The Linderniaceae and Gratiolaceae are further Lineages Distinct from the Scrophulariaceae (Lamiales)

R. Rahmannzadeh1, K. Müller2, E. Fischer3, D. Bartels1, and T. Borsch2
1 Institut für Molekulare Physiologie und Biotechnologie der Pflanzen, Universität Bonn, Kirschallee 1, 53115 Bonn, Germany
2 Nees-Institut für Biodiversität der Pflanzen, Universität Bonn, Meckenheimer Allee 170, 53115 Bonn, Germany
3 Institut für Integrierte Naturwissenschaften – Biologie, Universität Koblenz-Landau, Universitätsstraße 1, 56070 Koblenz, Germany

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Abstract: The Lamiales are one of the largest orders of angiosperms, with about 22,000 species. The Scrophulariaceae, as one of their most important families, has recently been shown to be polyphyletic. As a consequence, this family was re-classified and several groups of former scrophulariaceous genera now belong to different families, such as the Calceolariaceae, Plantaginaeae, or Phrymaceae. In the present study, relationships of the genera Craterostigma, Lindernia and its allies, hitherto classified within the Scrophulariaceae, were analyzed. Sequences of the chloroplast trnK intron and the matK gene (~2.5 kb) were generated for representatives of all major lineages of the Lamiales and the former Scrophulariaceae. Bayesian and parsimony analyses revealed two isolated lineages, one of which consists of Lindernia and its allies, the other of Gratiola and allies. Gratiola was previously assumed to be related to Lindernia and was therefore included here. It is proposed to treat the two clades as separate families, Linderniaceae and Gratiolaceae. For the Linderniaceae, several morphological synapomorphies exist in addition to molecular data, such as conspicuous club-shaped stamen appendages.

Key words: Lamiales, Scrophulariaceae, poikilohydric plants, Linderniaceae, Gratiolaceae, phylogeny.

Introduction

With more than 22,000 species, the Lamiales are one of the most diverse orders of angiosperms and include widely known plants such as mints, foxgloves, and snapdragons. The Lamiales are monoecyhyphytic and comprise important families such as the Acanthaceae, Gesneriaceae, Lamiales, Lentibulariaceae, and Scrophulariaceae. Moreover, they are of scientific interest because desiccation-tolerant plants, such as Craterostigma (Gaff, 1971), occur within this lineage. The present analyses were in part motivated in order to provide a phylogenetic context that can be used to reconstruct the evolution of characters involved in desiccation tolerance.

Traditionally, Craterostigma, Lindernia and their relatives have been treated as members of the family Scrophulariaceae in the order Lamiales (e.g., Takhtajan, 1997). Although it is well established that the Plocospermataceae and Oleaceae are their first branching families (Bremer et al., 2002; Hilu et al., 2003; Soltis et al., 2000), little is known about the evolutionary diversification of most of the order’s diversity. The Lamiales branching above the Plocospermataceae and Oleaceae are called “core Lamiales” in the following text. The most recent classification by the Angiosperm Phylogeny Group (APG2, 2003) recognizes 20 families. However, the recent literature (e.g., Olmstead et al., 2001; Fischer, 2004) shows that concepts of several families are in flux, as the number of genera increases that are included in phylogenetic analyses and as the addition of molecular characters continues to produce new results. The genera Craterostigma and Lindernia have so far not been included in any phylogenetic analysis.

Wettstein (1891) included Craterostigma and Lindernia into a tribe Gratiolaeae, and this was also followed by other authors (e.g., Takhtajan, 1997). However, the Linderniaceae were already recognized as a separate tribe of the Scrophulariaceae (Reichenbach, 1831) and as a subtribe of the Gratiolaeae (Bentham, 1846). These two authors based their entity on corolla shape and geniculate stamens to originally comprise the genera Arctanema, Curanga Juss. (= Picria Lour.), Torenia L. (incl. Craterostigma Hochst. as a section), Vandellia L., Lindernia All., Ilysanthes Rafin., Bonnaya Link et Otto, Peplidium Del., Micranthemum Rich., and Hemianthus Nutt. Urban (1884) lumped Ilysanthes and Bonnaya, with two fertile stamens, on the one hand, and Lindernia and Vandellia, with four fertile stamens, on the other. This concept with two genera, Lindernia and Ilysanthes, was also presented by v. Wettstein (1891). Principally due to arguments giving weight to particular characters, the classification system within the Linderniaceae continued to be modified. The genera Torenia and Craterostigma were separated out by Engler (1897), now using the winged calyx of Torenia as a main character. As a consequence, most species were included in a large genus Craterostigma. Pennell (1935) united Lindernia and Ilysanthes and argued that a reduction of stamens cannot be used as a generic character. Yamazaki (1954) tried to adopt characters such as seed anatomy and suggested division of Lindernia into Lindernia s.str., with non-alveolated seeds, and Vandellia, with bothrosporous seeds. However, this concept was ignored by most subsequent authors. Moreover, the monotypic Chamaegeigas was included in Lindernia.
by Obermeyer (1967). The genus *Crepidorhopalón* Eb. Fisch. was described as including former members of *Craterostigma*, *Lindernia*, and *Torenia* which share flax-like and characteristically hairy flowers on the lower corolla lip (Fischer, 1989). A further genus, *Harttiella*, was described during a revision of the Linderniae in Africa (Fischer, 1992).

The Scrophulariaceae in the circumscription of Bentham (1846, 1876) and Wettstein (1891) was traditionally treated as the largest family in the Lamiales. Other families, like the Orobanchaceae, Lentibulariaceae, Globulariaceae, or Plantaginaceae, were recognized based on morphological differences, but were considered to be derived from an ancestral stock of the Scrophulariaceae. Only Hallier (1905) included the latter families into the Scrophulariaceae, whereas most authors followed Wettstein in the circumscription and division of the Scrophulariaceae by dividing the family into the three subfamilies the Pseudoslanae, Antirrhinoideae, and Rhinanhoideae. Nevertheless, the Scrophulariaceae sensu Wettstein appeared as a heterogeneous and perhaps not natural group in detailed morphological studies (e.g., Fischer, 1992). The first molecular phylogenetic analysis (Olmedast and Reeves, 1995) was based on rbcL and ndhF sequences and indicated that the Scrophulariaceae are polyphyletic. Using a broader sampling and three genes, Olmedast et al. (2001) divided the Scrophulariaceae into several distinct families: (I) Scrophulariaceae s.str., comprising the Hemimerideae, Aptosimeae, Verbasceae, Manuleae, Selagineae, and also *Buddleja* (Buddlejeae) and *Myoporum* (Myoporaceae); and (II) the Veronicaceae, including the Gratiolae (without *Mimulus*), Antirrhineae, Clerodinae (without *Halleria*), Hemimerideae, Digitalideae, and also Callitriche (Callitrichaceae), Hippuris (Hippuridaceae), Globularia (Globulariaceae), and Plantago (Plantaginaceae). This latter clade is now referred to as the Plantaginaceae (APG II, 2003). Olmedast et al. (2001) included a core of the Gratiolae (*Gratiola, Amphianthus* and *Bacopa*) plus *Angelonia* (Hemimerideae) into the Plantaginaceae because it appears as sistergroup to the remaining Plantaginaceae in their analysis, although lacking support. The third division (III) comprises the Orobanchaceae, uniting all parasitic and semi-parasitic Scrophulariaceae, as outlined above. *Paulownia* is sister to the Orobanchaceae. Whereas v. Wettstein (1891) classified it within the Antirrhinoideae-Cheloneae, Olmedast et al. (2001) followed Nakai (1949) who described it in a separate family, the Paulowniaeae. The clade of *Calceolaria* and *Jovellana* (also comprising *Poroditia*) was found to be the most isolated among all scrophulariaceous lineages by Olmedast et al. (2001), so that these authors formally established the family name Calceolariaceae.

Hitherto, molecular analyses with a broad sampling of the Lamiales revealed *Mimulus* (tribe Gratiolae in v. Wettstein, 1891) in an isolated but unsupported position close to the Lamiales (Olmedast et al., 2001). A more detailed study (Beardsley and Olmedast, 2002) showed that the genus *Mimulus* is not monophyletic but revealed a well-supported clade comprising species of *Glossostigma*, *Peplidium*, *Leucocarpus*, *Berendtiella*, *Hemichaena*, *Mazus*, *Lancea*, and *Phryma*. This clade can be recognized as an extended family, the Phrymaeaceae.

All subsequent phylogenetic analyses with a broad representation of the Lamiales (Bremer et al., 2002; Hiu et al., 2003; Müller et al., 2004) confirmed the polyphyly of the Scrophulariaceae. Only some progress has been made with respect to resolving the backbone of core Lamiales, even when combining six different genomic regions (Bremer et al., 2002) or employing Bayesian approaches for tree inference (Müller et al., 2004). However, the Gesneriaceae and Plantaginaceae appear to be the first branching in both studies, although their relative positions are unsupported. The fossil record of the Lamiales only dates back to the Tertiary (Magallon et al., 1999). Therefore, the Lamiales represents a young angiosperm lineage, which possibly underwent rapid radiation in the Tertiary. This would imply that during early branching of the main lineages few mutations were fixed, which may also be one of the reasons for the difficulty to infer Lamiales relationships.

This study builds upon a set of complete sequences of the trnL intron (including the matK gene) that was originally generated to infer relationships of the Lentibulariaceae (Müller et al., 2004). The matK gene (approx. 1600 bp) codes for a maturation and is located within domain IV of the trnL intron (approx. 700 bp), upstream of psbA in the chloroplast genome large single copy region (Chiba et al., 1996; Neuhaus and Link, 1987). The aims of this contribution are to infer the phylogenetic relationships of *Lindernia* and its allies, in particular, testing their affinities to the Scrophulariaceae s.str. and the Plantaginaceae. Of particular relevance are those genera included into the Gratiolae (Wettstein, 1891). Molecular-based phylogenetic hypotheses are then discussed in relation to morphological characters in the context of an updated classification system.

Materials and Methods

Plant material

Most plants sequenced for this study were either cultivated in the Botanical Gardens of Bonn University or collected in the field by E. F. For detailed information see Table 1.

Amplification and sequencing

DNA was isolated from fresh or silica gel-dried tissues using a CTAB buffer method (Doyle and Doyle, 1990). Purification of genomic DNA was achieved using QiaQuick columns (Qiagen Inc., Valencia, California). For PCR amplification of the trnL intron, 25 μl volumes were used, containing 15 μl DNA template, 3.3 μl dNTP mix (1.25 mM each), 0.5 μl of each primer, and 1 u Taq Polymerase (Promega). The PCR profile was 1:30 min at 96°C, 1 min at 50°C, 1:30 min at 72°C, 35 cycles of: 0:30 min at 95°C, 1 min at 50°C, 1:30 min at 72°C, and a final extension of 10 min at 72°C. In most taxa, the region was amplified in two overlapping fragments. For primers used for DNA amplification and sequencing see Müller et al. (2004).

Alignment and coding of indels

The alignment of DNA sequences was done by eye using the program QuickAlign (Müller and Müller, 2003), which is designed for optimal manual sequence manipulation. Specific alignment rules were followed, as presented in Borsch et al. (2003). These alignment rules follow the principle to consider microstructural changes (e.g., simple sequence repeats) as single events, rather than limiting homology assessment to similarity-based searches using only single nucleotides as characters. This approach more closely reflects processes of sequence
Table 1  Taxa used in the present study, including familial affiliation, voucher information (Acronyms of Herbaria according to Index Herbariorum), and GenBank accession numbers. All sequences generated for this study are indicated as such. Classification of families, except for the Gratiolaceae and Linderniaceae, follows APG II (2003)

<table>
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Evolution in length variable genomic regions (e.g., Graham et al., 2000; Kelchner, 2000). Indel information was incorporated into parsimony analyses by producing a separate binary indel matrix according to the “simple indel coding” approach of Simmons and Ochoterena (2000), with the addition of coding adjacent gaps separately (Löhne and Borsch, in press) using the program SeqState (Müller, in press).

Maximum parsimony analyses

All parsimony analyses were done with PAUP version 4.0b10 (Swofford, 1998). In the heuristic searches, 1000 random addition cycles were carried out, applying TBR branch swapping, saving multiple trees, and assuming accelerated transitions. Bootstrapping was used to address confidence in individual clades. Swapping on up to 100 trees, starting from a simple-addition-tree, was performed for each of 1000 pseudoreplicates. This results in 95% confidence intervals of ±2% for bootstrap proportions of ≥95%, thus providing sufficient accuracy for conclusions to be drawn. Nodes gaining less than 50% were regarded as unsupported and collapsed in the majority rule consensus tree. Bremer support was calculated with the help of PRAP (Müller, 2004), applying the parsimony ratchet during searches under topological constraints.

Bayesian inference of phylogeny

The model of molecular evolution to be assumed in the Bayesian analysis was selected using the Akaike information criterion (Akaike, 1974), which rewards models for good fit but imposes a penalty for surplus parameters that may increase both computational effort and sensitivity to random error (Swofford et al., 1996). AIC values were calculated and the optimal model was selected with help of the program Modeltest (Posada and Crandall, 1998). The model chosen was a sub-model of the general time reversible model (GTR) (Rodriguez et al., 1990), with the two transitional rates united (TVM, Posada and Crandall, 1998). In additional hierarchical likelihood ratio tests, also performed with Modeltest, the assumption of two unequal transitional rates yielded an insignificant increase in likelihood scores over the null hypothesis of equal transitional rates. Moreover, among site rate variation following a gamma distribution (estimated shape parameter, 4 rate categories represented by the mean) and an estimated proportion of invariable sites were incorporated into the model (GTR+I+I). Using this model, Bayesian inference of phylogeny was performed with the program MrBayes 3 (Ronquist and Huelsenbeck, 2003). The a priori probabilities supplied were those specified in the default settings of the program. Posterior probability distributions of trees were created using the approach of a Metropolis-coupled Markov chain Monte Carlo and following recommended search strategies (Huelsenbeck et al., 2002; Huelsenbeck and Ronquist, 2001; Huelsenbeck et al., 2002). Four chains were run simultaneously, with the temperature of one heated chain set to 0.2. Chains were sampled every 10 generations and the respective trees were written to a tree file. Such a run of four chains was repeated three times, each time comprising 100,000 generations. In all cases, the probabilities converged onto the same stable value after about 25,000 generations, and calculation of the consensus tree and of the posterior probability of clades was based upon the trees sampled thereafter. Consensus tree topologies and posterior probabilities based on the different analyses were found to be essentially identical. A negligible divergence of clade probabilities between the three trees was restricted to few nodes.

Kishino-Hasegawa tests

To test hypotheses for particular nodes against the trees found in our phylogenetic analyses, we performed maximum likelihood-based two-tailed Kishino-Hasegawa tests using normal approximation and the GTR+I+I substitution model, with all parameters estimated from the data.

Results

The trnK intron in the Lamiales

trnK intron sequences were obtained and compiled for a total of 53 taxa (Table 1). On average, the sequenced fragment was about 2500 bp long, resulting in an alignment of 3508 positions. Lengths of the two non-coding parts of the trnK intron and of matK are provided in Table 2. Positions 238–255, 282–293, 457–468, 752–782, 910–931, 1417–1454, and 3032–3131 were excluded as hotspots because no robust positional homology could be established. Furthermore, the matrix was truncated at the beginning (positions 1 to 39) and the end (positions 3442 to 3508) to account for different positional extensions of primer reads, leaving 3169 characters for the phyloge-
netic analyses. Sequence divergence for all character partitions was around 10% and the ti/tv ratio was near 1.0 (Table 2).

Out of 291 distinguished indels, two thirds were less than 10 bp in length, but still about 4% were longer than 50 bp (up to a 127 bp insertion in *Jasminum*). Some indels were found to be synapomorphies for those clades discussed in more detail below, e.g., a deletion at pos. 150–163 for the Linderniaceae (cf. Fig. 3), and a deletion at pos. 199–202 for the Gratiolaceae.

Patterns of nucleotide and length mutations in the *trnK* intron in the Lamiales are basically those previously outlined (Müller et al., 2004, using a dataset less representative of the Lamiales and focusing on the Lentibulariaceae), the general picture did not change with the inclusion of further Lamiales lineages. Among the high number of indels found, many turned out to be synapomorphies when plotted on the tree (not shown; see examples above under “results”).

**Phylogenetic trees and topological tests**

Consensus trees derived from parsimony and Bayesian analysis largely agree with each other, although the Bayesian tree is better resolved (Figs. 3, 4). Both show strong support for a clade comprising *Craterostigma, Lindernia, Artanema, Torenia*, and *Crepidorhopolon* (annotated as the Linderniaceae in Fig. 4). Bremer support for this node is the highest in the whole tree, if the in-group node is ignored. Similarly, a highly supported Gratiolaceae clade consisting of *Gratiola, Otacanthus*, and *Bacopa* is found in both analyses, as well as a separate clade comprising representatives of the Scrophulariaceae sensu stricto (including former the Buddlejaceae and Myoporaceae). Further major clades resolved with *matK/trnK* are the Plantaginaeae (albeit only found in the Bayesian tree), and a clade with a successive branching of the Phrymaceae, Paulowniaceae, and Orobanchaceae. A very large terminal group within core Lamiales comprising the Phrymaceae, Paulowniaceae, Orobanchaceae, Acanthaceae, Lamiales, Martyniaceae, Verbenaceae, Pedaliaceae, Bignoniaceae, and Lentibulariaceae appears with high confidence in the Bayesian tree.

While the parsimony analysis remains inconclusive with respect to the relationship between the Linderniaceae, Gratiolaceae, and Scrophulariaceae s.str., the Bayesian approach reveals a position of the Linderniaceae clearly distinct from the Scrophulariaceae s.str. (posterior probabilities for relevant nodes are in bold). The position of the Gratiolaceae is not resolved in the Bayesian tree. Nevertheless, the Gratiolaceae together with the Calceolariaceae, Gesneriaceae, and Plantaginaceae are excluded with high probability from the remainder of the core Lamiales. Since the consensus of the trees found with parsimony (Fig. 3) is inconsistent with the hypothesis inferred with Bayesian statistics, likelihood-based two-tailed Kishino-Hasegawa tests were utilized, assuming a sister group relationship of the Scrophulariaceae and Gratiolaceae. The results show that a sister group of the Gratiolaceae and Scrophulariaceae is considerably less likely than the topology of all 20 shortest trees found (maximum unconstrained – in likelihood score 29,577,392,49, constrained 29,710,451,60, p = 0.000; all other: p < 0.05). Likewise, a clade uniting the Linderniaceae and Gratiolaceae is highly significantly less probable (constrained – in likelihood score 29,589,20034, p = 0.000; all other: p < 0.05).

The inclusion of indels in the parsimony analyses resulted in strict consensus and bootstrap trees not differing significantly from those obtained without indel information. Some clades received somewhat stronger bootstrap support, while bootstrap support decreased slightly for a similar number of nodes. In the Linderniaceae and Gratiolaceae clades on which this study focuses, Bremer support was generally raised when indel information was included. For example, support for the Gratiolaceae clade increased from 40 to 45, for *Oncanthus + Gratiola* from 15 to 16, while in the *Craterostigma + Lindernia* clade nodes received 7, 10, and 5 instead of 5, 9, and 4 (not shown).

**Discussion**

**Major lineages and relationships in the Lamiales**

Both Bayesian and parsimony approaches provide convincing support for the monophyly of the lineage encompassing the genera *Craterostigma, Lindernia, Artanema, Torenia*, and *Crepidorhopolon*. This lineage is annotated as the Linderniaceae in Fig. 4. A clade consisting of members of the former Gratiolaceae (*Gratiola, Otacanthus*, and *Bacopa*, but not *Mitimus*), here labelled as the Gratiolaceae, is also unequivocally resolved. The Bayesian tree shows clear evidence for the Linderniaceae being a lineage separate from the clade comprising the Scrophulariaceae sensu stricto (in the sense of Olmstead et al., 2001), as do our Kishino-Hasegawa tests. A monophyletic assemblage of the Gratiolaceae and Scrophulariaceae sensu stricto is also clearly rejected by Kishino-Hasegawa tests, which supports both as distinct lineages.

There are some backbone nodes of the Lamiales which are resolved for the first time with confidence: a major clade gaining 98% posterior probability (PP) is inferred that unites the Phrymaceae, Paulowniaceae, Orobanchaceae, Acanthaceae, Lamia-
Fig. 1 Linderniaceae – plant habit and seed morphology. (1) Craterostigma pumilum; (2) Crepidiorthalon whytei; (3) Lindernia microcalyx; (4) Lindernia philcoxii; (5) Lindernia nummularifolia, bothospermous seed; (6) Crepidiorthalon whytei, aulacospermous seed; (7) Lindernia microcalyx, non-alveolated seed.

Fig. 2 Abaxial stamens of various Linderniaceae showing geniculate filaments. (1) Craterostigma lanceolatum; (2) Crepidiorthalon whytei; (3) Lindernia microcalyx; (4) Lindernia philcoxii.
The Linderniaceae and Gratiolaceae are further Lineages Distinct from the Scrophulariaceae (Lamiales)

Fig. 3 Phylogenetic relationships in the Lamiales, as inferred by maximum parsimony analysis of the trnK intron, including the matK gene. Strict consensus tree of the 20 most parsimonious trees. Bootstrap percentages are indicated above, Bremer support values below the branches.

cae, Martyniaceae, Verbenaceae, Pedaliaceae, Bignoniacae, and Lentibulariaceae. Moreover, it is substantiated by the Bayesian tree of this matK/trnK dataset that the Gratiolaceae, Calceolariaceae, Gesneriaceae, and Plantaginaceae are early branching in core Lamiales. Parsimony analysis of three non-coding and three coding chloroplast regions combined by Bremer et al. (2002) found the same result, although their sampling was narrower (Gratiolaceae and Linderniaceae not included). Compared to Bremer et al. (2002), this study only includes about 20% of the characters. As found in the matK/trnK Bayesian analysis of Müller et al. (2004), the Scrophulariaceae s.str. come next, with high confidence, after these early branching families. The case is interesting because the respective nodes were not altered by the inclusion of additional taxa, which may be interpreted as additional evidence for their existence in the true phylogeny.
The circumscription and relationships of the Plantaginaceae appears to be in need of further clarification, in addition to the exclusion of the Gratiolaceae, as advocated here. Compared to \textit{rbcL+ndhF+ps2} (Olmstead et al., 2001), the position of \textit{Antirrhinum} is incongruent as inferred with \textit{matK/trnK}. A sister group relationship of \textit{Antirrhinum} and \textit{Chelone} appears with Bayesian and parsimony methods of phylogeny inference, contrary to a position as sister to a large clade of the Digitalaceae, Plantaginaceae, Globulariaceae, Haloragidaceae, and Callitrichaceae. Increased sampling of further species, along with the analysis of additional characters, will help to solve these problems. Recent results from analysis of \textit{Veronica} relationships (Albach and Chase, 2001) underscore the importance of broad taxon sampling, as even currently accepted larger genera may not be monophyletic and may hold surprises of hitherto un-predicted relationships.

\textbf{Monophyly of the Linderniaceae and Gratiolaceae}

Although the Gratiolaceae are united in a single tribe by Wettstein (1891), the available phylogenetic data provide evidence for two independent lineages. One of these lineages comprises \textit{Lindernia} and its allies, the other lineage is a core of the Gratiolaceae. For the former, the sampling in this study is quite representative. For the latter, much more detailed work will be required because not all the non-Linderniaceae genera formerly included into the Gratiolaceae by Wettstein (1891) are also close relatives of \textit{Gratiola}.
All genera that are resolved in the Linderniaceae clade (Lindernia, Crepidoraphalan, Torenia, Artanema, Craterostigma; Figs. 3, 4) share a special type of stamens in which the abaxial filaments are conspicuously geniculate, zig-zag-shaped or spurred (Craterostigma type and Crepidoraphalon type of geniculation, Fischer, 1992; Fig. 2). An origin of this feature can be reconstructed to have arisen in the common ancestor of the Linderniaceae, for which it resembles a synapomorphy, as shown in Fig. 4. Mostly, these geniculations have a knob- or club-shaped outgrowth and are covered with blue to yellow glandular hairs mimicking an anther with pollen, or are otherwise optically attractive (Fischer, 1992; Magin et al., 1989). Morphology therefore fully agrees with the Linderniaceae clade found based on molecular data. In addition, this clade shares the largest number of synapomorphies in the trnK/matK data when compared to other lineages within the core Lamiales. At this point, not all genera of the Linderniaceae, such as Hartliella, Camaegigas, Piceria, Scorophyllum etc., could be included into our molecular analysis because of limitations in material availability. However, they also share the characteristic stamen morphology.

Based on the above-mentioned morphological synapomorphy, the tribe Linderniaceae, as accepted by Fischer (2004), which corresponds to the Linderniaceae at the family level, is considered to comprise the genera listed in Table 3. Diversity centres are tropical Africa, with seven genera and 89 species, and Southeast Asia, with eight genera and 84 species. While the Asian representatives mainly occur in rain forest areas, the African Linderniaceae are mainly found in specialized habitats, such as seasonally water-filled rock pools, inselbergs, and heavy metal (copper or cobalt) soils.

The tribe Gratiolaceae was originally created by Bentham for Gratiola, Herpestis (= Bacopa) and its relatives. It is difficult to define the Gratiolaceae in terms of traditional morphology, but some features can help to separate it from other “Scrophulariaceae”. The most important characters occur in ovule and seed anatomy. Intermediate layers of ovule integument are 1–3, endothelial cells are large, transversally elongated, arranged in 6–8 longitudinal rows, and thickened only towards the endosperm; the endosperm of mature seeds is smooth or furrowed, the seeds have longitudinal ridges, and the testa cells show hook-like wall thickenings. Fischer (2004) recognizes 36 genera (excl. Linderniaceae) that can be assigned to the Gratiolaceae and belong to three tribes: Gratiolae, Stemediaceae, and Limoselleae (see below). The tribe Angeloniaceae comprising the South American genera Angelonia Bonpl. (25 spp.), and Monopera Barringer (2 spp.) appears closely related to the Gratiolaceae according to Olmstead et al. (2001). It differs, however, in the corolla tube which is bisaccate with 2 or 1 gibbous spurs abaxially and the intermediate layers of ovule integument which are 3–10. It remains to be clarified whether Angelonia (not sampled here) is sister to the Gratiolae, and thus should be included into the Gratiolaceae. The combined parsimony analysis of rbcl + ndhF + rps2 by Olmstead et al. (2001) would be in favour of such a treatment, although the low bootstrap value of 58% requires caution.

The genera that are classified within the Gratiolaceae based on morphology are provided in Table 3. Some of the genera that were included into the Gratiolaceae by Wettstein (1891) can be excluded based on the currently available phylogenetic data.

**Table 3** Genera of the Linderniaceae and Gratiolaceae, including their distribution and species numbers. Genera not sampled in the molecular analyses are assigned based on morphology.

**Linderniaceae**
- *Artanema* D. Don (4 spp.) in tropical Africa and Asia
- *Picria* Lour. (= Curanga [juss.] (1 sp.) in Indomalesia
- *Pierranthus* Bonati (1 sp.) in Southeast Asia
- *Schizotorenia* T. Yamaz. (2 spp.) in Southeast Asia
- *Legazpin Blanco* (1 sp.) in East Asia, Micronesia, and New Guinea
- *Scrophylleum* T. Yamaz. (2 spp.) in Southeast Asia
- *Hemiarheno* Benth. (1 sp.) in tropical Northwestern Australia
- *Chamaeigias* Dinter (1 sp.) in Namibia
- *Craterostigma* Hochst. (9 spp.) in tropical and South Africa, Arabia, Yemen, and India
- *Crepidoraphalon* Eb. Fisch. (28 spp.) in tropical and South Africa to Madagascar (1 sp.)
- *Hartliella* Eb. Fisch. (4 spp.) in Central Africa (Kongo, Katanga)
- *Torenia* L. (40 spp.) in Southeast Asia with 6 species in Africa and Madagascar
- *Lindernia* All. (incl. *Vandellia* L., *Bonnaya* Link and Otto, *Ilysanthes* Raf.) (ca. 100 spp.) in Europe (2 spp.), tropical Africa (40 spp.), Madagascar (17 spp.), Southeast Asia (40 spp.), North America (5 spp.), and Central and South America (7 spp.).

**Gratiolaceae**
- *Tribus Gratiolae*
- *Amphiathanus* Torr. (1 sp.) in the USA
- *Bacopa* Aubl. (c. 60 spp.) pantropical
- *Benjaminia* Mart. ex Benjam. (1 sp.) from Venezuela to Brazil
- *Boelkeei Rossov* (1 sp.) in Bolivia
- *Braunblanquetia* Eskuche (1 sp.) in Argentina
- *Caparria* L. (4 sp.) in tropical America
- *Deinostema* T. Yamaz. (2 sp.) in East Asia
- *Dopatrium* Buch.-Ham. ex Benth. (12 sp.) from tropical Africa to Asia
- *Gratiola* L. (20 sp.) in North America, Europe, and Asia
- *Hydrotiche* Zucc. (4 sp.) in Madagascar
- *Limnophila* R. Br. (36 spp.) in tropical Africa and Asia
- *Maevielia* Rossov (1 sp.) in South America
- *Mecardonia* Ruiz and Pav. (15 sp.) in temperate and tropical America
- *Philcoxia* P. Taylor and V. C. Souza (3 sp.) in Brazil
- *Scaparia* L. (20 sp.) in tropical America
- *Sophronanthe* Benth. (1 sp.) in Eastern USA
- *Stemediaceae*
- *Achetaria* Cham. and Schltdl. (5 sp.) in Brazil and the Caribbean
- *Adenosma* R. Br. (15 sp.) in China, Indomalesia, and Australia
- *Cheilophyllum* Pennell ex Britton (8 sp.) in the Caribbean
- *Conoea* Aubl. (7 sp.) in tropical America
- *Dcerya* B.L. Turner and Cowan (3 sp.) in Costa Rica and from Panama to Colombia
- *Dizygostemon* (Benth.) Radlk. ex Wettst. (2 sp.) in Brazil
- *Leucospora* Nutt. (1 sp.) in Eastern North America
- *Lindenbergia* Lehm. (12 sp.) in tropical Northeast Africa, Arabia, and tropical Asia
- *Morgania* R. Br. (4 sp.) in Australia
- *Oxacanthus* Lindl. (6 sp.) in Brazil
- *Schistophragma* Benth. ex Endl. (2 sp.) from Western North America to Central America
- *Schirosepala* G.M. Barroso (1 sp.) in Brazil
- *Stemodia* L. (56 spp.) in tropical America, Asia, and Africa
- *Stenodiplos* Engl. (6 sp.) in tropical Africa
- *Tetrulacium* Turcz. (1 sp.) in Brazil
- *Limoselleae*
- *Limosella* L. (15 sp.) in temperate Europe, Asia, North America, and tropical mountains
This applies to *Mimulus* which is now placed into the Phrymaecea (Beardsley and Olmstead, 2002), and to *Lindenbergia*. The position of *Lindenbergia*, however, is questionable. In several studies it forms a clade together with the parasitic Scrophulariaceae (Young et al., 1999; Olmstead et al., 2001). This is not supported by morphological data, which show a close resemblance to the tribe Stemodiae (Hjertson, 1995). The activation can vary from rhinaitoid to antheriod, even on a single plant (Hartl, 1955), and cannot be used for systematic placement. Generally, petal activation which was used as a major character by Wettstein (1891) is not very useful for delimitation of any lineage. Further molecular studies with increased taxon sampling are needed to test the relationships of the genera that could not be included in the present analysis.

**Family level classification of the Lindeniaceae and Gratiosiaceae**

The following new families have to be introduced:

**Lindeniaceae** (Rchb.) Borsch, K. Müller, and Eb. Fisch. stat. nov.
- Subtribe: Lindeniinae Benth., Prodr. 10: 343, 407 (1846)
- Type: *Lindernia* All.

**Gratiosiaceae** Martynov, Tekhno-Bot. Slovar: 249 (1820)
- Tribe: Gratiosiaceae Benth., Bot. Reg. 21; ad t. 1770 (1835)
- Subtribe: Gratiosiaceae Benth., Prodr. 10: 341, 367 (1846)
- Type: *Gratiola* L.

Based on genera that were included within the tribe Gratiosiaceae (Wettstein, 1891), two families have been published. The oldest name is Gratiosiaceae Martynov (1820), whereas Limosellacea J. Agardh (1858) is younger.

**Relationships within the Lindeniaceae and Gratiosiaceae**

The relationships within the Lindeniaceae as inferred with trnK/matK sequence data are well resolved and supported. Seed anatomy supports the recognition of three clades within the Lindeniaceae (Fig. 4). When a protraction of all endothelial cells is restricted to the central part of the cell, the endosperm surface becomes aleoelated (bothrosporous), as shown earlier by Fischer (1992) and Hartl (1959) to occur in the Craterostigma, part of *Lindernia*, *Artanema*, and *Torenia* (Fig. 1). By fusion of endosperm alveoles, longitudinally furrowed seeds arise. These are called aulacosporous (Fischer, 1992; Hartl, 1959) and occur in *Crepidorhapalton* and *Harttiella* (Fischer, 1992). The first clade of the Lindeniaceae comprises the genera with aleoelated, bothrosporous seeds (e.g., *Craterostigma*, *Lindernia* p.p. with *L. acicularis*, *L. philoxei*, *L. nummularifolia*, *L. subracemosa*, *Torenia*, and *Artanema*), the second clade comprises the genera with non-aleoelated endosperm (e.g., *Lindernia* s.str. with *L. rotundifolia*, *L. microcalyx*). In the third clade, the genera with aleoelated aulacosporous seeds (e.g., *Crepidorhapalton*) are included.

As the type species of *Lindernia*, *L. procumbens*, shares the same morphological characters with *Lindernia rotundifolia* and *L. microcalyx*, i.e., non-aleoelated seeds, these taxa remain in *Lindernia* s.str. *Lindernia procumbens* is a native and highly endangered species in Central Europe, for example occurring in the Rhine valley (Philippi, 1996), which unfortunately was not available for sampling. Some of the *Lindernia* species with bothrosporous seeds, which are resolved in the first clade together with *Craterostigma*, *Torenia*, and *Artanema*, might have to be transferred to *Vandellia*. Its type species, *Vandellia diffusa* L., is closely related to *L. nummularifolia* and *L. subracemosa*. However, further sampling is needed before any decision can be made along this line because several *Lindernia* species appear in a grade basal to *Craterostigma*. Altogether approximately 100 species are included into *Lindernia* sensu Pennell (1935), from which only a small fraction could be analyzed here, the division of *Lindernia* s.l. into two genera, i.e., *Lindernia* s.str. with smooth endosperm and *Vandellia* with aleoelated bothrosporous endosperm, was proposed by Yamazaki (1954). Furthermore, the trnK/matK tree confirms the generic concept of *Craterostigma* (type species *C. plantagineum*) proposed by Hepper (1987a) and Fischer (1992, 2004). Morphological characters of *Craterostigma* are the rosulate growth form with truncate inflorescence and the red to yellow root colour in the intercellular space of root cortex. The tree also confirms that *Crepidorhapalton* is not related to *Torenia*, as suggested by Hepper (1987b).

The sampled Gratiosiaceae are part of two tribes, the Gratiosiaceae and Stemodiae, which have also been recognized by Fischer (2004). A third and monotypic tribe, the Limosellacea with *Limosella*, could not be sampled, but probably also belong here. For an assessment of generic relationships, expanded sampling will be required.

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T. Borsch
Nees-Institut für Biodiversität der Pflanzen
Universität Bonn
Meckheimer Allee 170
53115 Bonn
Germany
E-mail: borsch@uni-bonn.de

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