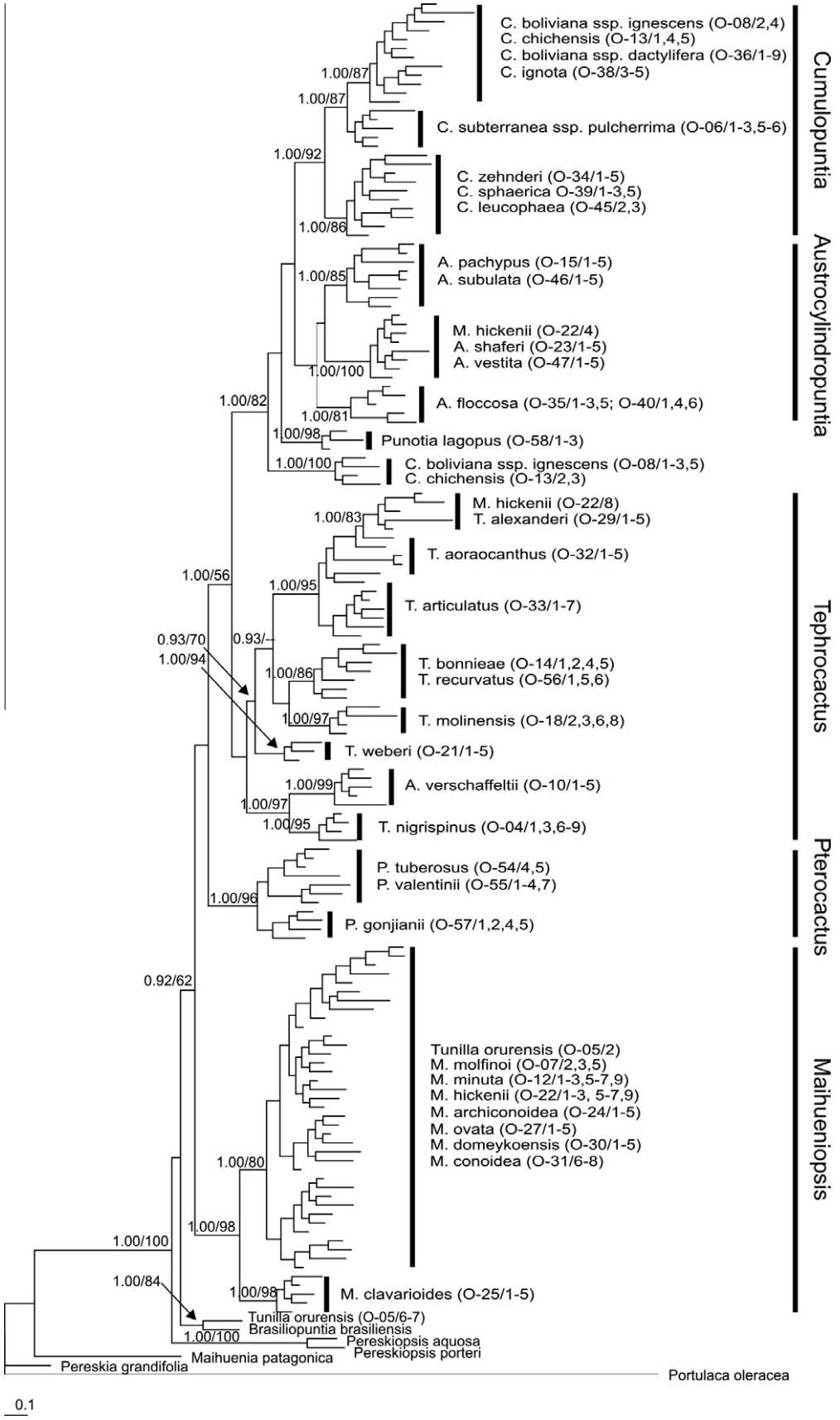

Fig. 3. Bayesian phylogeny based on exon 1 region of the *phyC* gene encoded by nuclear DNA. Posterior probabilities (PP) higher than 0.9 and Maximum Likelihood Bootstrap percentages (BS) higher than 70% are given above branches (PP/BS). Statistical support for copies of the same species within each clade is not shown.

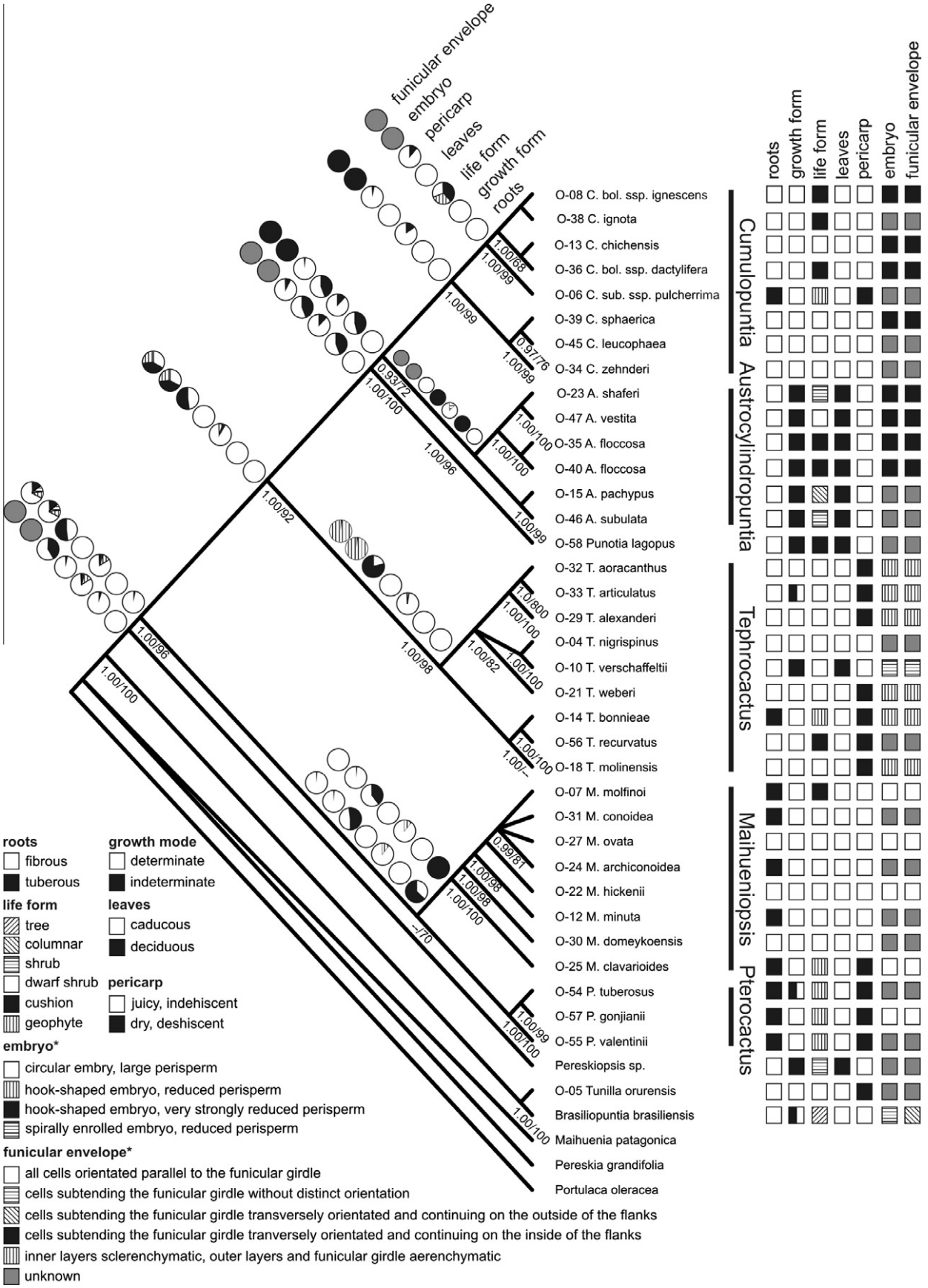

Fig. 4. Bayesian phylogeny based on combined sequences of the *trnK/matK* region and *phyC* exon 1 region. Posterior probabilities (PP) higher than 0.9 and Maximum Likelihood Bootstrap percentages (BS) higher than 70% are given below branches (PP/BS). Character states of seven morphological characters are present in boxes right of the taxa. Estimates of ancestral character states for internal nodes of the tree are presented in pie charts. Characters marked with an asterisk are taken from Stuppy (2002). Abbreviations of species names: C. bol. = *Cumulopuntia boliviana*; C. sub. = *Cumulopuntia subterranea.*

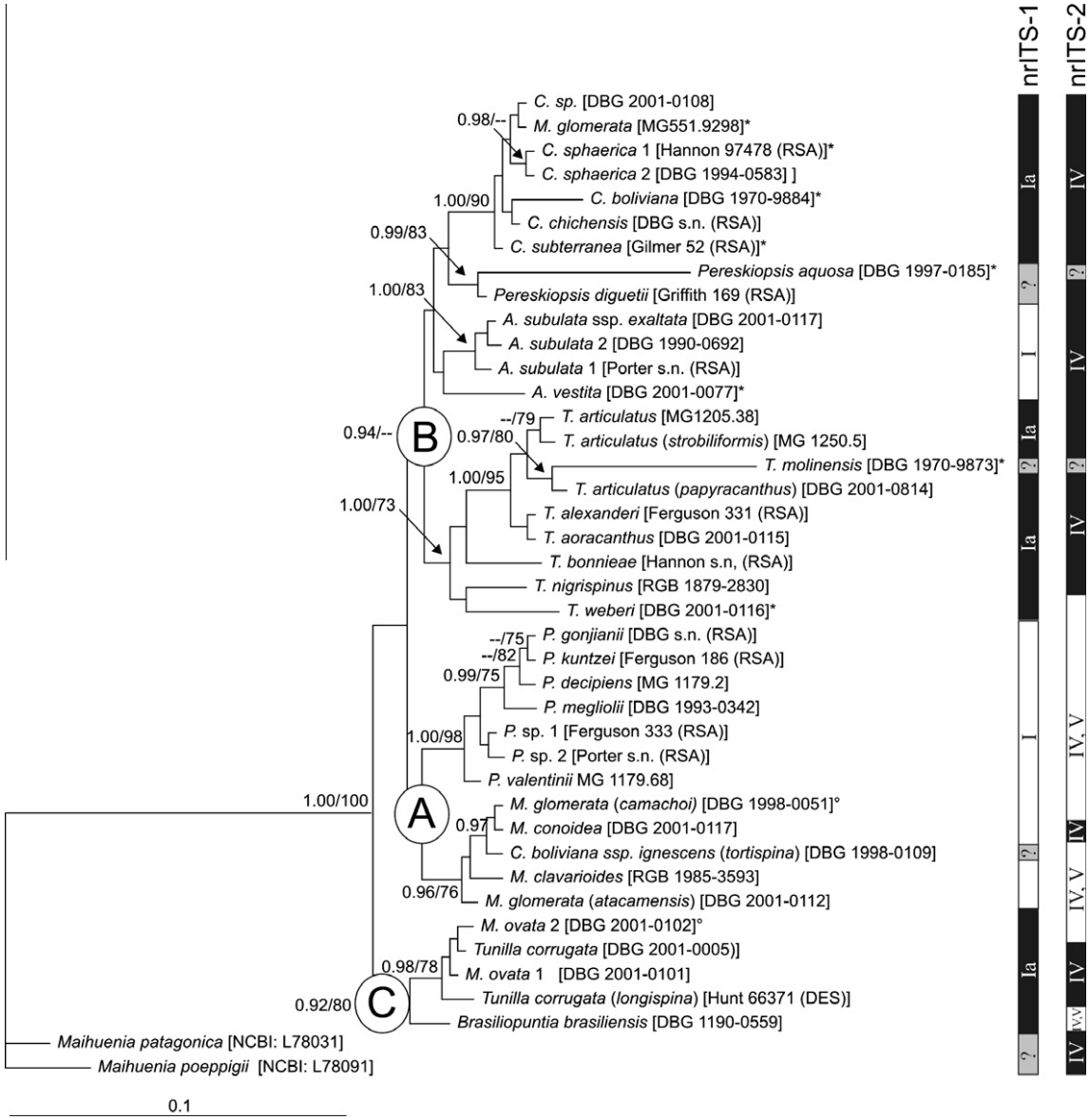

Fig. 5. Bayesian phylogeny based on *nrITS* region (*nrITS*-1, 2, 5.8SrDNA) re-analysed from the study of Griffith and Porter (2009). Posteriori probabilities (PP) higher than 0.9 and Maximum Likelihood Bootstrap percentages (BS) higher than 70% are shown above branches (PP/BS). Genera were abbreviated by first letter: A. = *Austrocylindropuntia*, C. = *Cumulopuntia*, M. = *Maihueniopsis*, P. = *Pterocactus*, T. = *Tephrocactus*. Original voucher information for each sequence is presented in rectangular brackets. Sequences that contained mismatches to conserved motifs within *nrITS-2* and 5.8S rRNA were marked with an asterisk (∗) or circle (°), respectively. Columns right of the phylogeny present data from secondary structure predictions of *nrITS-1* and *nrITS-2*. Within *nrITS-1* structures we found two major types, one containing helix II and the other containing helix Ia (Table S2, Fig. 6). Within *nrITS-2* we also observed two main structures: one type consists of four helices and the second type of five helices (Table S2, Fig. 6). A question mark is assigned to structures, which could not be confined to one of these types.

contained three major clades (A–C), that were not congruent with the clades in Figs. 2–4, because *Pereskiopsis* was sister to *Cumulopuntia* and the genus *Maihueniopsis* was polyphyletic (Fig. 5).

3.2. Reconstruction of morphological traits

Assignment of the morphological traits of each species and results from the reconstruction of ancestral character states are presented in Fig. 4. Thus, according to our analyses, the ancestor of the Tephrocacteae was probably a dwarf shrub (0.84 PP) with determinate shoot segments (1.00 PP), caducous leaves (1.00 PP) and fibrous roots (0.98 PP). The ancestors of *Tephrocacteae* had probably a curved embryo with large amounts of perisperm (0.68 PP), and the cells of the funicular envelope were orientated parallel to the funicular girdle (0.69 PP; i.e. parallel to the funicular vascular bundle that runs inside the girdle; see also sketches of seed structures of *Maihueniopsis* and *Pterocactus* in Fig. 2). Reconstructions also imply that tuberous roots originated independently in the *Maihueniopsis–Pterocactus* clade, in *Tephrocactus* and in *Cumulopuntia*.

3.3. Structural analysis of the nrITS region

Sequence lengths of *nrITS-1* and *nrITS-2* taken from Griffith and Porter (2009) were not substantially variable and ranged within the ingroup from 188 to 191 bp and from 221 to 227 bp, respectively. Sequence variability within the ingroup was low (app. 3%, Table 1); but sequences of *Tephrocactus molinensis* and *Pereskiopsis aquosa* deviated app. 7% from the other *nrITS* sequences. Mean and standard deviation of the GC-content was 0.68 ± 0.01 for both

nrITS-1 and *nrITS-2*. The lowest GC-content was observed in *Tephrocactus molinensis* (0.66 for *nrITS-1* and *nrITS-2*; Table S2 in the Supplementary material).

We found no deviation from the conserved *nrITS-1* motif GCCRY-(4-7n)-GYGYCAGGAA published by Liu and Schardl (1994). We detected mismatches in four of six conserved motifs within the *nrITS-2* region (Harpke and Peterson, 2008b; Hershkovitz and Zimmer, 1996; Table S3 in the Supplementary material). One of three conserved motifs within the 5.8S region differed from published consensus sequences (Harpke and Peterson, 2008a; Jobes and Thien, 1997, Table S3).

Secondary structure models resulted in mean free energy values of -78.78 ± 3.52 for *nrITS-1* and -109.47 ± 4.79 for *nrITS-2* (Table S3). The highest values for *nrITS-1* were observed in *T. articulatus* (*papyracanthus*) and *T. articulatus* (*strobiliformis*; −70.33 kcal/mol) and for *nrITS-2* in *T. molinensis* (−95.65 kcal/mol). Within secondary structures of *nrITS-1*, we detected two major types: one consisting of the helices I, II, III and the other of helices Ia, II, III, with the substructure Ia consisting of two smaller helices (Table S3, Fig. 6). Sequences forming helix II are confined to clade A and to the genus *Austrocylindropuntia* in the *nrITS* based phylogeny (Fig. 5). Secondary structures containing helix Ia were found in clade C, and in the genera *Cumulopuntia* and *Tephrocactus* (Fig. 5). The secondary structure of *Tephrocactus molinensis* was very dissimilar to all other investigated structures and did not contain one of the detected helices (data not shown). Nuclear *rITS-2* sequences formed structures with four or five helices, which were concordant with common angiosperm structures (Fig. 6, Mai and Coleman 1997). Except for *Brasiliopuntia brasiliensis* and *Maihueniopsis ovata* 2, *nrITS-2* sequences forming five helices were found in clade A in the *nrITS*-based phylogeny (Fig. 5). Nuclear *rITS-2* structures of *Tephrocactus molinensis* and *Pereskiopsis aquosa* could not be confined to one of the found *nrITS-2* types (data not shown).

4. Discussion

4.1. Relationships and evolution of morphological traits within Tephrocacteae

Our results and those of others studies clearly imply that the genera *Austrocylindropuntia*, *Punotia*, *Cumulopuntia*, *Tephrocactus*, *Pterocactus* and *Maihueniopsis*, now circumscribed as tribe Tephrocacteae (Hunt, 2011) represent a distinct lineage from the remaining terete-stemmed opuntias (tribe Cylindropuntieae sensu Hunt et al., 2006), and the mainly flat-stemmed genera (tribe Opuntieae sensu Hunt et al., 2006; Bárcenas et al., 2011; Edwards et al., 2005; Hernández-Hernández et al., 2011; Wallace and Dickie, 2002). Within the Tephrocacteae, we detected four strongly supported clades representing the genera (1) *Pterocactus*, (2) *Maihueniopsis*, (3) *Tephrocactus* and (4) *Punotia*, *Cumulopuntia* and *Austroclindropuntia* (Figs. 2–4).

Reconstruction of morphological character states implies that the morphological traits that have been traditionally considered as plesiomorphic in the Opuntioideae, are not ancestral within the Tephrocacteae. Wallace and Dickie (2002) assumed that precursors of Opuntioideae shared many characters with *Austrocylindropuntia* because they interpreted its indeterminate growth and the persistent leaves as plesiomorphic analogous to the ancestral position of the leafy trees of the genus *Pereskia* (Britton and Rose, 1919; Edwards et al., 2005). Our results suggest that *Austrocylindropuntia* and *Cumulopuntia* are sister to *Tephrocactus* and the ancestors of them probably developed determinate segments and caducous leaves (Fig. 4). Within *Punotia*, *Austrocylindropuntia* and *Cumulopuntia* a stepwise reduction of the size of leaf rudiments is observed. *Punotia* and *Austrocylindropuntia* have conspicuous leaves (reaching 7 cm or more in length in *A. subulata*), which persist for at least one growing season whereas leaves of *Cumulopuntia* are vestigial and soon caducous. The loss and regain of leafiness occurred evidently several times independently in Opuntioideae (Edwards and Donoghue, 2006; Griffith, 2004). These findings are supported by the observation that rudimentary leaves are present at early developmental stages in many species of subfamily Cactoideae (Mauseth, 2007).

Different life forms are very homoplastic within the Tephrocacteae (Fig. 4). Geophytes evolved independently in all major clades (Fig. 4) from ancestors that were probably dwarf shrubs. The geophytes *Maihueniopsis clavarioides*, *Tephrocactus bonnieae* and *Cumulopuntia subterranea* are not closely related (Figs. 2–4) and so cannot be grouped together as the separate genus *Puna* (Kiesling, 1982). Though *Maihueniopsis* and *Cumulopuntia* are not very closely related, their morphological and ecological similarities are striking; both form dwarf shrubs or dense cushions with determinate stem-segments and mesotonic to sub-acrotonic branching and they occur often in sympatry. Their seeds do, however, provide useful diagnostic characters, both morphological and anatomical (Fig. 2; Iliff, 2002; Stuppy, 2002). The so-called funicular envelope, one of the features unique to the Opuntioideae, is of particular interest. Effectively taking over the function of the seed coat (i.e. mechanical protection), this aril-like structure encasing the seed does not originate from the integument but from the funiculus, which completely surrounds and covers the ovule already during early development. Its central vascular bundle together with a sheath of longitudinally orientated sclerenchymatic cells forms a protruding ridge named funicular girdle (Stuppy, 2002). The tissue of the funicular envelope subtending the funicular girdle usually consists of transversely orientated fibres but in *Maihueniopsis*, these and all other sclerenchymatic cells of the funicular envelope are orientated in parallel to the funicular girdle (Fig. 2; Stuppy, 2002).

The ancestral character state reconstructions within the Tephrocacteae corroborate hypotheses on the evolution of embryo shape and volume of perisperm within opuntioid seeds (Fig. 4; Stuppy, 2002). Seeds of *Maihueniopsis* and *Pterocactus* contain a comparably large amount of perisperm tissue surrounded by a ring-shaped embryo, which is also most likely the character state for the ancestor of Tephrocacteae (Figs. 2 and 4). The enlargement of the embryo at the expense of the perisperm derived from nucellar tissue found in *Austrocylindropuntia*, *Cumulopuntia* and *Tephrocactus* (Fig. 2) are probably advantageous because the storage tissue is directly incorporated in the embryo thus saving energy for transporting nutrients at the time of germination (Stuppy, 2002).

4.2. Relationships within major clades of Tephrocacteae

4.2.1. Maihueniopsis

Contrary to the results of Griffith and Porter (2009) and to the *nrITS* based phylogeny based on sequences taken from the study of these authors (Fig. 5), the genus *Maihueniopsis* appears to be a strongly supported monophyletic group (Figs. 2–4). Possible reasons for the incongruence of genetic markers are discussed in the section 'nuclear ITS evolution'.

The geophytic species *Maihueniopsis clavarioides* is sister to the remaining species of *Maihueniopsis* and morphologically very similar to *Pterocactus*, which is distributed in the Southern Andes in Northern Argentina. In contrast to the wide distribution of the rest of the *Maihueniopsis* clade, which is found from the eastern Andes to the Atlantic coast (Fig. 1), *M. clavarioides* is also distributed in the southern Andes in northern Argentina. Interestingly, the next species which branches off after *M. clavarioides* is *M. domeykoensis*. This species is found in the western Andes of northern Chile, and thus we assume that the ancestors of *Maihueniopsis* and *Pterocactus*

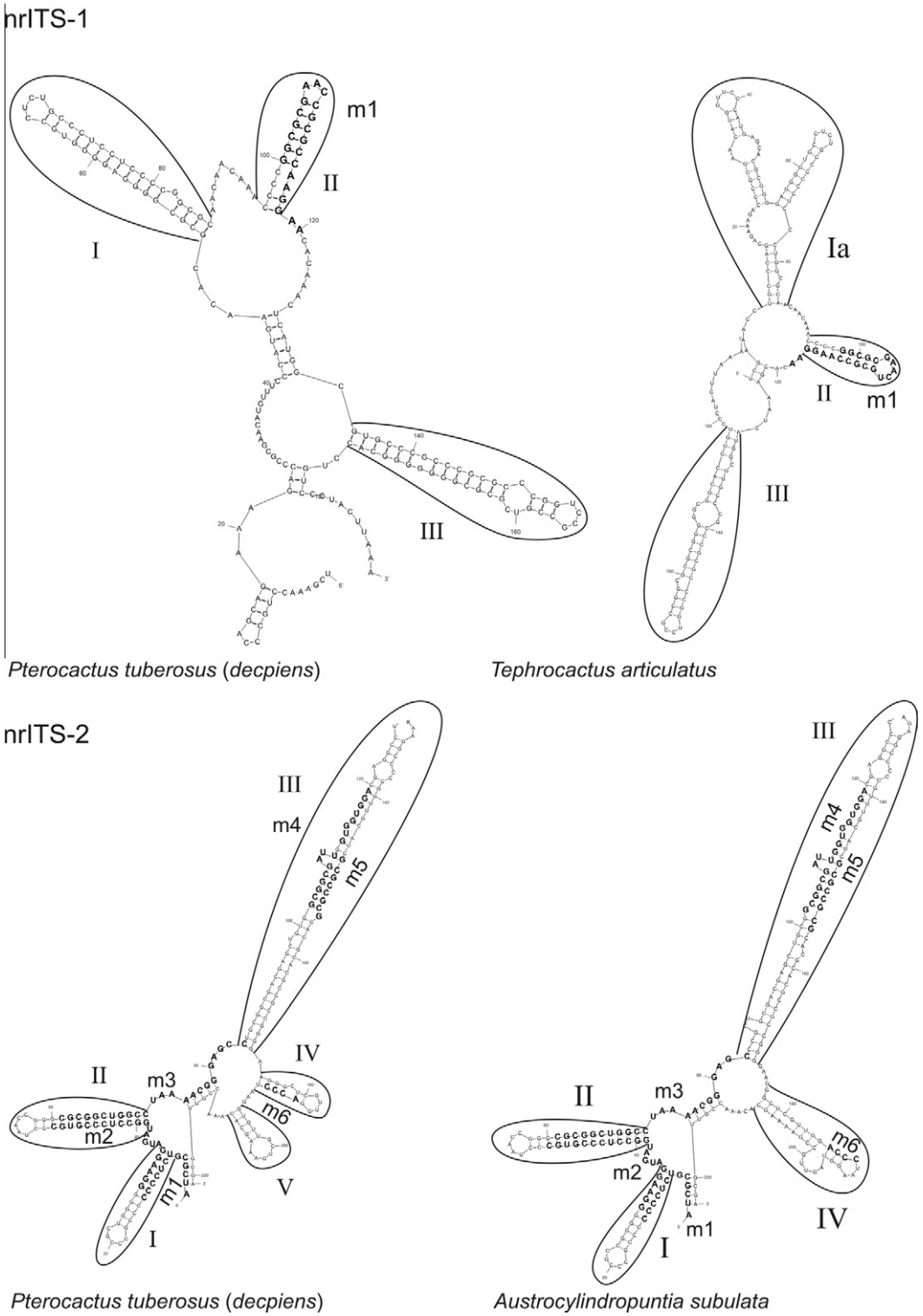

Fig. 6. Examples for secondary structure predictions of the *nrITS* region. Within *nrITS-1* sequences, we observed two major types of secondary structures. The first type (represented by the *nrITS-1* structure of *Pterocactus tuberosus* (*decipiens*) contains the helices I to III. The second type (represented by the *nrITS-1* structure of *Tephrocactus articulatus* consists of the helices Ia, II and III. The conserved *nrITS-1* motif (Liu and Schardl, 1994) is presented in bold letters and marked with "m1". Within *nrITS-2* sequences we observed either structures consisting of five helices, e.g. *Austrocylindropuntia subulata* or of four helices, e.g. *Pterocactus tuberosus* (*decipiens*). All helices found in *nrITS-2* are concordant to structures found in flowering plants by Mai and Coleman (1997) Six conserved *nrITS-2* motifs (Hershkovitz and Zimmer, 1996) are presented in bold letters and marked with "m1-m6".

originated in the Southern Andes, followed by an early dispersal of *Maihueniopsis* to the west, and a further colonisation of *Maihueniopsis* over its entire range.

Specimens formerly treated as *M. minuta* (G. Charles, pers. comm.) were not closely related with each other (O-11, O-12, O-20 in Fig. 2). Thus, the plant material was re-evaluated and a new species, *M. glochidiata* G.Charles, was described (Hunt, 2011). This taxon is part of a clade (0.98 PP) consisting of species with very small segments (Fig. 2). The samples O-22, O-48, O-49 did not cluster together but were treated as *M. darwinii* in the

collection of G. Charles and the Royal Botanical Gardens Kew, respectively (Fig. 2). A close examination of the plants revealed that none of them matched the description of the type of *M. darwinii*, thus the species *M. hickenii* and *M. platyacantha* were re-established (Hunt, 2011).

Within *Maihueniopsis hickenii* and *Tunilla orurenis* we detected *phyC* copies which did not cluster within the clade of the respective genus (O-05/2; O-22/4, O-22/8 in Fig. 3). Present hybridisation events seem to be an improbable reason for this peculiar pattern because all above mentioned species are spatially isolated from each other. A likely scenario for the presence of paralogous alleles in these species is ancient hybridization between the ancestors of the genera combined with a loss of alleles in some taxa because nuclear ribosomal DNA data resulted also in a conflicting phylogeny (see section 'nuclear *rITS* evolution'). Although the intraindividual sequence polymorphism among *phyC* clones was high in some taxa (2–25 bp) we do not expect that the paralogous *phyC* copies originated by *Taq* polymerase and cloning errors because sequences did not contain unprecedented mutations like premature stop codons as similarly shown by Thornhill et al. (2007). Nevertheless, more detailed research including the determination of the ploidy level of investigated plants, the exact copy number of the *phyC* gene in the genome and more sequence information from other nuclear markers are needed to disentangle possible explanations for paralogy ranging from ancient hybridisation, gene duplication and incomplete lineage sorting.

4.2.2. Tephrocactus

The incorporation of *Tephrocactus nigrispinus*, *T. verschaffeltii* and *T. recurvatus* in this genus, suggested by molecular data (Figs. 2–4) and also assumed by Backeberg and Knuth (1936), Backeberg (1963) and Nyffeler and Eggli (2010) for the latter two species, complicates the morphological description of *Tephrocactus* because the otherwise characteristic aerenchymatic funicular girdle of the seeds is not developed in these species. This suggests that these species are not dispersed by wind and water but probably by endozoochory (Fig. 2; Stuppy, 2002). However, species of *Tephrocactus* are characterised by acrotonic branching resulting in typical chains of segments, and their glochids are sunken into cavities with small openings ('encrypted glochids'; Stuppy, 2002).

The species with tiny joints, *Tephrocactus bonnieae*, *T. recurvatus* and *T. molinensis* are closely related (Figs. 2–4). *Tephrocactus recurvatus* was originally described by Backeberg as *T. curvispinus* Backeb. (Backeberg, 1963), but this turned out to be an invalid name because it was based on living material. Later Gilmer and Thomas described this species validly and transferred it to *Cumulopuntia* because of similar seed characters (Gilmer and Thomas, 2000). However, they also mentioned the intermediate morphology between *Tephrocactus* and *Cumulopuntia* and suspected a hybrid origin of the species (Gilmer and Thomas, 2000). Our results do not support a hybrid origin, but only copy of the *phyC* gene was sampled (Fig. 3). Interestingly, *T. recurvatus* was found to be tetraploid but contained only two nucleolar organiser regions (Las Peñas et al., 2009), thus it could be an allopolyploid species which has lost one of the parental ribosomal loci.

Tephrocactus nigrispinus is sister to *T. verschaffeltii* (Figs. 2–4). The latter species was treated as *Austrocylindropuntia* (Backeberg, 1966). Stuppy's detailed analysis of seed anatomy already revealed that seed structures of *T. verschaffeltii* are exceptional within *Austrocylindropuntia*, but nevertheless he considered them to be typical for *Austrocylindropuntia* because of their rudimentarily developed funicular girdle (Stuppy, 2002). *Tephrocactus nigrispinus* and *T. verschaffeltii* share a number of morphological characters that are unusual for the genus. Both species have widely opened orange to red flowers, whereas the remaining species have mostly white or pink flowers. Both species are distributed in high altitudes in the eastern Andes in contrast to the other *Tephrocactus* species which are usually found at medium to low elevations in the eastern Andes (Fig. 1).

The close relationship between *Tephrocactusaoracanthus*, *T. alexanderi* and *T. articulatus* (Figs. 2–4) is supported by morphology; these plants form apically branching shrubs up to 30 cm in height consisting of rather large moniliform segments, which easily detach from the plants.

Tephrocactus weberi is not included in any of the above mentioned clades (Figs. 2–4) and is characterised by a variety of flower colours including yellow flowers which are not found in *Tephrocactus* elsewhere (Eggli and Leuenberger, 1998).

4.2.3. Punotia and Austrocylindropuntia

Austrocylindropuntia as treated in Hunt et al. (2006) is not monophyletic. *Austrocylindropuntia lagopus*, which has now been referred as a monotypic genus *Punotia* (Hunt, 2011), is sister to the remaining species of *Austrocylindropuntia* and *Cumulopuntia* (Figs. 2–4). *Austrocylindropuntia* and *Punotia* are characterised by indeterminate growth, persistent leaves and fruits containing pulp, whereas *Cumulopuntia* develops determinate segments with soon caducous leaf rudiments and pulp-free fruits. Thus, leafiness, which was regained by the ancestors of the *Austrocylindropuntia–Cumulopuntia–Punotia* clade, was again lost during the evolution of *Cumulopuntia* (Fig. 4). Besides leafiness and indeterminate growth, *Austrocylindropuntia* is well characterised by seed characters because the vascular bundle of the funiculus is not covered by the tissue of the funicular girdle ('naked vascular bundle'; Stuppy, 2002).

The close relationship of *Austrocylindropuntia subulata* and *A. pachypus* (Figs. 2–4) was also assumed by Iliff (2002). Both species have cylindrical upright stems but the columnar *A. pachypus* has smaller leaves than the shrubby *A. subulata*. These two sister species have a vicariant distribution: *A. pachypus* is distributed in low altitudes of western Andes, whereas *A. subulata* is naturally found at high elevations east of the Andean continental divide, but is also cultivated in large parts of South America.

Austrocylindropuntia vestita, A. shaferi and *A. floccosa* are closely related (Figs. 2 and 4). This is supported by morphology because these species are characterised by hairy, cylindrical stems forming shrubs, dwarf shrubs or cushions.

4.2.4. Cumulopuntia

Phylogenetic reconstructions revealed two major clades within *Cumulopuntia*: the *C. boliviana* clade and the *C. sphaerica* clade (Figs. 2–4). Both clades are well differentiated by morphology and distribution (Fig. 7). They mostly correspond to the informal species groups defined by Iliff (2002) and Nyffeler and Eggli (2010) suggested treating them as separate genera. The species of the *C. boliviana* clade form hemispheric cushions or dwarf shrubs consisting of ovoid joints whose areoles are clustered towards the apex and their seeds have lateral ridges on the funicular envelope (Stuppy, 2002). The species of the *C. sphaerica* clade are characterised by non-tuberculate, easily detaching segments with evenly distributed areoles and their seeds lack lateral ridges on the funicular envelope (Stuppy, 2002). The species of the *C. sphaerica* clade and *C. corotilla*, which is sister to the remaining species of the *C. boliviana* clade, grow at low to relatively high elevations on the western side of the Andes (0–3400 m, Fig. 7). This suggests the genus *Cumulopuntia* originated in this region and the *C. boliviana* clade spread to higher elevations (2500–4400 m) of the eastern Andes.

We sequenced *phyC* copies from *C. chichensis* and *C. ignescens*, which were sister to each other but did not cluster within the *C. boliviana* clade and appeared at the base of the *Austrocylindropuntia–Cumulopuntia* clade (Fig. 3). This indicates a common origin of

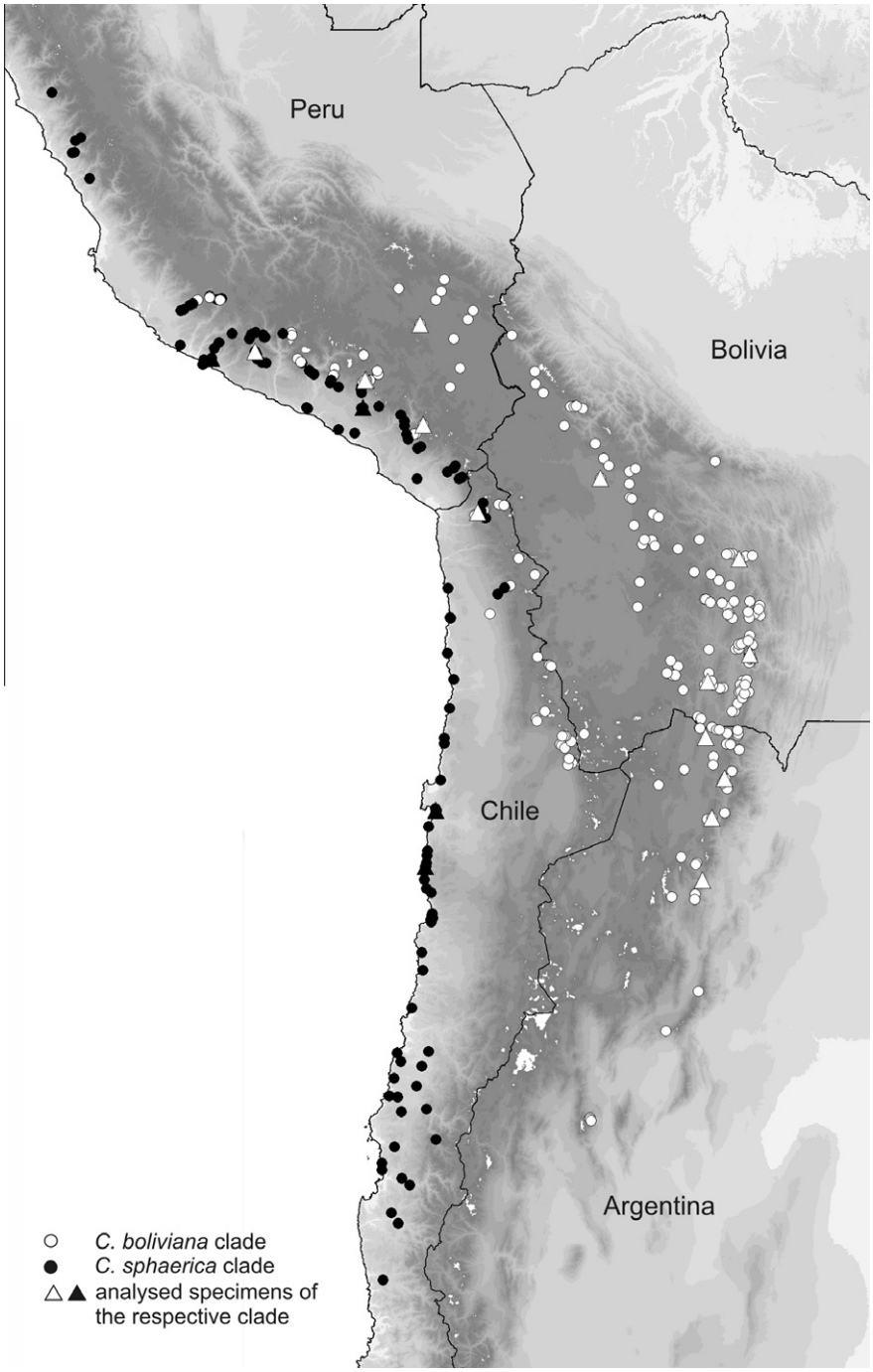

Fig. 7. Distribution area of the genus *Cumulopuntia*. Localities known to the authors are presented as dots, localities of specimens analysed during this study are presented as triangles. Species of the *C. sphaerica* clade are found at low to higher elevations at the western side of the Andes, whereas species of the *C. boliviana* clade are mainly distributed at higher elevations at the eastern side of the Andes.

these alleles before speciation within the clade. However, as outlined above, more information on ploidy level of species and copy number of the *phyC* gene in Opuntiodeae is needed to investigate whether these results arose from ancient hybridisation combined with a loss of alleles in related taxa or from incomplete lineage sorting.

4.3. Nuclear rITS evolution

The phylogeny based on a subset of *nrITS* sequences taken from Griffith and Porter (2009) is congruent to results of these authors based on a combined data set of *nrITS* and *trnL-trnF* intergenic spacer sequences but contradicts the topology of trees in Figs. 2–4. The *nrITS* based phylogeny found *Pereskiopsis* within the clade containing *Austrocylindropuntia* and *Cumulopuntia* (Fig. 5), whereas our data assign this genus to the outgroup. The genus *Maihueniopsis* turned out to be monophyletic in Figs. 2–4, but *nrITS* sequences of this genus were scattered within all major clades of the *nrITS* based phylogeny. This conflict between phylogenies is caused either by the inclusion of misidentified plant material as assumed for the study of Griffith and Porter (2009) by Nyffeler and Eggli (2010) or by the inclusion of paralogous *nrITS* sequences in the analysis. It is unlikely that misidentification is responsible for all conflicting positions because 12 samples from the Tephrocacteae

appeared in the deep lineages of the Opuntioideae and 20 samples in a derived clade of terete-stemmed Opuntiodeae (Griffith and Porter, 2009). To investigate whether this topology resulted from locus-specific properties of the ribosomal rRNA region we analysed sequence features of the *nrITS* region. We did not observe large differences between GC-content, minimum free energy of secondary structures and found no deviations from the *nrITS-1* conserved motif and only few deviations from *nrITS-2* conserved motifs (Tables S2 and S3). Among three conserved motifs within the 5.8S rDNA sequences, which are suited to test for the presence of pseudogenes (Harpke and Peterson, 2008b), we found one deletion within motif 2 in two samples (A- instead of AA, Table S3), which might be prone to misinterpretation reading the sequencing file. These results imply that the *nrITS* data set did not comprise a large proportion of highly degraded pseudogenes as found in other Cactaceae, e.g. *Mammillaria*, *Lophocereus* (Harpke and Peterson, 2006, 2007; Hartmann et al., 2001, 2002). However, we suspect that the *nrITS* sequences of *Tephrocactus molinensis* and *Pereskiopsis aquosa* are pseudogenes because they differed approximately 7% from the other ingroup sequences, which is twice as high as the average sequence divergence in the ingroup. Their considerably lower GC-contents and the substantially deviating secondary structures at higher minimum free energy values compared to the other samples (Table S2) also indicate pseudogenization (Mayol and Rossello, 2001). These putative pseudogenes themselves are not found at unexpected positions in the *nrITS* phylogeny (Fig. 5). Thus homoplasy due to pseudogenization is probably not responsible for incongruency of phylogenies.

The secondary structure predictions of *nrITS-1* and *nrITS-2* sequences revealed two major types in each region. Nuclear ribosomal *ITS-1* sequences formed both helix II and III, and helix II consisted of a conserved motif in angiosperms (Liu and Schardl, 1994; Fig. 6). The structures differed in the assembly of helix one, which forms two smaller stem structures (Ia) in approximately half of the sequences (Fig. 6). Nuclear ribosomal *ITS-2* sequences were either assembled to structures consisting of four or five helices, which were found to be conserved in angiosperms (Mai and Coleman, 1997). Interestingly, the structure types of both spacer sequences did not vary within major clades of the phylogeny (Fig. 5), thus secondary structure predictions contained an obvious phylogenetic signal as was also shown by other studies (Grajales et al., 2007; Keller et al., 2010, 2008; Young and Coleman, 2004). This consistency and the incongruence of the tree to other phylogenies (Figs. 2–4) implies that the *nrITS* phylogeny might represent a gene tree, which is not inevitably identical to the species phylogeny because it is based on a mixture of orthologous and paralogous *nrITS* sequences. Paralogous sequences emerge by gene or genome duplication, the latter is often connected with hybridization (homeologous genes in allopolyploids). Recent studies on chromosome numbers revealed that genome duplication occurred frequently within the evolution of cacti and especially within Opuntioideae (Arakaki et al., 2007; Las Peñas et al., 2009; Pinkava, 2002). Moreover, Las Peñas et al. (2009) mapped the physical location of the nucleolar organiser regions (NOR), which include the *nrITS* region, in Cactaceae and observed one NOR-bearing chromosome pair in diploid species. Interestingly, different results were yielded from tetraploid species: tetraploid samples of *Maihuenia poeppigii* Speg. contained four NOR-bearing chromosomes as expected, but in tetraploid samples of *Tephrocactus recurvatus* only two NOR regions were found implying that two NOR loci were lost after duplication. These results strongly support the existence of paralogous *nrITS* loci within Opuntioideae. More detailed Fluorescent in situ Hybridization (FISH) and cloning experiments are needed to understand the fates of duplicates ribosomal DNA loci in these cacti. The investigation of intra-individual *nrITS* polymorphisms can elucidate whether paralogous sequences have been maintained (Ritz et al., 2005; Sang et al., 1995), were homogenised to one parental sequence type in certain lineages (Kovařik et al., 2005; Wendel et al., 1995) or were recombined to new *nrITS* types (Kovařik et al., 2004). It is generally difficult to assess whether paralogy is caused by incomplete lineage sorting (ancient sequence polymorphism predating speciation) or whether it is caused by hybridization followed by maintenance or loss of homeologs. We assume that the situation observed here probably emerged from ancient hybridization events because the presence of multiple and not closely related alleles of the independent nuclear marker *phyC* in e.g. *Maihueniopsis* and *Cumulopuntia* (Fig. 3) and the observed polyploidy in the group (Las Peñas et al., 2009) point also to genome whole doubling coming along with hybridization. However, the phylogeny based on nuclear low copy gene *phyC* was largely congruent to the chloroplast phylogeny, and it was much better suited to detect conflicting phylogenetic signals because paralogous sequences were simultaneously sampled, which is accordance to previous suggestions on the use of molecular markers in plant phylogeny (Alvarez and Wendel, 2003; Small et al., 2004).

Acknowledgments

We thank British members of the International Organization of Succulent Plant Study (IOS) for financial support of this study. We thank L. Csiba (Jodrell Laboratory, Royal Botanic Gardens Kew, UK) for supplying us DNA samples from their collection and F. Kattermann (Wantage, USA) for kindly providing locality data for the maps. We thank H. Krufzik, J. Föller and J. Spies (Justus-Liebig-University Gießen, Germany) for excellent technical support and V. Wissemann (Justus-Liebig-University Gießen, Germany) for many helpful comments during the study and the possibility to work in his lab. We thank U. Eggli (Sukkulentensammlung, Zürich, Switzerland) and R. Nyffeler (University Zürich, Switzerland) for fruitful discussions during planning this study, N. Korotkova (Freie Universität Berlin, Germany), D. Harpke (Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany), K. Wesche (Senckenberg Museum of Natural History Görlitz, Germany) and the two anonymous reviewers for very helpful comments on the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.07.027.

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