Caryophyllales phylogenetics: disentangling Phytolaccaceae and Molluginaceae and description of Microteaceae as a new isolated family

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Abstract


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The Caryophyllales are one of the major lineages of angiosperms, including some 12 000 species and well known families such as Amaranthaceae, Cactaceae, Caryophyllaceae, Droseraceae, Nyctaginaceae and Polygonaceae. Phylogenetic hypotheses based on molecular characters have led to their circumscription and have considerably improved our understanding of interfamilial relationships. For this study, we generated a data set of the non-coding and rapidly evolving chloroplast petB-petD region, consisting of a transcribed spacer and a group I intron for 87 taxa of Caryophyllales and 22 outgroups. In addition, we analysed a complementary matK data set with complete sequences of the coding region. Trees obtained from both markers were well resolved and especially petD data yielded a well supported backbone for the Caryophyllales. The order is constituted by two sister clades, caryophyllids and polygonids, the latter containing a carnivorous subclade. Both Molluginaceae and Phytolaccaceae had been considered as polyphyletic, but not as severely as is now evident from this study with improved taxon sampling. As a great surprise, the hitherto unsampled genus Microtea is found with high support in an isolated position as the fourth branch in the caryophyllid clade. On the other hand, Lophiocarpus as the second genus of the Phytolaccaceae subfamily Microteoideae is sister to an Aizoaceae-Nyctaginaceae-Phytolaccaceae lineage. In line with their morphological distinctness, Microteaceae are described as a new family. Our data further resolve a distinct Mollugo clade, whereas Hypertelis appears to have affinities with Limeum, suggesting an expanded Limeaceae.

Additional key words: eudicots, angiosperm classification, molecular phylogeny, petD, matK, neotropical plant families

Introduction

With about 12 000 species, Caryophyllales are one of the largest eudicot orders. Many of them are adapted to dry or saline habitats and a number of economically important plants (such as spinach, quinoa) or ornamentals (such as cacti and carnations) are found in this order. The core of the Caryophyllales has long been considered as a natural group based on their basal or free-central placenta, which led to the name Centrospermae (Braun 1864; Eichler 1875–78). The circumscription of Caryophyllales reflecting the Centrospermae was basically upheld until the 1990ies and is also reflected in major pre-phylogenetic classification systems of angiosperms (Dahlgren 1980; Takhtajan 1987; Cronquist 1988; Thorne 1992). A detailed presentation of the classification history was provided by Cronquist & Thorne (1994).

These Caryophyllales in the strict sense comprise the only betalain families (Aizoaceae, Amaranthaceae, Basellaceae, Cactaceae, Chenopodiaceae, Didiereaceae, Halophytaceae, Hecorellaceae, Nyctaginaceae, Phytolaccaceae, Portulaccaceae and Stigmospermae) in angiosperms (Clement & Mabry 1996b). In Achatocarpaceae, however, pigments have never been analysed (Clement...
Following Ehrendorfer (1976), this group, the Chenopodiinae, was considered to be monophyletic with the idea that their common ancestor lost anthocyanin biosynthesis. Ehrendorfer (1976) laid out a scenario in which the loss of anthocyanin biosynthesis was associated with a shift to anemophily. Molluginaceae and Caryophyllaceae were known to possess the mutually exclusive anthocyanins (Stafford 1994) as red pigments like other flowering plants (the Caryophyllineae). Caryophyllales were further shown to have subtype-P3 sieve element plastids (Behnke 1993, 1994) as a characteristic feature.

First molecular phylogenetic studies of angiosperms using rbcL indicated the close relationship of Polygoneae and Plumbaginaceae to the centrospermous families (Giannasi & al. 1992; Chase & al. 1993). The same was shown for the carnivorous families Anisostropladaeae, Dioncophyllaceae, Droseraceae and Nepenthaceae (Albert & al. 1992; Chase & al. 1993), which were formerly classified as Droserales and Nepenthales (e.g., Takhtajan 1997). Genera such as Simmondsia and Rhabdodendron were then positioned in Caryophyllales when adding further rbcL and also atpB sequences (Savolainen & al. 2000a), although their internal positions did not yet receive significant statistical support. The results were substantial for recognising an expanded order Caryophyllales. We also refer to this circumscription here.

Although several molecular phylogenetic analyses dealt specifically with the Caryophyllales previous to this study (Giannasi & al. 1992; Manhart & Retting 1994; Downie & Palmer 1994; Downie & al. 1997; Meimberg & al. 2000; Cuénoud & al. 2002) and most recently Brockington & al. (2009), a number of questions on internal relationships of the Caryophyllales remain. The parsimony analysis of rbcL sequence data by Manhart & Retting (1994) already indicated that betalains could also have arisen twice, once in Amaranthaceae-Chenopodiinae and once in a clade comprising other Centrospermae except Caryophyllaceae. However, Mollugo appeared within this clade in the rbcL tree. Later analyses with better statistical support on Caryophyllales relationships confirmed the non-monophyly of Chenopodiinae, indicating a more complex pattern of betalain evolution (Cuénoud & al. 2002).

In terms of taxon sampling Cuénoud & al. (2002) provided the so far most comprehensive tree based on a matK fragment (127 taxa). This extended taxon sampling surprisingly showed genera such as Lophiocarpus (classified as Phytolaccaceae; Rohwer 1993) and Limeum (classified as Molluginaceae; Endress & Bittrich 1993) to be in isolated positions distinct from the core of their respective families. A combined analysis of atpB+rbcL+matK+nr18S of a reduced taxon set (Cuénoud & al. 2002) yielded only slightly improved confidence into hypothesised deep nodes within Caryophyllales. Moreover, their results rendered families such as Phytolaccaceae and Portulacaceae as para- or polyphyletic. Evolutionary relationships of the so-called “portulacaceous cohort” became only better understood recently, using an again extended taxon sampling and sequence data from multiple rapidly evolving and non-coding genomic regions (Nyffeler 2007; Nyffeler & Eggli in press).

The latest phylogenetic analysis of Caryophyllales (Brockington & al. 2009) includes only 36 species but many characters (12 000 nt from plastid genes, 5000 nt from nuclear genes and 24 000 nt from the plastid inverted repeat). The improvement over the two and four-gene analyses of Cuénoud & al. (2002) mostly regards to deep nodes. On the other hand, important deep nodes (such as for placement of Limeum and Stegnosperma) remain unsupported in the maximum parsimony strict consensus tree, while relationships within shallower clades such as the “portulacous cohort” and the “rhipide clade” are generally not well clarified. Also, most of the putatively polyphyletic or paraphyletic families were undersampled (e.g., Phytolaccaceae) and some families were not included at all (Agastidaceae, Petiveriaceae). Non-coding and rapidly evolving cp DNA has recently been shown to be a valuable tool for inferring plant phylogenetic relationships even on levels far deeper than genera (Borsch & al. 2003; Löhne & Borsch 2005; Müller & al. 2006). A comprehensive approach to sequence and analyse the mutational dynamics of non-coding genomic regions from the chloroplast genome for a set of about 500 genera of eudicots has been carried out within the Eudicot project (www.eudicots.de). Worberg & al. (2007) were able to get high statistical support for the early branches of eudicots with a combined matK+petD+trnL-F data set and similar improvement of tree resolution and support was gained for the rosids (Worberg & al. 2009).

In the present study, the chloroplast petB-petD region, consisting of a transcribed spacer and a group II intron, and the matK coding region, were analysed with the aim to test the phylogenetic utility of these markers and further illuminate evolutionary relationships in Caryophyllales. Special attention was payed to a careful and reasonably dense sampling of taxa.

With respect to taxonomy, this study aims at a revised classification of Microtea. This genus has usually been treated under Phytolaccaceae but doubts as to its position have been expressed for decades. Interestingly, the study of Microtea in the Berlin-Dahlem Botanical Garden and Museum has a history of more than 120 years, starting with Urban (1885) and continued by Eckardt (1954, 1964, 1974). Complementing herbarium, morphological and anatomical work with molecular phylogenetics, the recognition of Microteaceae provides a nice example of integrating modern phylogenetic approaches and plant classification.

Material and methods

Taxon sampling and material. — In total 87 species from Caryophyllales, representing nearly all families, and
22 species from outgroups were sampled in this study. A complete list of taxa with their sources of origin and voucher information is given in Appendix 1. If available, fresh plant material was silica dried, otherwise herbarium samples were used. Fresh material was obtained primarily from the living collections of the Botanic Garden and Botanical Museum Berlin-Dahlem and from Bonn University Botanical Gardens. All petD sequences for Caryophyllales and of several outgroups were generated for this study; other outgroup sequences were taken from Worberg & al. (2007). For matK, 19 complete coding sequences of Caryophyllales taxa were generated newly; others were taken from Müller & Borsch (2005) and from the Caryophyllales subset of the angiosperm data set published by Hilu & al. (2003). In case own complete sequences were available for a genus only represented by partial matK sequences in the latter data set, these partial sequences were ignored.

DNA extraction, amplification and sequencing. — Plant material was homogenized using the Mixer Mill (MM 200, Retsch) and was then extracted with CTAB following the protocol described in Borsch & al. (2003) or with the AVEGene Plant DNA extraction kit (Avegene, Korea). The fragment amplified contains the petB-petD intergenic spacer, the petD 5'-exon and the petD group II intron. Both for PCR amplification and sequencing, the primers pipetB1411F and pipetD738R (Löhne & Borsch 2005) were used. If amplification of the entire fragment did not succeed, the region was amplified in two overlapping halves using the primers CapetD324R (5'-ATC CCY TGT TTC ACT CCC ATA G-3') and CapetD194F (5'-CAG GCT CCG TAA RAT CCA G-3') in combination with one of the primers mentioned above. PCR reaction was performed in 50 µl volume containing 5 units of SAWADY Taq polymerase (PeqLab), 8 µl dNTPs (each 1.25 mM), 5 µl 5 x Taq buffer (PeqLab), 2 µl of each forward and reverse primer, 4 µl genomic DNA and H₂O to 50 µl. For matK a broad spectrum of mostly family-specific internal amplification primers was used to amplify the whole trnK intron including the CDS coding sequence in two overlapping halves. Primer trnKF-bryo (Wicke & Quandt 2009) served as forward primer for the upstream fragment, with the reverse primers ACMatK1401R (this study; 5'-ATG GAT TCG TAT TCA CAT AC-3') for Aizoaceae, Cactaceae, Didiereaceae, Nyctaginaceae, Portulacaceae; ACMatK1400R (Müller & Borsch 2005) for Amaranthaceae and Basellaceae; CARYmatK1440R (this study; 5'-AKC GTC GAT GAG AGG ATT G-3') for Caryophyllaceae; PLUMmatK1401R (this study, 5'-ATG GAT TGA TAT TCA CAC AC-3') for Plumbaginaceae and POLYmatK1401R (this study, 5'-ATG GAT TCG TAT TCA CAC AC-3') for Polygonaceae. Thermal cycling was performed on a T3 thermocycler (Biomera, Göttingen) with an initial denaturation step (90 s) at 96 °C followed by 34 cycles of 30 s at 95 °C, 60 s at 50 °C, 90 s at 72 °C, and a final extension step of 20 min at 72 °C. Fragments were visualised using the Flu-o-blue system (Biozym, Hamburg, Germany) and excised from a 1.5 % agarose gel (NEOE-Agarose, Roth, Germany). The DNA was then purified using the AVEGene Gel Extraction Kit (Avegene, Korea) according to the manufacturer’s protocol. PCR products were directly sequenced using the DCTS Quick Start Kit (Beckman Coulter). The reaction mix contained 3 µl DCTS Quick Start Kit (Beckman Coulter), 0.5 µl primer (20 pm/µl), 0.5–6.5 µl DNA template and ultrapure water to obtain a total volume of 10 µl. The cycle sequencing temperature profile consisted of 30 cycles of 96 °C for 20 s, 50 °C for 20 s and 60 °C for 4 min, on the thermocycler mentioned above. Samples were run on an automated capillary sequencer (CEQ 8000 Genetic Analysis System, Beckman Coulter). Alternatively, cleaned fragments were sequenced via Macrogen Inc. (Seoul, South Korea; all new matK and petD sequences from BGBM). Pherograms of the latter source (ABI 3730 capillary sequencer) were usually clean and well readable until 850 nt, allowing to sequence the whole trnK intron with four primers. Pherograms were edited manually using the software PhyDE v0.995 (Müller & al. 2005+).

Sequence alignment. — Beside substitutions, non-coding chloroplast DNA shows a high number of length mutational events. Correct homology assessment and gap placing has to take into account the different kinds of length mutations. Alignment followed rules described in detail in Löhne & Borsch (2005). Where detected, inversions were reverse-complemented and aligned to the rest and treated as homologous.

Parsimony tree search. — Parsimony ratchet analysis using PAUP* (Swofford 1998) and PRAP (Müller 2004) was carried out using ten random addition cycles of 200 ratchet iterations with 25 % of the positions being reweighted. A strict consensus was computed from the shortest trees found. Tree evaluation was done via bootstrapping with 10 000 replicates with keeping only one tree in memory. For Maximum Parsimony (MP) analysis, length mutations were coded according to a modified simple indel coding method (Müller 2006), which resulted in a matrix of 100 indel characters.

Bayesian Inference. — Bayesian Inference of phylogeny was conducted using MrBayes (Ronquist & Huelsenbeck 2003) employing the GTR+Γ+I model. Two runs of four MCMC chains were run simultaneously for two million generations, sampling the chains every 100th generation. Trees were summarised with the burn-in conservatively set to the first 25 % of generations.
Fig. 1A–B. Maximum Parsimony tree (strict consensus) of *Caryophyllales* based on the *petD* region including a matrix of coded microstructural changes. – Values above branches are bootstrap percentages.
Results

PetD data set and trees

For the Caryophyllales and outgroup taxa sequenced in this study, the petB-petD region ranged from 711 to 1281 nucleotides in length and resulted in an aligned matrix of 2787 characters. Several mutual hotspots both in the petB-petD spacer and the petD intron were excluded from phylogenetic analyses due to ambiguous homology assessment. In total, 880 characters had to be marked as mutational hotspot. Alignment and matrix are available from the eudicot website (www.eudicots.de) and from the authors. For petD, parsimony ratchet search calculated a strict consensus of 972 shortest trees (length: 4735 steps, CI: 0.413, RI: 0.673, RC: 0.278). Bayesian Inference (BI) of phylogeny (considering only substitutions) resulted in a tree topology largely congruent to that from MP analysis. If differences were observed, they were soft incongruities (i.e., inconsistencies), lacking convincing support. However, resolution and support values are higher in BI compared to MP, and interestingly BI finds evidence for monophyly of Caryophyllales II (polygonids).

MatK data set and trees

Only characters from the matK CDS were used in this study, although the flanking trnK intron sequences were also generated here and will be analysed and published elsewhere. The matK data set consisted of 1718 characters; the sequence length range was between 540 and 1524 nucleotides due to incomplete sequences. There were no characters excluded from phylogenetic analyses. Modified simple indel coding resulted in 253 coded indels. Complete sequence statistics are given in Table 1. For the matK data set, 32 equally short trees were found (5348 steps, CI: 0.411, RI: 0.596, RC: 0.245); a strict consensus tree was calculated (not shown). Bayesian analysis of the matK data set yielded a tree topology mostly congruent to that from MP analysis. If differences were observed, they were soft incongruities (i.e., inconsistencies), lacking convincing support. However, resolution and support values are higher in BI compared to MP, and interestingly BI finds evidence for monophyly of Caryophyllales II (polygonids).

Combined data set and trees

Results from MP tree searches for each petD and matK with coded indels are shown in Fig. 1 and 2, respectively. Analyzing a data set that combined nucleotide data of those taxa for which both petD and matK were available with the MP approach resulted in only 8 shortest trees (7421 steps, CI: 0.440, RI: 0.624, RC: 0.275); the strict consensus tree of those trees is shown in Fig. 4.

Discussion

Two major clades within monophyletic Caryophyllales

The monophyly of Caryophyllales found in previous studies is confirmed with maximum confidence by both petD and complete matK sequences (Fig. 1–3), although the matK gene tree inferred with MP has only moderate support for this (not shown; 79 % JK). Partial matK sequences in Cuénoud & al. (2002) and Hilu & al. (2003) had no or weaker support, indicating the significantly enhanced usefulness of complete matK data sets. Multi-gene analyses (Cuénoud & al. 2002; Brockington & al. 2009) converge with petD group II intron (and petD+matK combined) analyses on maximum support for the Caryophyllales in a broad sense.

The Caryophyllales consist of two major clades. They are congruently depicted by this study (non-coding and rapidly evolving plastid regions) and the trees in Brockington & al. (2009). The four-gene (18S, rbcL, atpB and partial matK) and two-gene (rbcL+partial matK) analyses by Cuénoud & al. (2002) were inconsistent in placing Rhabdodendron as sister to the remainder of all Caryophyllales. BI (Fig. 3) of matK also shows improved confidence over MP (tree not shown, Cuénoud & al. 2002, Fig. 3) for Rhabdodendron and Simmondsia to be members of the core Caryophyllales. This “core” of the Caryophyllales clade (sensu Cuénoud & al. 2002) includes the Centrospermae and corresponds to the Caryophyllales I in Hilu & al. (2003). Considering the emerging overall agreement for the existence of this clade, we call it the caryophyllid clade. It can be easily distinguished from the polygonid clade within Caryophyllales (Fig. 2, 4). The latter corresponds to the so-called “non-core Caryophyllales” (Cuénoud & al. 2002; Brockington & al. 2009) or Caryophyllales II (Hilu & al. 2003).

For the circumscription of the polygonids, the position of the Frankeniaceae-Tamaricaceae clade requires some discussion. Support for the monophyly of polygonids is weak in petD data alone using the parsimony approach. The lineage is placed inconsistent in various positions depending on taxon sampling. Using Bayesian Inference, polygonids reach a posterior probability of 0.86, and Frankeniaceae and Tamaricaceae together

Table 1. Sequence statistics of petD and matK datasets of Caryophyllales analysed in this study. For the petD matrix, parts of the sequences (hotspots) could not be included due to uncertain homology. Values calculated from the matrix (underlined) are thus not based on all nucleotides.

<table>
<thead>
<tr>
<th>Character set</th>
<th># characters</th>
<th>length range</th>
<th>mean % divergence</th>
<th>ti/tv</th>
<th>% variable</th>
<th>% informative</th>
<th>% GC</th>
<th>% A</th>
<th>% C</th>
<th>% G</th>
<th>% T</th>
</tr>
</thead>
<tbody>
<tr>
<td>petD data set</td>
<td>1907</td>
<td>711–1281</td>
<td>1009.39</td>
<td>13.8</td>
<td>1.154</td>
<td>42.108</td>
<td>30.309</td>
<td>33.746</td>
<td>32.64</td>
<td>15.565</td>
<td>18.18</td>
</tr>
</tbody>
</table>
are sister to the remainder of polygonids. *Frankeniaceae* and *Tamaricaceae* generally appear in a clade with high support. It also has been found using coding markers as *rbcL* or *matK* (Nandi & al. 1998; Meimberg & al. 2000; Savolainen & al. 2000b; Solis & al. 2000; Cuénoud & al. 2002; Hilu & al. 2003; Brockington & al. 2009). As an alternative hypothesis derived from *matK* and *trnK/matK*, respectively, a clade comprising the *Frankeniaceae-Tamaricaceae* lineage and *Plumbaginaceae* plus *Polygonaceae* was found (Meimberg & al. 2000; Cuénoud & al. 2002; Hilu & al. 2003) and is also evident from Bayesian Inference of complete *matK* sequences in this study (Fig. 3). Brockington & al. (2009) resolve the *Frankenia-Tamarix* clade sister to *Plumbaginaceae-Polygonaceae* within polygonids.

**Relationships within polygonids**

*Plumbaginaceae* and *Polygonaceae* are both monophyletic and sister groups. Both *Plumbaginaceae* and *Polygonaceae* are recovered with *petD* sequences, and also their sister group relationship, which received high statistical support elsewhere (Nandi & al. 1998; Meimberg & al. 2000; Solis & al. 2000; Cuénoud & al. 2002; Hilu & al. 2003; Brockington & al. 2009). A sister group relationship between the *Frankeniaceae-Tamaricaceae* clade and the *Plumbaginaceae-Polygonaceae* clade, as suggested earlier (Meimberg & al. 2000; Cuénoud & al. 2002; Hilu & al. 2003; Brockington & al. 2009), is not supported by *petD* data.

The carnivorous clade with the families *Ancistrocladaceae*, *Dioncophyllaceae*, *Droseraceae*, *Drosophyllaceae* and *Nepenthaceae* is recovered by *petD* sequences plus coded indels with bootstrap support comparable to previous studies (Meimberg & al. 2000; Cuénoud & al. 2002; Brockington & al. 2009). Within this clade, *Ancistrocladaceae* and *Dioncophyllaceae* are sisters with maximum support as depicted earlier (Meimberg & al. 2000; Cuénoud & al. 2002; Hilu & al. 2003). *Drosophyllum lusitanicum* as sister to the former clade is supported in the present study comparable to Meimberg & al. (2000), Cuénoud & al. (2002) and Hilu & al. (2003). Monophyly of both *Droseraceae* and *Nepenthaceae* are recovered in the *petD* data set, but their placement to each other and to the clade of *Ancistrocladaceae*, *Dioncophyllaceae* and *Drosophyllaceae* is not resolved. Even far larger character sets (Brockington & al. 2009) could not clarify this question.

**Relationships within caryophyllids**

The caryophyllid clade was discovered by Cuénoud & al. (2002) and Hilu & al. (2003) using *matK* sequence data. In the most recent study on *Caryophyllales* phylogeny, Brockington & al. (2009) could substantiate the inclusion of *Simmondsia* and *Rhabdodendron* as the first branching taxa into this clade with a large number of sequence data from coding genes. Based on *petD* data alone, the sister group relationship between *Simmondsia chinensis* and the remaining caryophyllids is supported with 100% bootstrap (BS), comparable to values from much larger data sets (Brockington & al. 2009). Previous data sets (Cuénoud & al. 2002; Brockington & al. 2009), including *Asteropeia* and *Physena*, found these two genera to constitute a monophylum branching after *Simmondsia* in *Caryophyllales*. This lineage is also evident in our *matK* tree (Fig. 3).

A surprising new result from the present study is the position of *Microtea* (*Phytolaccaceae* subfam. *Microteoideae*) that is found as a successive sister to all other caryophyllids after the grade of *Simmondsia*, *Rhabdodendron* and *Asteropeia-Physena*. Thus, *Microtea* is not even closely related to other *Phytolaccaceae* and rather represents an isolated lineage in *Caryophyllales* (see below).

The placement of *Stegnosperma cubense* as inferred from *petD* sequences differs from other analyses. *Stegnosperma* is the next branch following *Microtea* in the *petD* MP tree (Fig. 1) or is unresolved between the CACA clade (Caryophyllaceae, Achatocarpaceae, Chenopodiaceae, Amaranthaceae) and the remainder of *Caryophyllales* (Bl. Fig. 2). Based on *rbcL*, *Stegnosperma* appeared together with *Cactaceae*, *Didiereaceae*, *Basellaceae*, *Molluginaceae*, *Phytolaccaceae*, *Nyctaginaceae*, *Gisekiaceae* and *Aizoaceae* (Manhart & Rettig 1993) and *Cactaceae*, *Didiereaceae* and *Basellaceae* (Rettig & al. 1992), respectively, but both topologies were lacking reliable support. The by now largest data set in terms of sequence characters (Brockington & al. 2009) places *Stegnosperma* sister to a so-called “globular inclusion clade”. In the two-gene analysis of Cuénoud & al. (2002) it is in the same position, although no *matK* sequence seems to exist (*Stegnosperma* is missing from Fig. 3 of Cuénoud & al. 2002; and no entry is in GenBank). Branches among *Stegnosperma*, *Limneum*, the CACA clade, the “raphide clade” and the “portulacaceous cohort” are extremely short in all studies existing so far. The respective nodes are completely unsupported in the MP tree of the total evidence data set of Brockington & al. (2009) and their maximum likelihood (ML) tree only provides support for a *Stegnosperma-Limneum* grade.

In line with previous studies employing different DNA markers (Manhart & Rettig 1993; Müller & Borsch 2005), monophyly of a group comprising *Amaranthaceae*, *Chenopodiaceae*, *Achatocarpaceae* and *Caryophyllaceae* is moderately to highly supported in this study. Using partial *matK* (Cuénoud & al. 2002), *Caryophyllaceae* were found branching after *Simmondsia/Rhabdodendron* and *Asteropeia*, albeit without any bootstrap support. Signal of complete *matK* (Fig. 3) is in line with *petD* (Fig. 1, 2) and multi-gene data sets (Brockington & al. 2009). Within this clade, *Amaranthaceae* and *Chenopodiaceae* together form a well supported monophylum, which is in line with all previous studies (Giannasi & al. 1992; Rettig & al. 1992; Manhart & Rettig 1993; Downie & Palmer 1994; Downie & al. 1997; Cuénoud & al. 2002; Hilu &
Fig. 2A–B. Phylogram of the Bayesian analysis of Caryophyllales based on the petD region. – Values above branches are posterior probabilities.
al. 2003; Kadereit & al. 2003; Müller & Borsch 2005). However, whereas the monophyly of Amaranthaceae has been shown with high confidence (e.g., Müller & Borsch 2005) the monophyly of Chenopodiaceae is still under debate. Hitherto existing multi-gene analyses only include Celosia and Spinacia (Cuénoud & al. 2002; Brockington & al. 2009), limiting insights into the monophyly of the respective families. Most interestingly, Amaranthaceae and Chenopodiaceae, respectively, appear monophyletic in gene trees of petD (Fig. 1, 2) and matK (Fig. 3) with the exception of an inconsistent position of Polycnemoideae (Nitrophila, Polycnemum). Earlier, using trnK/matK the monophyly of Amaranthaceae was affirmed (100 % BS, Müller & Borsch 2005); Chenopodiaceae were found to be paraphyletic, with Chenopodiaceae subfam. Polycnemoideae being sister to Amaranthaceae. Relationships of Amaranthaceae and Chenopodiaceae need further investigation, especially to test the circumscription of Chenopodiaceae. Achatocarpaceae as sister to the Amaranthaceae-Chenopodiaceae alliance is strongly supported here (Fig. 4). This relationship has been suggested by various studies before (Manhart & Rettig 1993; Cuénoud & al. 2002; Kadereit & al. 2003; Müller & Borsch 2005).

The clade consisting of Cactaceae, Portulacaceae, Basellaceae, Didiereaceae, Halophytaceae, Lophiocarpus and Mollugo corresponds to the “higher core II” clade (Hilu & al. 2003). Resolution and support are, compared to that of matK data (Cuénoud & al. 2002; Hilu & al. 2003), relatively weak within this clade. Cactaceae (58 % BS, pp = 1.00) were found in a clade (pp = 1.00) together with Talinella, Anacampseros and Portulaca, which is in line with earlier findings (Applequist & Wallance 2001; Applequist & al. 2006; Nyffeler 2007; Nyffeler & Eggli in press) using ndhF sequences. Combining the chloroplast genes ndhF, matK and the mitochondrial nad1, Anacampseros s.l. was depicted sister to Cactaceae (78 % BS and 0.72 pp in Bayesian Inference; Nyffeler 2007). However, it is noteworthy that phylogenetic hypotheses contradict each other depending on the genome analysed. In mitochondrial data, parts of Portulaca are sister to Cactaceae, while in chloroplast data, Anacampseros s.l. is found sister to Cactaceae with a Bayesian approach. Relationships among Cactaceae, Anacampseros s.l. and Portulaca remain unclear (Fig. 4) with matK and petD alone. Nevertheless, we follow the more extensive analyses of Nyffeler & Eggli (in press) in recognising Anacampserotaceae and Talinaceae separate from Portulacaceae (here only represented by Portulaca).

Both Claytonia species do not cluster with the other Portulacaceae in the current study (Fig. 1, 2), supporting a classification under a separate family Montiaceae. As reported previously (Applequist & Wallace 2001; Applequist & al. 2006; Nyffeler 2007). The circumscription of Didiereaceae has been revised based on molecular data (Applequist & Wallance 2003) including now the genera Portulacaria, Ceraria and Calyptrotheca (all formerly Portulacaceae). In our petD data set, a clade comprising Didiereaceae and Portulacaria was found with support, too. Usage of ndhF (Applequist & Wallance 2001) could not resolve the relationship between those two families. Combining three genes representing all three genomes (Nyffeler 2007), Basellaceae were found sister to a clade including Didiereaceae, Portulacaceae and Cactaceae, but this topology yielded only 65 % BS and 0.56 pp. Positions of Basellaceae and Didiereaceae within higher core II remained unclear. The position of Halophyton ameghinoi (Halophytaceae) as sister to a Basellaceae plus Didiereaceae clade was not supported in Brockington & al. (2009). However it is resolved congruently in this study (Fig. 2). Halophyton was not included in most studies dealing with Cactaceae and their nearest relatives (Applequist & Wallance 2001; 2003; Applequist & al. 2006; Nyffeler 2007). Halophytaceae belong to the “portulacaceous cohort”, but understanding their position requires further work.

**Polyphyly of Molluginaceae and complex evolution of betalain families**

In line with earlier findings (Cuénoud & al. 2002), Limeum (Limeaceae) is inconsistently placed apart from remaining Molluginaceae. A statistically well supported hypothesis of the systematic position of Limeum is still missing. Even a data set with over 42 000 nucleotides did not result in a reliable placement of that taxon (Brockington & al. 2009). Limeaceae were validated by Shipunov ex Rave in 2005, Corbichonia was already shown to be distant from Mollugo (Cuénoud & al. 2002), rendering Molluginaceae polyphyletic. It was recently included into Lophiocarpaceae along with Lophiocarpus (formerly Platylocaceae) (Doweld & Rave 2008). Using matK (Cuénoud & al. 2002), the Molluginaceae genera Pharmaceum, Suesenguthiella, Adenogramma, Glischrothamnus and Glinus formed a moderately supported group sister to the portulacaceous cohort but Mollugo was not sampled. In our study Mollugo is resolved in the same position with high confidence (Fig. 1–4). Furthermore, the genera Coelanthum, Macarthuria, Polypoda, Psammothrofa and Telephium have so far not been sampled in any molecular phylogenetic study. For a better understanding of the evolution of pigments, a complete sampling of Molluginaceae in combination with pigment

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Fig. 3. Phylogram of the Bayesian analysis of Caryophyllales based on the matK CDS. – Values above branches are posterior probabilities. Some nodes that gained significantly less support in the MP analysis (strict consensus not shown here) are marked with an asterisk. Respective bootstrap values are provided below branches. Branches inconsistently resolved in MP include Drosera (sister to Nepenthes), the Frankenia-Plumbago lineage (sister to the carnivorous clade), Simmondsia and Rhododendron (in a polytomy), Nyctuginaeae and Sarcobatus (in a polytomy), Basellaceae and Halophyton (in a polytomy).
data and insights into possible deviations of biosynthetic pathways are needed.

The clade including Aizoaceae, Gisekiaceae, Hyperbelis bowkeriana (Molluginaceae), Nyctaginaceae, Petiveriaceae and Phytolaccaceae corresponds to higher core I Caryophyllales (Hilu & al. 2003) or the raphide clade (Brockington & al. 2009). Surprisingly, Hyperbelis was placed sister to Gisekiaceae based on petD data, but this position lacks convincing support. Hyperbelis was not included in earlier studies, underscoring that a dense taxon sampling among Molluginaceae is needed to clarify if there are separate lineages within this family and how they are composed. In any case, betalain evolution in Caryophyllales is much more complex than previously thought. Rather than assuming a single origin of betalain biosynthesis in a common ancestor of all betalain taxa, multiple shifts between anthocyanin and betalain pathways may have to be considered in light of the current phylogenetic trees. Although their biosynthesis is mutually exclusive to anthocyanins (Stafford 1994), the anthocyanin taxa in Caryophyllaceae, Lophiocarpaceae, Limeaceae and Molluginaceae are scattered over a betalain producing radiation.

Disentangling Phytolaccaceae

The taxonomic history of Phytolaccaceae shows us how the family was disentangled step by step over the last decades. Whereas Heimerl (1934) included Agdestis, Barbeuia and Stegnosperma, the latter was raised to family level by Nakai (1942), a treatment supported by a morphological study of Bedell (1980). The first rbcL data (Manhart & Rettig 1994) did not support a placement of Stegnosperma within Phytolaccaceae. Further molecular phylogenetic analyses (Cuénoud & al. 2002) provided robust evidence of Stegnosperma being distant from other Phytolaccaceae in core Caryophyllales (Cuénoud & al. 2002), although the deep nodes in their analyses remained unsupported (however, there seems to be no matK fragment available in GenBank from Cuénoud & al. 2002). The multi-gene analysis of Brockington & al. (2009) and the petD data of this study clarified the isolated position of Stegnosperma in Caryophyllales, supporting its classification in a family of its own.

The three respective families (Agdestidaceae, Barbeuiaceae, Stegnospermacaeae) are now generally recognised (e.g., APG III). Lophiocarpaceae were published recently by Doweld & Reveal (2008) based on the results of Cuénoud & al. (2002) and contain two genera, Corbichonia and Lophiocarpus. They represent the most distant lineage as depicted in all molecular phylogenetic analyses (Cuénoud & al. 2002; Brockington & al. 2009; this study, Fig. 1–4).

Close relatives of Phytolaccaceae are in the so-called “raphide clade” (Brockington & al. 2009) that also has been recovered by most previous analyses (Cuénoud & al. 2002) and is corroborated here (Fig. 4). The presence of raphid crystals in vegetative tissues of members of this group of taxa was initially described by Rodman (1994). Families within this clade are Agdestidaceae (not sampled by Brockington & al. 2009), Aizoaceae, Gisekiaceae, Nyctaginaceae, Petiveriaceae (not sampled by Brockington & al. 2009), Phytolaccaceae and Sarcobataceae. The last family was separated by Behnke (1997) from Chenopodiaceae considering form and size of sieve-element plastids and first rbcL data (Clement & Mabry 1996a).

Nyctaginaceae are recovered in the present study with maximum support in both MP and BI trees, which is slightly higher than earlier [85 % BS and 96 % JK respectively (Cuénoud & al. 2002; Hilu & al. 2003)]. Petiveria as nested within Phytolaccaceae s. fam. Rivinoideae was found previously with only medium confidence (Cuénoud & al. 2002) but is recovered here also with petD data (no complete matK CDS for Petiveria is yet available). With Phytolacca being sister to a clade of Sarcobatus and Agdestis, it is obvious that the familial circumscription of Phytolaccaceae even only including Agdestidoideae, Phytolaccoidae and Rivinoideae currently does not represent a monophyletic group. It will not only be necessary to broaden the taxon sampling among Phytolaccaceae s. str, but also to carry out a thorough study of both molecular and morphological characters. Based on matK (Cuénoud & al. 2002) data, Sarcobatus was found sister to Nyctaginaceae (unsupported) and Agdestis sister to Phytolaccaceae s. fam. Rivinoideae. In the same study, after combining sequences from rbcL and matK, Agdestis and Sarcobatus appear as sisters with 77 % BS support, congruent to findings in Hilu & al. (2003). PetD trees of this study give another hint for the close relationship of these two families (Fig. 1, 2).

Microtea as isolated lineage and description of Microteaeae as a new family

The isolated phylogenetic position of Microtea is highly supported statistically in all our trees (Fig. 1–4). The same regards to a very distant relationship of Microtea and Lophiocarpus. The latter is sister to Corbichonia (formerly classified within Molluginaceae; Endress & Bittrich 1993) in an early-branching position of the so-called “globular inclusion clade”. Microtea and Lophiocarpus have long been suspected as being deviant members of different families. Heimerl (1934) thought the two genera share characters of Chenopodiaceae and Phytolaccaceae. The organisation of the ovary was considered more similar to Amaranthaceae/Chenopodiaceae than to Phytolaccaceae (Eckardt 1954, 1964, 1974). The subfamily Microteoideae of Phytolaccaceae was formally recognised by Nowicke (1968). Although this...
Fig. 5. Microtea portoricensis Urb. – holotype in the herbarium of the Botanic Garden and Botanical Museum Berlin-Dahlem (B).
treatment was accepted by Rohwer (1993), he mentioned that Microteoideae may eventually be better classified within Chenopodiaceae. Behnke (1993) also considered Microtea to have an unclear familial position. Previous molecular phylogenetic studies of Caryophyllales (e.g., Cuénoud & al. 2002) or Chenopodiaceae (e.g., Kadereit & al. 2003) did not sample Microtea. Its inclusion in the current study thus yields clear data for an unequivocal reclassification.

Microtea was described by Swartz in 1788. Unique to this genus are the muricate to spiny achenes (Urban 1885), whereas the single-ovuled ovaries are shared with Lophiocarpus (Urban 1885; Eckardt 1964; Nowicke 1968). The latter character was also the reason to describe Phytolaccaceae subfam. Microteoideae Eckardt ex Nowicke (1968), comprising those two genera. All species of Microtea are annual herbs with small flowers in racemiform thyrsoid inflorescences (see Fig. 5). Several species were described in this genus by Moquin (1849), then by Urban (1885) from the Antilles, but also under Lophiocarpus [e.g., M. burchelii (Hook.f.) N.E. Br.], and more recently by Marchiorotto & deSiqueira (1998) from Brazil. There are 21 species names and six additional names of infraspecific taxa. In the absence of a modern monograph, the diversity of Microtea and the Microteaceae may be estimated to encompass a dozen species. Microteaceae has a distribution ranging from Central America and the Antilles throughout South America and is thus one of the families restricted to the Neotropics.

Microteaceae Schäfferhoff & Borsch, fam. nov.
Type: Microtea Swartz, Prodr. Ind. Occ. 4: 53. 1788.
Herbae annuae, raro basi suffrutescentes; caulibus erectis vel ascendentibus, ramosis, usque ad 25(–60) cm altis; foliis alternis, sessilibus, lanceolatis vel ovato-ellipticis, glabris; inflorescentia spiculata vel racemosa; floribus hermaphroditis, actinomorphis, pedicellatis aut sessili- glabris; inflorescentia spiculata vel racemosa; floribus aequilongis vel brevioribus; tepalis 5 (vel 4), aequalibus, ellipticis vel lineari-ellipticis, membranaceis, flore aequilongis vel brevioribus; tepalis 5 (vel 4), aequabilibus, ellipticis vel lineari-ellipticis, uninervibus; staminibus 5–8, filamentis filiformibus subhypogynis imoque calycis insertis, 5 exterioribus cum ejusdem laciniae alternis; pollinis graminibus pantoporatatis et aque microspinulosis; ovario simplex, uniloculari; ovula campylotropa; stylis 3–5, distinctis; stigmatibus piloso-penicilliformibus; achenesi laevibus, subreticulato-tuberculatis vel projecturis spiniformibus dense tectis; seminibus lenticularibus vel subglobosis, testa nigra, crustaceae.

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Appendix 1.

Plant material used in this study

Appendix 1. Plant material used in this study. Collections made in the field first list country and locality in the case of both herbarium specimens and silica gel dried samples. Samples obtained from the living collections of the Botanic Garden Berlin-Dahlem and Bonn University Botanical Gardens first list the garden accession number and then country and locality data in square brackets. Collector and collection number are given in italics, the herbarium abbreviation in parentheses. Information on the specific history of the plant individual sampled can be obtained from the label that goes with the herbarium voucher. A unique identifier is given for every DNA isolate; it follows the specimen data and precedes the herbarium voucher. The herbarium abbreviation in parentheses is also included. Sequences of outgroups are completely from the matrix of Worberg et al. (2007). For sequences generated from other material than the isolated here, the respective publication is indicated (see below).


Caryophyllales: Achatocarpaceae: Achatocarpus praecox Grieseb., BGBM 142-78-94-10 [Argentina], Leuenberger, Arroyo-Leuenberger & Eggl 4345 (B), AC073, petD FN598616 (this study), matK AY514845. Phaulothamnus spinescens A. Gray, USA, Texas, Borsch, Müller & Pratt 3446 (B, ISC), AC060, petD FN598617 (this study), matK AY514846. Agdestis-ticideae: Agdestis elmentidea Moc. & Sessé, Mexico, Sáenz V. 5583 (MEXU), AC418, petD FN598638 (this study); Cuénoud & al. (2002), matK AY042538. Aizoaceae: Delosperma cooperi L. Bolus, BG Bonn 3632 [without locality data], Schäferhoff s.n. (BONN), AC331, petD FN598633 (this study). D. echinatum Schwantes, Cuénoud & al. (2002), matK AY042575. Lampranthus blands Schwantes, BGBM 016-19-82-70 [South Africa], Cubr 28489 (B-Gartenherbar), AC642, petD FN598634 (this study), matK FN597631 (this study). Amaranthaceae: Alternanthera caracasana Kunth, USA, Texas, Borsch, Müller & Pratt 3433 (B, ISC), AC058, petD FN598619 (this study). A. flavescens H.B.K., Sage & al. (2007), matK AM887484. A. sessili R.Br., Müller & Borsch (2005), matK AY514796. Amaranthus graggii S. Watson, USA, Texas, Pratt, Müller, Borsch 207 (B, ISC), AC059, petD FN598622 (this study). A. viridis L., Sage & al. (2007), matK AM887488. Bosea yervanora L., BG Meise 75-2966 [without locality data], no voucher, AC029, petD FN598623 (this study). Iresine palmeri Standl., USA, Texas, Borsch, Müller & Pratt 3445 (B, ISC), AC054, petD FN598618 (this study). A. alternifolia (Unile & W. L. Bray) S. Watson, Borsch & al. (unpubl. data), matK AM887490. Piletopetalum sprucei Standl., BG Bonn 16484 [Venezuela], Borsch 3996 (B), AC020, petD FN598621 (this study), matK AF542596. Pitolius exaltatus Nees, BGBM 231-15-74-80 [Australia], Cubr 46421 (B-Gartenherbar), AC644, petD FN598620 (this study). Anacampserotaceae: Anacampseros vulcanensis A. von., BG Bonn, Borsch 3595 (BONN), AC049, petD FN598660 (this study), matK AF542597. Anisterculaceae: Anistocladus abbreviatus Airy Shaw [without locality data], BG Bonn 16430, Schäferhoff 5 (BONN), AC291, petD FN598602 (this study); Meimberg & al. (2001), matK AF315939. Asteropeaceae: Asteropea micraster Hallier f., Cuénoud & al. (2002), matK AY042549. Barbeuiaceae: Barbeuia madagascariensis Steud., Cuénoud & al. (2002), matK AY042552. Basellaceae: Anredra brachystachys (Moq.) Sperling, BGBM 185-03-98-33 [Ecuador], Cubr 46782, AC546, petD FN598654 (this study), matK FN597626 (this study). Baselia alba L., BG Bonn 03671 [without locality data]: Schäferhoff 8 (BONN), AC299, petD FN598653 (this study); Cuénoud & al. (2002), matK FN598653. Cactaceae: Browningia hertlingiana (Backerb.) Buchb., BG Bonn 2416, [no voucher yet], petD FN598666 (this study). Harrisia pomanensis (F.A.C. Weber ex K. Schum.) Bbriton & Rose, 160-37-86-10 [Argentina, La Rioja], Leuenberger 3608 (B), AC632, petD FN598664, matK FN597629 (this study). Harrisia sp., BGBM 304-08-99-10 (Dominican Republic, Pedernales), Cubr 46781 (B), AC639, petD FN598665 (this study), matK FN597627 (this study). Opuntia microdasys (Lehm.) Pfeiff., without locality data, Schäferhoff s.n. (BONN), AC312, petD FN598667 (this study). Pereikhailov DEC, BG Bonn 22, [Guadeloupe]. Schäferhoff s.n. (BONN), AC300, petD FN598662 (this study). P. ziniiflora DEC, BGBM 261-19-93-60/1 [Cuba], Cubr 40527 (B-Gartenherbar), AC645, petD FN598663 (this study), matK FN597625 (this study). Rhipsalis paradoxa Salm-Dyck, Worberg & al. (2007), petD AM396555;
Nyffeler (2002) matK AM396551. Caryophyllaceae: Corrigiola litoralis L., BG Bonn 03472 [without locality data], Schäferhoff s.n., petD FNS98610 (this study). Illecebrum verticillatum L., Germany, Borch & Müller 3541 (B), AC063, petD FNS98612 (this study), matK AY514849. Paronychia canariensis Juss., BGBM 041-36-00-102 Spain, Teneriffa, Dürrbye 1126 (B), AC641, petD FNS98614 (this study), matK FNS97636 (this study). Pollichia campesiris Aiton, South Africa, Müller 850 (B), AC353, petD FNS98613 (this study). Scleranthus perennis L., Germany, North Rhine Westfalia, Eifel, Borsch 3389 (B), AC034, petD FNS98609 (this study), matK AY514847. Silene oiters Sm., Netherlands, Borsch 3495 (B), AC071, petD FNS98608 (this study), matK AY514848. Stellararia holostea L., BG Bonn 14501, Germany, Eifel, Krätin & s.n. (BONN), AC293, petD FNS98611 (this study). Chenopodiaceae: Allenrolfae vaginata Kunzke, BG Bonn 2488 [Argentina], Schäferhoff 3994 (B, BONN), AC017, petD FNS98625, matK AY514828. Atriplex elegans (Moq.) D. Dietr., USA, Borsch, Pratt & Müller 3425 (B, KAS, ISC), petD FNS98628 (this study), matK AY514830. Chenopodium hircinum L., Germany, North Rhine Westfalia, Eifel, Schäferhoff 10351 (B, JUa, lPB), ac661, petD fn598643 (this study), matK fn597635 (this study). Portulacaria afra Lam. f., South Africa, Swaziland, C. Afri cana Cape, Wondafrash 2105 (B, LBP), AC662, petD FNS98607 (this study), matK FNS97533. Molluginaeae: Hypertelis bowkeriana Sond., BG Bonn 9964 [without locality data], Schäferhoff 54 (BONN), AC314, petD FNS98635 (this study). Mollugo brevipes Urb., Cuba, Borsch, Rankin, Urquiola & Acosta 3947 (B, HAJB, HPFR), AC628, petD FNS98649 (this study). M. cerviana (L.) Ser., South Africa, Müller 847 (B, PRE), AC352, petD FNS98648 (this study). M. verticillata L., Fior & al. (2006), matK AY936330. Montiaceae: Claytonia arctica Adam, Russia, Verchwoye reki Opi Oiskoje plato Scheben, Andrianoova s.n., AC332, petD FNS98650 (this study). C. perfoliata Donn. ex Willd., Germany, Bochum, Schäferhoff s.n. (BONN), AC333, petD FNS98651 (this study). Montia parvifolia (Moq. ex DC.) Greene, Cuénoud & al. (2002), matK AY042616. Nepenthaceae: Nepenthes lowii Hook. f., BG Bonn 09957 [without locality data], Schäferhoff s.n. (BONN), Foto, AC302, petD FNS98599 (this study). N. madagascariensis Poir., BG Bonn 25236 [Madagascar]: Barthlott s.n. (BONN), AC335, petD FNS98598 (this study). Nyctaginaceae: Bougainvillia glabra Choisy, BG Bonn 18196 [without locality data], Schäferhoff 2 (BONN), AC295, petD FNS98646 (this study), Cuénoud et al. (2002), matK AY042560. Guapira riedeliania (Fisch.) Lundell, BGBM 060-95-74-8013 [without locality data], Schäferhoff 25465 (B), AC630, petD FNS98647 (this study), matK FNS97630 (this study). Mirabilis jalapa L., BG Bonn 03624 [without locality data], Schäferhoff (BONN), AC305, petD FNS98645 (this study), Cuénoud & al. (2002), matK AY042614. Oxypus acuminata Sweet, Cuénoud & al. (2002), matK AY042624. Petiveriaeae: Petiveria alliacea L., BG Bonn 8687 [without locality data], Schäferhoff 6 (BONN), AC309, petD FNS98644 (this study). Phytolaccaceae: Phytolaca dioica L., BG Bonn 17776 [Spain, Teneriffa]. Seehauer s.n. (BONN), AC296, petD FNS98640 (this study), Cuénoud & al. (2002), matK AY042631. Rivina humilis L., BG Bonn 9865 [without locality data], Borsch 3542 (B, BONN), AC203, petD FNS98642 (this study), matK AY514850. Sequi era aculeata Jacq., BGBM 05-43-74-83-00, Cab 20984 (B-Gartenherbar), AC659, petD FNS98641 (this study), matK FNS97634. Trichostigma peruvianum (Moq.) H. Walter, BGBM 136-79-74-833 (B-Gartenherbar), AC661, petD FNS98643 (this study), matK FNS97635 (this study). Plum-
baginaceae: Acantholimon lycopodioides (Girard) Boiss., BGBM 009-25-84-13/4 [Pakistan, Gilgit], Cabbr 27249 (B-Gartenherbar), AC640, petD FN598587 (this study), matK FN597642 (this study), L. monniera monotetal Boiss., BGBM 109-08-01/10-2 [France, Aude], Cabbr 43356 (B-Gartenherbar), AC643, petD FN598588 (this study), matK FN597641 (this study). L. latifolium (Sm.) O. Ktze., BG Bonn 03678 [without locality data], Müller 883 (BONN), AC127, petD FN598585 (this study), matK AY514861. P. bagoi aurea (G. & C.) Buch.-Ham. ex Bunge, BGBM 146-35-74-80 [without locality data], AC632, L., BGBM 16603 [without locality data], Schäfferhof 3 (BONN), AC297, petD FN598586 (this study).

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