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

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A B S T R A C T

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.

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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; Backebeg, 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 (Bárdenas et al., 2011; Edwards et al., 2005; Griffith and Porter, 2008; 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 (Bárdenas 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 (Backebeg, 1958–1962). Current classifications recognise about 15 genera (Anderson, 2001, 2011; Hunt...
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 Spog., 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 Opuntioideae 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.,

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**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.
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 nrITS 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 Kaderreit, 2001), because the high copy numbers of the tandemly repeated ribosomal DNA arrays in the nuclear organisar 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 Holsford, 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 errone- ous 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 Tephrocactaceae 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 Tephrocactaceae. 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 Tephrocactaceae 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 Perocactus, the monotypic genus Puntia 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 from this study 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., Maihueniopsis sen- su Hunt et al., 2006) and the mainly flat-stemmed Opuntioideae (Opuntiaceae sensu Hunt et al., 2006) and (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 Opuntioideae. 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 nrITS 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 Kaderreit, 2001), because the high copy numbers of the tandemly repeated ribosomal DNA arrays in the nuclear organisar 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 Holsford, 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 errone- ous 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).

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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 Solits, 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 MgCl2 (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 (Qiagquick Gel extraction Kit, Qiagen, Hilden, Germany) were cloned into vector pjet1 (Clonetec PCR Cloning Kit, Thermo Fisher Scientific, Schwerte, Germany). Ligation products were electroporated into E. coli DH5α.

PCR products purified with ExosoAP-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).
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 JLD 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 hypocotyl tissue); (2) growth mode (indeterminate, determinate); (3) life form (tree; columnar; shrub; dwarf shrub; cushion; geophytes); (4) leaf rudiments (persistent; cadaceous); (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 orurensis and Pereskiopsis sp.

2.5. GC-content and secondary structure models of nrITS

In order to test the presence of pseudogenes within the nrITS data set taken from Griffith and Porter (2009) we determined the GC-content of nrITS sequences using Paup v. 4.0b10 (Swofford, 2002). Minimum free energy secondary structures of nrITS-1 and nrITS2 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). Intra-individual 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. bonneae* formerly assigned to *Puna* (Figs. 2–4). The genera *Austrocylindropuntia* and *Cumulopuntia* including both sub-species 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 alexandri* (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

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**Table 1**

Sequence information for the different sequence data sets.

<table>
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<tr>
<th>Alignment characteristics</th>
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<th>nrITS region</th>
<th>Combined trnK/matK and phyC</th>
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<td>29</td>
</tr>
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<td>HRY + G (GTR + G)</td>
<td>GTR + G</td>
</tr>
</tbody>
</table>

* Best-fit model according to Akaike information Criterion as implemented in MrModeltest (Nylander, 2004).
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; 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 presented 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.
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 oriented 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 Tephrocadotheca 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)-GCGYACGGAA published by Liu and Schardl (1994). We detected mismatches in four of six conserved motifs within the nrITS-2 region (Harpe and Peterson, 2008b; Hershkovitz and Zimmerman, 1996; Table S3 in the Supplementary material).

One of three conserved motifs within the 5.8S region differed from published consensus sequences (Harpe and Peterson, 2008a; Jobes and Thien, 1997, Table S3).

Secondary structure models resulted in mean free energy values of \(-78.78 \pm 3.52\) for nrITS-1 and \(-109.47 \pm 4.79\) for nrITS-2 (Table S3). The highest values for nrITS-1 were observed in \(T. articulatus\) (\(papycanthus\)) and \(T. articulatus\) (\(stromatiformis\); \(-70.33\) 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, and III and the other of helices Ia, II, III, with the substructure Ia consisting of the remaining 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 nrITS-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, nrITS-2 sequences forming five helices were found in clade A in the nrITS-based phylogeny (Fig. 5). Nuclear nrITS-2 structures of Tephrocactus molinensis and Ptereskoiopsis 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 Tephrocactae

Our results and those of others studies clearly imply that the genera Austrocylindropuntia, Punotia, Cumulopuntia, Tephrocactus, Pterocactus and Maihueniopsis, now circumscribed as tribe Tephracteae (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 Tephrocactae, we detected four strongly supported clades representing the genera (1) Pterocactus, (2) Maihueniopsis, (3) Tephrocactus and (4) Punotia, Cumulopuntia and Austrocylindropuntia (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 Tephrocactae. 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 leaves 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 homoplasic within the Tephrocactae (Fig. 4). Geophytes evolved independently in all major clades (Fig. 4) from ancestors that were probably dwarf shrubs. The geophytes Maihueniopsis clavarioides, Tephrocactus bonneae 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-acrotic branching and they occur often in sympathy. 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 sclerencymatic 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 sclerencymatic cells of the funicular envelope are orientated in parallel to the funicular girdle (Fig. 2; Stuppy, 2002).

The ancestral character state reconstructions within the Tephrocactae 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 Tephrocactae (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 Tephrocactae

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
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. darwini* in the
The species with tiny joints, Tephrocactus bonnieae 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 aereophytic fleshy 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 acrotenic branching resulting in typical chains of segments, and their glochids are sunken into cavities with small openings (‘encrypted glochids’; Stuppy, 2002). The latter species was treated as validly and transferred it to Cumulopuntia 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 nuclear 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 fleshy 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 Tephrocactusaurantacanthus, 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 fleshy 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) suggesting 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 fleshy 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 fleshy envelope (Stuppy, 2002). The species of the C. sphaerica clade and C. corollina, 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...
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 Opuntiodoeae 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 Pereskia 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 Tephroctaeae

![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. sphenoica 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.](image-url)
appeared in the deep lineages of the Opuntioideae and 20 samples in a derived clade of terete-stemmed Opuntioidae (Griffith and Porter, 2009). To investigate whether this topology resulted from locus-specific properties of the ribosomal RNA 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 aquisa 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-content 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 homoplasies due to pseudogenization is probably not responsible for incongruence 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, 2005; Sullivan et al., 2003). Among 112 sequences, 98% had 11 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, 2002). Moreover, 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 in accordance to previous suggestions on the use of molecular markers in plant phylogeny (Alvarez and Wendel, 2003; Small et al., 2004).

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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.

References


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