

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

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Abstract: The Lamiales are one of the largest orders of angiosperms, with about 22000 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, Plantaginaceae, 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 22000 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 monophyletic and comprise important families such as the Acanthaceae, Gesneriaceae, Lamiaceae, 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 Gratioloae, and this was also followed by other authors (e.g., Takhtajan, 1997). However, the Lindernieae were already recognized as a separate tribe of the Scrophulariaceae (Reichenbach, 1831) and as a subtribe of the Gratioloae (Bentham, 1846). These two authors based their entity on corolla shape and geniculate stamens to originally comprise the genera *Artanema* D. Don, *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 Lindernieae 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 bothrospermous seeds. However, this concept was ignored by most subsequent authors. Moreover, the monotypic *Chamaegigas* was included in *Lindernia*

by Obermeyer (1967). The genus *Crepidorhopalon* Eb. Fisch. was described as including former members of *Craterostigma*, *Lindernia*, and *Torenia* which share aulacospermous seeds and characteristic hairs on the lower corolla lip (Fischer, 1989). A further genus, *Hartliella*, was described during a revision of the Lindernieae 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 (1903) 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 Pseudosolaneae, Antirrhinoideae, and Rhinanthoideae. 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 (Olmstead and Reeves, 1995) was based on *rbcl* and *ndhF* sequences and indicated that the Scrophulariaceae are polyphyletic. Using a broader sampling and three genes, Olmstead et al. (2001) divided the Scrophulariaceae into several distinct families: (I) Scrophulariaceae s.str., comprising the Hemimerideae, Aptosimeae, Verbasceae, Manuleae, Selagineae, and also *Buddleja* (Buddlejaceae) and *Myoporum* (Myoporaceae); and (II) the Veronicaceae, including the Gratiroleae (without *Mimulus*), Antirrhineae, Cheloneae (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). Olmstead et al. (2001) included a core of the Gratiroleae (*Gratiola*, *Amphianthus* and *Bacopa*) plus *Angelonia* (Hemimeridaceae) 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, Olmstead et al. (2001) followed Nakai (1949) who described it in a separate family, the Paulowniaceae. The clade of *Calceolaria* and *Jovellana* (also comprising *Porodittia*) was found to be the most isolated among all scrophulariaceous lineages by Olmstead 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 Gratiroleae in v. Wettstein, 1891) in an isolated but unsupported position close to the Lamiaceae (Olmstead et al., 2001). A more detailed study (Beardsley and Olmstead, 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 Phrymaceae.

All subsequent phylogenetic analyses with a broad representation of the Lamiales (Bremer et al., 2002; Hilu et al., 2003; Müller et al., 2004) confirmed the polyphyly of the Scrophulariaceae. Only some progress has been made with respect to re-

solving 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 *trnK* 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 maturase and is located within domain IV of the *trnK* group II 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 Gratiroleae (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 *trnK* 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 (Akronyms 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 APG2 (2003)

Species	Section/family	Origin (field/garden)	Voucher	GenBank accession number	Authors
<i>Avicennia germinans</i> L.	Acanthaceae	Bot. Gard. Bonn	T. Borsch and V. Wilde 3094 (FR)	AF531771	Müller et al. (2004)
<i>Dipteracanthus portellae</i> (Hook f.) Boom	Acanthaceae	Bot. Gard. Bonn	K. Müller 734 (BONN)	AF531773	Müller et al. (2004)
<i>Thunbergia alata</i> Boj. ex Sims	Acanthaceae	Bot. Gard. Bonn	K. Müller 740 (BONN)	AF531811	Müller et al. (2004)
<i>Campsis radicans</i> Seem.	Bignoniaceae	Bot. Gard. Bonn	K. Müller 701 (BONN)	AF531775	Müller et al. (2004)
<i>Kigelia africana</i> Benth.	Bignoniaceae	Bot. Gard. Bonn	–	AF051988	Young et al. (1999)
<i>Byblis gigantea</i> Lindl.	Byblidaceae	Bot. Gard. Bonn	K. Müller 733 (BONN)	AF531774	Müller et al. (2004)
<i>Calceolaria falklandica</i> Kraenzl.	Calceolariaceae	Bot. Gard. Bonn	F. Borsch 3788 (BONN)	AY667457	this study
<i>Streptocarpus bindseilii</i> Eb. Fisch.	Gesneriaceae	Bot. Gard. Bonn (Rwanda)	E. Fischer 1006 (KOBL, BR)	AF531810	Müller et al. (2004)
<i>Bacopa monnieri</i> (L.) Pennell	Gratiolaceae	Bot. Gard. Bonn	E. Fischer s.n. (BONN)	AY667458	this study
<i>Gratiola officinalis</i> L.	Gratiolaceae	Bot. Gard. Bonn	K. Müller 710 (BONN)	AF531777	Müller et al. (2004)
<i>Otacanthus coeruleus</i> Lindl.	Gratiolaceae	Bot. Gard. Bonn	F. Borsch 3789 (BONN)	AY667459	this study
<i>Lamium maculatum</i> L.	Lamiaceae	Bot. Gard. Bonn	K. Müller 737 (BONN)	AF531780	Müller et al. (2004)
<i>Genlisea aurea</i> St. Hil.	Lentibulariaceae	Bot. Gard. Bonn	K. Müller 748 (BONN)	AF531814	Müller et al. (2004)
<i>Genlisea uncinata</i> P. Taylor and Fromm-Trinta	Lentibulariaceae	Bot. Gard. Bonn	K. Müller 730 (BONN)	AF531819	Müller et al. (2004)
<i>Pinguicula agnata</i> Casper	Lentibulariaceae	Bot. Gard. Bonn	L. Legendre 1021 (NSW)	AF531782	Müller et al. (2004)
<i>Pinguicula alpina</i> L.	Lentibulariaceae	Bot. Gard. Bonn	J. Steiger s.n. (JE)	AF531783	Müller et al. (2004)
<i>Utricularia multifida</i> R. Br.	Lentibulariaceae	Bot. Gard. Bonn	K. Müller 719 (BONN)	AF531848	Müller and Borsch (in press)
<i>Utricularia vulgaris</i> L.	Lentibulariaceae	Bot. Gard. Bonn	K. Müller 743 (BONN)	AF531831	Müller and Borsch (in press)
<i>Artanema fimbriatum</i> D. Don	Linderniaceae	Bot. Gard. Bonn	F. Borsch 3790 (BONN)	AY667460	this study
<i>Craterostigma hirsutum</i> S. Moore	Linderniaceae	Bot. Gard. Bonn	E. Fischer 9003 (KOBL)	AF531776	Müller et al. (2004)
<i>Craterostigma plantagineum</i> Hochst.	Linderniaceae	Rwanda or S-Afrika	E. Fischer s.n.	AY667461	this study
<i>Craterostigma pumilum</i> Hochst.	Linderniaceae	Kenia	E. Fischer 8759 (KOBL)	AY667462	this study
<i>Crepidorhopalonwhitei</i> (Skan) Eb. Fisch.	Linderniaceae	Rwanda	E. Fischer 7657 (KOBL)	AY667463	this study
<i>Diascia barbarae</i> Hook. f.	Scrophulariaceae	Bot. Gard. Bonn	F. Borsch 3791 (BONN)	AY667464	this study
<i>Lindernia acicularis</i> Eb. Fisch.	Linderniaceae	Rwanda	E. Fischer 4230 (KOBL)	AY667465	this study
<i>Lindernia microcalyx</i> Stehlé	Linderniaceae	Bot. Gard. Mainz	–	AY667468	this study
<i>Lindernia nummulariifolia</i> (Benth.) Wettst.	Linderniaceae	Rwanda	E. Fischer 1410/87 (KOBL)	AY667467	this study
<i>Lindernia philcoxii</i> Eb. Fisch.	Linderniaceae	Rwanda	E. Fischer 553/87 (KOBL)	AY667466	this study
<i>Lindernia rotundifolia</i> (L.) Alston	Linderniaceae	Rwanda	E. Fischer 7320 (KOBL)	AY667469	this study
<i>Lindernia subracemosa</i> De Willd.	Linderniaceae	Rwanda	E. Fischer 1350 (KOBL)	AY667470	this study
<i>Torenia vagans</i> Roxb.	Linderniaceae	Bot. Gard. Edinborough	E. Fischer s.n. (KOBL)	AF531812	Müller et al. (2004)
<i>Ibicella lutea</i> v. Eselt	Martyniaceae	Bot. Gard. Bonn	K. Müller 735 (BONN)	AF531778	Müller et al. (2004)
<i>Proboscidea louisianica</i> (Mill.) Thell.	Martyniaceae	Bot. Gard. Bonn	K. Müller 706 (BONN)	AF531809	Müller et al. (2004)
<i>Jasminum nudiflorum</i> Lindl.	Oleaceae	Bot. Gard. Bonn	K. Müller 736 (BONN)	AF531779	Müller et al. (2004)
<i>Lathraea clandestina</i> L.	Orobanchaceae	–	–	AF051989	Young et al. (1999)
<i>Melampyrum sylvaticum</i> L.	Orobanchaceae	–	–	AF051991	Young et al. (1999)
<i>Orobanche caryophyllacea</i> Sm.	Orobanchaceae	–	–	AF051992	Young et al. (1999)
<i>Paulownia tomentosa</i> Steud.	Orobanchaceae	–	–	AF051997	Young et al. (1999)
<i>Pedicularis sylvatica</i> L.	Orobanchaceae	Bot. Gard. Bonn	K. Müller 744 (BONN)	AF531781	Müller et al. (2004)
<i>Uncarina grandideri</i> (Baill.) Stapf	Pedaliaceae	Bot. Gard. Bonn	K. Müller 707 (BONN)	AF531813	Müller et al. (2004)
<i>Mimulus guttatus</i> DC.	Phrymaceae	Bot. Gard. Bonn	F. Borsch 3792 (BONN)	AY667471	this study
<i>Antirrhinum majus</i> L.	Plantaginaceae	–	–	AF051978	Young et al. (1999)
<i>Chelone lyonii</i> Pursh	Plantaginaceae	Bot. Gard. Bonn	F. Borsch 3793 (BONN)	AY667472	this study
<i>Globularia nudicaulis</i> L.	Plantaginaceae	Bot. Gard. Bonn	F. Borsch 3794 (BONN)	AY667473	this study

continued →

Table 1 continued

Species	Section/family	Origin (field/garden)	Voucher	GenBank accession number	Authors
<i>Plantago media</i> L.	Plantaginaceae	Bot. Gard. Bonn	F. Borsch 3795 (BONN)	AY667474	this study
<i>Tetranema roseum</i> (Mart. and Gal.) Standley and Steyerem.	Plantaginaceae	Bot. Gard. Bonn	F. Borsch 3796 (BONN)	AY667475	this study
<i>Buddleja alternifolia</i> Maxim.	Scrophulariaceae	Bot. Gard. Bonn	K. Müller 732 (BONN)	AF531772	Müller et al. (2004)
<i>Myoporum montanum</i> R. Br.	Scrophulariaceae	Bot. Gard. Bonn	K. Müller 738 (BONN)	AF531808	Müller et al. (2004)
<i>Nicotiana tabacum</i> L.	Solanaceae	–	–	NC001879	Young et al. (1999)
<i>Solanum tuberosum</i> L.	Solanaceae	–	–	Z11741	Du Jardin, unpubl.
<i>Halleria tetragona</i> Baker	Stilbaceae	–	E. Fischer 108 (KOBL)	AY667476	this study
<i>Verbena rigida</i> Spreng.	Verbenaceae	Bot. Gard. Bonn	K. Müller 742 (BONN)	AF531820	Müller et al. (2004)

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 $\ll \pm 2\%$ for bootstrap proportions of $\geq 90\%$, 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+ Γ +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., 2001). 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 1000 000 generations. In all cases, the probabilities converged onto the same stable value after about 25000 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+ Γ +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 3442–3508) to account for different positional extensions of primer reads, leaving 3169 characters for the phyloge-

Table 2 Sequence characteristics for the 5' and 3' non-coding parts of the *trnK* intron and the *matK* coding region in the sampled Lamiales taxa. S.D. standard deviation; S.E. standard error; ti/tv transition–transversion ratio; % divergence = overall uncorrected *p* distance × 100; % variable/informative: percentage of variable/informative positions in alignment

Character set	Number of characters ¹	Length range	Mean length	S.D.	% divergence ¹	S.E. ¹	ti/tv ¹	S.E. ¹	% variable ¹	% informative ¹
5'	853	681– 741	716.925	15.487	10.028	0.445	1.088	0.098	50.762	31.301
<i>matK</i>	1 725	1445– 1596	1529.26	20.781	11.636	0.336	1.093	0.057	62.377	40.522
3'	591	188– 512	280.377	36.995	10.204	0.916	1.13	0.321	20.474	12.183

¹ Calculated after exclusion of hotspots and truncation of 5' and 3' end

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 *Crepidiorhopalon* (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 Plantaginaceae (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, Lamiaceae, 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 – ln likelihood score 29577.39249, constrained 29710.45160, $p \approx 0.000$; all other: $p \ll 0.05$). Likewise, a clade uniting the Linderniaceae and Scrophulariaceae is highly significantly less probable (constrained – ln likelihood score 29589.20034, $p \approx 0.000$; all other: $p \ll 0.05$).

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 *Otacanthus* + *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 *Crepidiorhopalon*. 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 *Mimulus*), 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, Lami-



Fig. 1 Linderniaceae – plant habit and seed morphology. (1) *Craterostigma pumilum*; (2) *Crepidorhopalon whytei*; (3) *Lindernia microcalyx*; (4) *Lindernia philcoxii*; (5) *Lindernia nummularifolia*, bothrospermous seed; (6) *Crepidorhopalon whytei*, aulacospermous seed; (7) *Lindernia microcalyx*, non-alveolated seed.

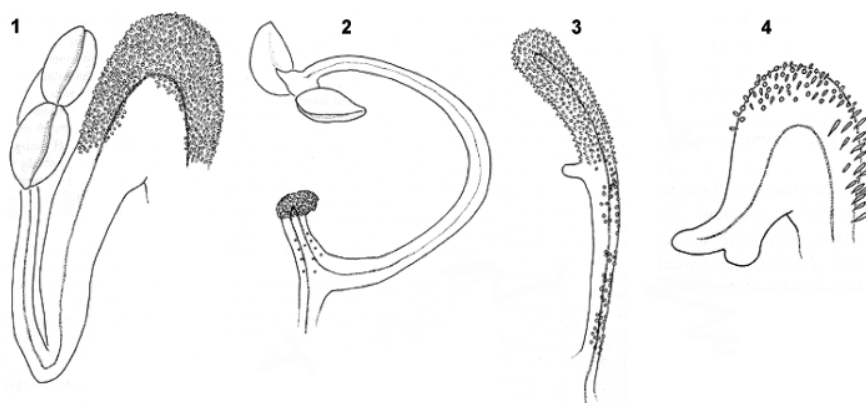


Fig. 2 Abaxial stamens of various Linderniaceae showing geniculate filaments. (1) *Craterostigma lanceolatum*; (2) *Crepidorhopalon whytei*; (3) *Lindernia microcalyx*; (4) *Lindernia philcoxii*.

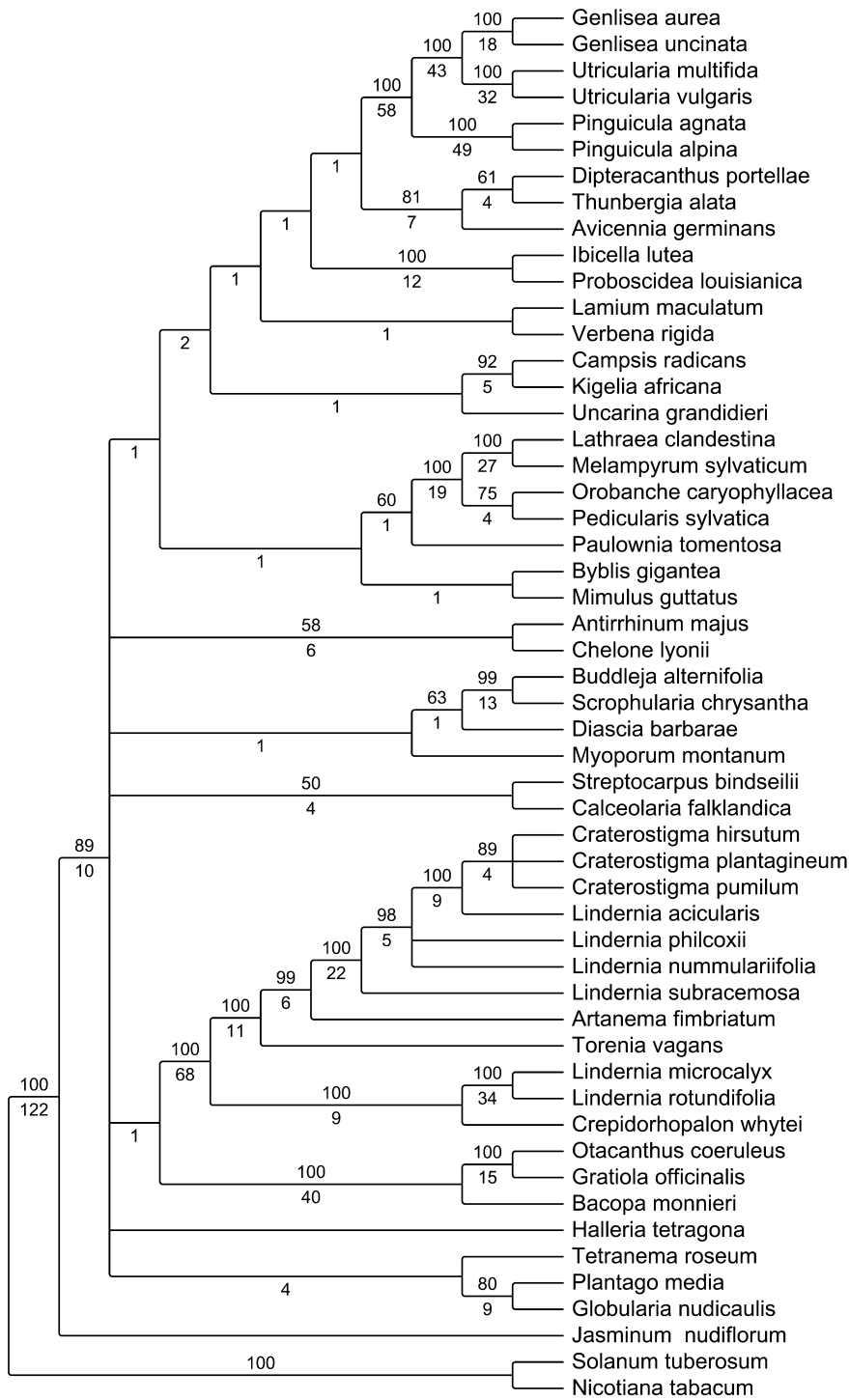


Fig. 3 Phylogenetic relationships in the Lamiales, as inferred by maximum parsimony analysis of the *trnK* intron, including the *matK* gene. Strict consensus tree of the 20 most parsimonious trees. Bootstrap percentages are indicated above, Bremer support values below, the branches.

ceae, Martyniaceae, Verbenaceae, Pedaliaceae, Bignoniaceae, 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.

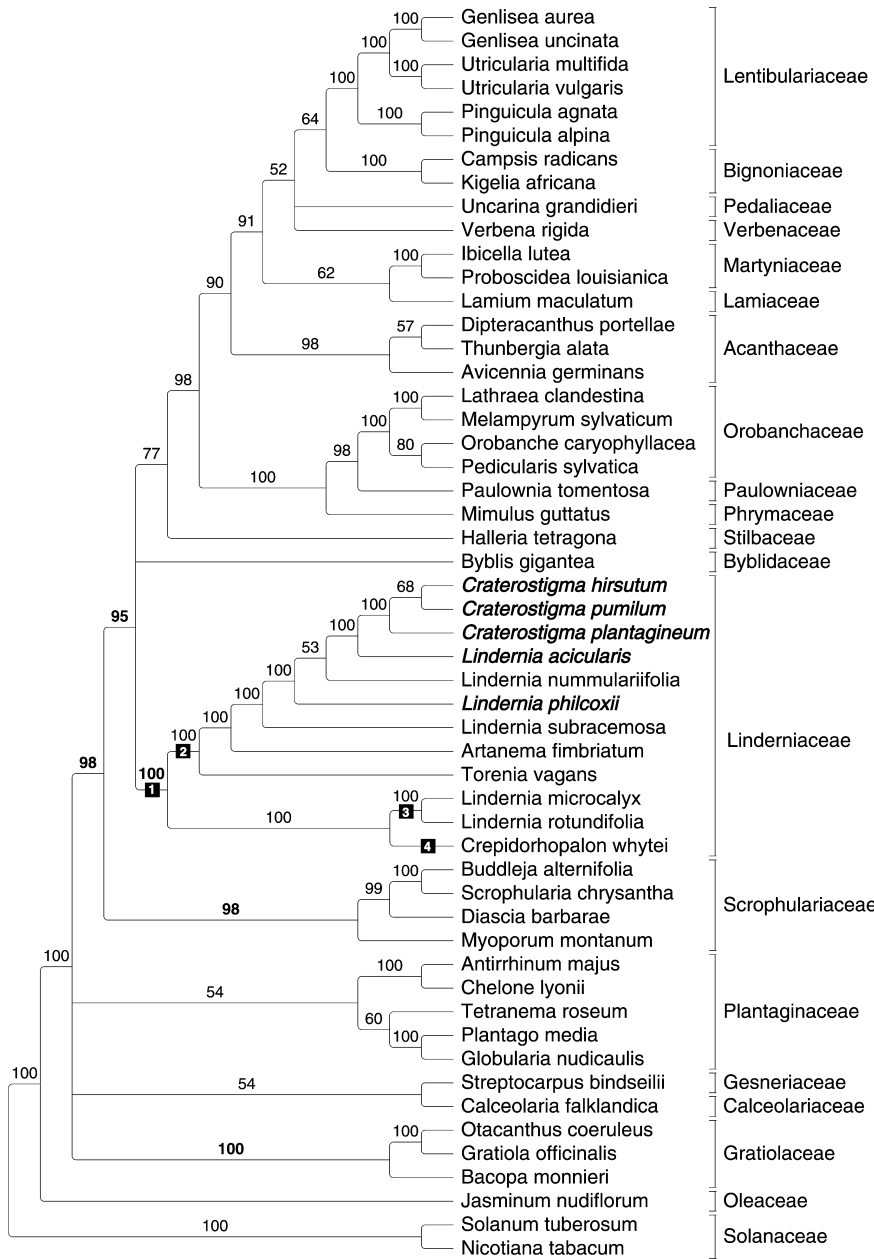


Fig. 4 Phylogenetic relationships in the Lamiales, as inferred by Bayesian analyses of the *trnK* intron, including the *matK* gene. Numbers above branches indicate posterior probabilities (%). Selected morphological features synapomorphic for particular clades are: (1) filaments of abaxial stamens geniculate, z-shaped or with spur-like appendages; (2) bothrospermous seeds; (3) non-alveolated seeds; (4) aulacospermous seeds. Family names follow APG2 (2003) except for the Linderniaceae and Gratiolaceae, which this study proposes to recognize. Desiccation-tolerant species are shown in red.

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 *rbcL+ndhF+rps2* (Olmstead et al., 2001), the position of *Antirrhinum* is incongruent as inferred with *matK/trnK*. A sister group relationship of *Antirrhinum* and *Chelone* appears with Bayesian and parsimony methods of phylogeny inference, contrary to a position as sister to a large clade of the Digitaleae, Plantaginaceae, Globulariaceae, Haloragidaceae, and Callitricaceae. Increased sampling of further species, along with the analysis of additional characters, will help to solve these problems. Recent results from analysis of *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 unpredicted relationships.

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 *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 *Gratiola*.

All genera that are resolved in the Linderniaceae clade (*Lindernia*, *Crepidorhaphalon*, *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 *Crepidorhaphalon* 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*, *Chamaegigas*, *Picria*, *Scolophyllum* 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 Lindernieae, 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 Gratiolaeae was originally created by Bentham for *Gratiola*, *Herpestis* (= *Bacopa*) and its relatives. It is difficult to define the Gratiolaeae 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. Lindernieae) that can be assigned to the Gratiolaceae and belong to three tribes: Gratiolaeae, Stemodieae, and Limoselleae (see below). The tribe Angelonieae comprising the South American genera *Angelonia* Bonpl. (25 spp.), and *Monopera* Barringer (2 spp.) appears closely related to the Gratiolaeae 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 Gratiolaeae, 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 Gratiolaeae 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 Indomalaysia
Pierranthus Bonati (1 sp.) in Southeast Asia
Schizotorenia T. Yamaz. (2 spp.) in Southeast Asia
Legazia Blanco (1 sp.) in East Asia, Micronesia, and New Guinea
Scolophyllum T. Yamaz. (2 spp.) in Southeast Asia
Hemiarrhena Benth. (1 sp.) in tropical Northwest Australia
Chamaegigas Dinter (1 sp.) in Namibia
Craterostigma Hochst. (9 spp.) in tropical and South Africa, Arabia, Yemen, and India
Crepidorhaphalon Eb. Fisch. (28 spp.) in tropical and South Africa to Madagascar (1 sp.)
Hartliella Eb. Fisch. (4 spp.) in Central Africa (Congo, 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 Gratiolaeae
Amphianthus Torr. (1 sp.) in the USA
Bacopa Aubl. (c. 60 spp.) pantropical
Benjaminia Mart. ex Benjam. (1 sp.) from Venezuela to Brazil
Boelckea Rossow (1 sp.) in Bolivia
Braunblanquetia Eskuche (1 sp.) in Argentina
Capraria L. (4 sp.) in tropical America
Deinostema T. Yamaz. (2 spp.) in East Asia
Dopatrium Buch.-Ham. ex Benth. (12 spp.) from tropical Africa to Asia
Gratiola L. (20 spp.) in North America, Europe, and Asia
Hydrotriche Zucc. (4 spp.) in Madagascar
Limnophila R. Br. (36 spp.) in tropical Africa and Asia
Maeviella Rossow (1 sp.) in South America
Mecardonia Ruiz and Pav. (15 spp.) in temperate and tropical America
Philcoxia P. Taylor and V. C. Souza (3 spp.) in Brazil
Scoparia L. (20 spp.) in tropical America
Sophranthe Benth. (1 sp.) in Eastern USA

Stemodieae

Achetaria Cham. and Schldtl. (5 spp.) in Brazil and the Caribbean
Adenosma R. Br. (15 spp.) in China, Indomalaysia, and Australia
Cheilophyllum Pennell ex Britton (8 spp.) in the Caribbean
Conobea Aubl. (7 spp.) in tropical America
Darcya B.L. Turner and Cowan (3 spp.) in Costa Rica and from Panama to Colombia
Dizygostemon (Benth.) Radlk. ex Wettst. (2 spp.) in Brazil
Leucospora Nutt. (1 sp.) in Eastern North America
Lindenbergia Lehm. (12 spp.) in tropical Northeast Africa, Arabia, and tropical Asia
Morgania R. Br. (4 spp.) in Australia
Otacanthus Lindl. (6 spp.) in Brazil
Schistophragma Benth. ex Endl. (2 spp.) from Western North America to Central America
Schizosepala G.M. Barroso (1 sp.) in Brazil
Stemodia L. (56 spp.) in tropical America, Asia, and Africa
Stemodiopsis Engl. (6 spp.) in tropical Africa
Tetraulacium Turcz. (1 sp.) in Brazil

Limoselleae

Limosella L. (15 spp.) in temperate Europe, Asia, North America, and tropical mountains

This applies to *Mimulus* which is now placed into the Phryma-
ceae (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 Scrophulari-
aceae (Young et al., 1999; Olmstead et al., 2001). This is not
supported by morphological data, which show a close resem-
blance to the tribe Stemodieae (Hjertson, 1995). The aestiva-
tion can vary from rhinantoid to antirrhinoid, even on a single
plant (Hartl, 1955), and cannot be used for systematic place-
ment. Generally, petal aestivation which was used as a major
character by Wettstein (1891) is not very useful for delimita-
tion of any lineage. Further molecular studies with increased
taxon sampling are needed to test the relationships of the gen-
era that could not be included in the present analysis.

Family level classification of the Linderniaceae and Gratiolaceae

The following new families have to be introduced:

Linderniaceae (Rchb.) Borsch, K. Müller, and Eb. Fisch.
stat. nov.

- Tribe: Lindernieae Rchb., Fl. Germ. Excurs. 1 (3): 385 (1831)
- Subtribe: Linderniinae Benth., Prodr. 10: 343, 407 (1846)
- Type: *Lindernia* All.

Gratiolaceae Martynov, Tekhno-Bot. Slovar: 249 (1820)

- Subfam.: Gratioloideae (Benth.) Leurss., Handb. Syst. Bot. 2: 993 (1882)
- Tribe: Gratioleae Benth., Bot. Reg. 21: ad t. 1770 (1835)
- Subtribe: Gratiolinae Benth., Prodr. 10: 341, 367 (1846)
- Type: *Gratiola* L.

Based on genera that were included within the tribe Gratioleae
(Wettstein, 1891), two families have been published. The old-
est name is Gratiolaceae Martynov (1820), whereas Limosella-
ceae J. Agardh (1858) is younger.

Relationships within the Linderniaceae and Gratiolaceae

The relationships within the Linderniaceae as inferred with
trnK/matK sequence data are well resolved and supported.
Seed anatomy supports the recognition of three clades within
the Linderniaceae (Fig. 4). When a protrusion of all endothelial
cells is restricted to the central part of the cell, the endosperm
surface becomes alveolated (bothrospermous), as shown ear-
lier by Fischer (1992) and Hartl (1959) to occur in the Cratero-
stigma, part of *Lindernia*, *Artanema*, and *Torenia* (Fig. 1). By fu-
sion of endosperm alveoles, longitudinally furrowed seeds
arise. These are called aulacospermous (Fischer, 1992; Hartl,
1959) and occur in *Crepidorphalon* and *Hartliella* (Fischer,
1992). The first clade of the Linderniaceae comprises the gen-
era with alveolated, bothrospermous seeds (e.g., *Craterostig-
ma*, *Lindernia* p.p. with *L. acicularis*, *L. philcoxii*, *L. nummulari-
ifolia*, *L. subracemosa*, *Torenia*, and *Artanema*), the second clade
comprises the genera with non-alveolated endosperm (e.g.,
Lindernia s.str. with *L. rotundifolia*, *L. microcalyx*). In the third
clade, the genera with alveolated aulacospermous seeds (e.g.,
Crepidorphalon) are included.

As the type species of *Lindernia*, *L. procumbens*, shares the
same morphological characters with *Lindernia rotundifolia*
and *L. microcalyx*, i.e., non-alveolated 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
bothrospermous seeds, which are resolved in the first clade to-
gether with *Craterostigma*, *Torenia*, and *Artanema*, might have
to be transferred to *Vandellia* L. Its type species, *Vandellia diffu-
sa* L., is closely related to *L. nummulariifolia* and *L. subracemosa*.
However, further sampling is needed before any decision can
be made along this line because several *Lindernia* species ap-
pear in a grade basal to *Craterostigma*. Altogether approxi-
mately 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., *Linder-
nia* s.str. with smooth endosperm and *Vandellia* with alveolat-
ed bothrospermous endosperm, was proposed by Yamazaki
(1954). Furthermore, the *trnK/matK* tree confirms the generic
concept of *Craterostigma* (type species *C. plantagineum*) pro-
posed by Hepper (1987a) and Fischer (1992, 2004). Morpho-
logical 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 *Crepidorphalon* is not related to *Torenia*, as
suggested by Hepper (1987b).

The sampled Gratiolaceae are part of two tribes, the Gratioleae
and Stemodieae, which have also been recognized by Fischer
(2004). A third and monotypic tribe, the Limoselleae with *Li-
mosella*, could not be sampled, but probably also belong here.
For an assessment of generic relationships, expanded sampling
will be required.

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