

PIECING TOGETHER THE “NEW” PLANTAGINACEAE¹

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Scrophulariaceae is one of the families that has been divided extensively due to the results of DNA sequence studies. One of its segregates is a vastly enlarged Plantaginaceae. In a phylogenetic study of 47 members of Plantaginaceae and seven outgroups based on 3561 aligned characters from four DNA regions (the nuclear ribosomal ITS region and the plastid *trnL-F*, *rps16* intron, and *matK-trnK* intron regions), the relationships within this clade were analyzed. The results from parsimony and Bayesian analyses support the removal of the Lindernieae from Gratiolaeae to a position outside Plantaginaceae. A group of mainly New World genera is paraphyletic with respect to a clade of Old World genera. Among the New World taxa, those offering oil as a pollinator reward cluster together. *Ourisia* is sister to this clade. Gratiolaeae consist of *Gratiola*, *Otacanthus*, *Bacopa*, *Stemodia*, *Scoparia*, and *Mecardonia*. Cheloneae plus *Russelia* and *Tetranema* together constitute the sister group to a clade predominantly composed of Old World taxa. Among the Old World clade, *Ellisiophyllum* and *Lafuentea* have been analyzed for the first time in a molecular phylogenetic analysis. The former genus is sister to *Sibthorpia* and the latter is surprisingly the sister to Antirrhineae.

Key words: ITS; *matK-trnK* intron; phylogeny; Plantaginaceae; *rps16* intron; Scrophulariaceae; *trnL-F*.

In the last 15 years, molecular systematists have reshaped our understanding of angiosperm evolution, culminating in the classifications of the Angiosperm Phylogeny Group (1998, 2003). Botanists are now faced with families whose circumscriptions have drastically changed relative to those of 20 years ago. One of those families is Scrophulariaceae, a family often considered to be heterogeneous and bound together by symplesiomorphies (Olmstead et al., 2001). Several characters have been used to delimit subfamilies in Scrophulariaceae, such as corolla aestivation (Bentham, 1846; Wettstein, 1895), anther morphology (Van Tieghem, 1903), and nectary morphology (Bellini, 1907). Certain genera (e.g., *Paulownia*; see Armstrong, 1985) have been moved back and forth between Scrophulariaceae and related families. It is therefore not surprising that DNA sequence analysis showed that Scrophulariaceae is polyphyletic. As first demonstrated by Olmstead and Reeves (1995), only a few genera grouped together with *Scrophularia* in their “scroph I” clade (hereafter called Scrophulariaceae sensu stricto [s.str.]), whereas some of the best known temperate genera of Scrophulariaceae, such as *Antirrhinum*, *Digitalis*, and *Veronica* were found in their “scroph II” clade. *Plantago*, *Hippuris*, and *Callitriche*, which traditionally were considered monogeneric families, were also included in “scroph II.” Further studies (Oxelman et al., 1999; Olmstead et al., 2001) have corroborated the results of Olmstead and Reeves (1995) and showed that other members of

the former Scrophulariaceae (hereafter, Scrophulariaceae sensu lato [s.l.]) either grouped with Orobanchaceae, Stilbaceae, and Phrymaceae or formed their own families (Paulowniaceae, Calceolariaceae). The “scroph II” clade was expanded by these analyses to include most of the well-known genera of Scrophulariaceae s.l. in the Northern Hemisphere and also Globulariaceae. The clade is now formally called Plantaginaceae although it is drastically expanded from what systematists considered it to be just a few years ago (APG, 1998). A proposal had been made to name this clade Antirrhinaceae or Veronicaceae in order to prevent confusion (Reveal et al., 1999), but Plantaginaceae, the oldest conserved name with priority, will be used here. With so many recent changes in family circumscription in angiosperms, Plantaginaceae constitutes yet another case in which botanists have to relearn a family circumscription.

Since the first report of the polyphyly of Scrophulariaceae (Olmstead and Reeves, 1995), several other large-scale analyses of Lamiales have also found good support for Plantaginaceae sensu APG (1998) but included few representatives of this clade (e.g., Oxelman et al., 1999; Olmstead et al., 2000; Albach et al., 2001; Bremer et al., 2002). Globulariaceae was first shown by Oxelman et al. (1999) also to belong to this clade. The analysis by Olmstead et al. (2001) markedly increased taxonomic sampling and included 15 genera in their “Veronicaceae.” Other analyses added additional genera (e.g., *Ourisia* and *Sibthorpia*; Albach and Chase, 2001). Oxelman et al. (in press) did a study in parallel to this, where they included also *Melosperma*, *Monttea*, *Stemodia*, *Mecardonia*, and *Campylanthus*, which all are shown to belong to Plantaginaceae. Together with their well-established relatives, this raises the number of genera in Plantaginaceae to about 92 (Table 1), with approximately 2000 species.

Despite the progress that has been made toward a new circumscription of Plantaginaceae via molecular phylogenetic studies, important questions remain. There are several genera that have not been assigned to one of the segregate families of Scrophulariaceae s.l. (such as Plantaginaceae, Orobanchaceae, Calceolariaceae, Phrymaceae) with any certainty and

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whose relationships to one another and within the family remain obscure. Whereas detailed intratribal analyses within the Plantaginaceae have been conducted, the intertribal relationships have not been addressed. Additionally, several enigmatic genera have been placed in surprising positions (e.g., *Aragoa* as sister to *Plantago*; Bello et al., 2002), which sheds new light on their evolution. Many other small genera have not been included in any analysis to date, and their addition may reveal unexpected new relationships. Therefore, reconstruction of a robust phylogenetic hypothesis of the “new” Plantaginaceae is important to clarify relationships within the family, characterize the family morphologically, and understand the evolution of its members and features such as secondary compounds and flower development.

We present here a phylogenetic hypothesis that focuses on Plantaginaceae based on four DNA markers that together comprise more than 3500 aligned characters: the nuclear ribosomal internal transcribed spacer (ITS) region, and the plastid *trnL-F* region, *rps16* intron, and *matK-trnK* intron. ITS is one of the most widely sequenced DNA regions in molecular systematics and has been used to assess intrageneric and intratribal relationships within Plantaginaceae in Veroniceae, *Plantago* and Cheloneae (Albach and Chase, 2001; Bello et al., 2002; Rønsted et al., 2002; Wolfe et al., 2002). The *trnL-F* region consists of the *trnL* intron, *trnL* 3' exon and the *trnL-F* spacer. It is one of the most frequently sequenced plastid markers used to assess intrageneric relationships, but it can also be used at the intergeneric level. Its utility has been demonstrated in Plantaginaceae by Rønsted et al. (2002) and Albach et al. (2004a). The *rps16* intron is another plastid DNA region, which has been employed in various groups (e.g., Oxelman et al., 1997; Samuel et al., 2001; Oxelman et al., in press). Within Plantaginaceae, however, it has so far been restricted to Veroniceae (Albach and Chase, 2004). The *matK-trnK* region, another frequently used plastid region (Hilu and Liang, 1997; Soltis and Soltis, 1998), has also been shown to be informative to assess relationships within and among genera of Plantaginaceae (Wolfe et al., 2002). All three plastid regions are not just informative among genera, but their utility has also been proven at higher taxonomic levels (e.g., the asterid subclass, Bremer et al., 2002). We therefore present here results from a rigorous phylogenetic analysis of 45 of the 92 genera considered to belong to Plantaginaceae, which will be used as a basis for discussion of the evolution of this diverse family and of some nonmolecular characters that may support the relationships found in our study.

MATERIALS AND METHODS

Taxon sampling—In our attempt to resolve relationships within Plantaginaceae, we have sampled 47 putative representatives (representing 45 genera) of the family (Appendix, see Supplemental Data accompanying online version of this article; Table 1). Our taxon sampling has been guided by previous morphology-based (e.g., Wettstein, 1895) and molecular-based (e.g., Olmstead et al., 2001) systematic studies. We tried to sample as many genera with unclear affinities as possible, while limiting the number of representatives of larger, well-circumscribed clades. We therefore included all likely genera of Plantaginaceae with the exception of some in well-defined groups such as Antirrhineae (sensu Sutton, 1988; 6/27 genera sampled), Veroniceae (sensu Albach et al., 2004b; 4/9 genera sampled), Cheloneae (sensu Wolfe et al., 1997; 4/10 genera sampled), and Lindernieae (sensu Fischer, 1992; 3/7 genera sampled). Only six of the approximately 27 genera of Gratiolateae were sampled here due to lack of material; further studies on this tribe will be necessary to ascertain its delimitation. All four markers were sequenced for all repre-

sentatives except for the *matK-trnK* intron in *Poskea* and *Hemiphragma*, the *trnL-F* region for *Globularia repens*, *Aragoa* and *Hemiphragma*, and ITS in *Angelonia*, due to the lack of sufficient DNA. Occasionally different accessions of the same species, or different species in the same genus, were sequenced for different markers (Appendix, see Supplemental Data accompanying online version of this article). Nine outgroup genera (Appendix, see Supplemental Data accompanying online version of this article) were chosen from a variety of lamialean families, for which some or all four DNA markers had already been sequenced and could thus be downloaded from GenBank, with an emphasis on Scrophulariaceae s.str. Thus, *Oreosolen* (Digitalideae sensu Wettstein, 1895), *Selago* and *Myoporium* were included even though at least one marker is missing for each of them, whereas all four markers were downloaded for the other six outgroups (Appendix, see Supplemental Data accompanying online version of this article). Among the 214 sequences used in our study, 130 are published for the first time (30 ITS, 24 *trnL-F*, 33 *rps16* intron, and 43 *matK-trnK* intron).

DNA extraction, amplification, and sequencing—Total genomic DNA was extracted from herbarium material or silica-gel-dried leaf samples according to the 2× hexacyclimethylammonium bromide (CTAB) procedure of Doyle and Doyle (1987) and then washed twice with 70% ethanol. DNA pellets were dried and resuspended in TE buffer. The *trnL-F* region (including the *trnL* intron, 3' exon, and *trnL-F* spacer) was amplified with primers c and f of Taberlet et al. (1991). ITS sequences (including a small section of 18S, ITS1, 5.8S, ITS2, and a small section of 26S rDNA) were amplified and sequenced using primers 17SE (Sun et al., 1994) or ITS1a (Downie and Katz-Downie, 1996) and ITS4 (White et al., 1991). Sequences of the *rps16* intron were amplified and sequenced using primers *rpsF* and *rpsR2* (Oxelman et al., 1997). The *matK-trnK*-intron was amplified and sequenced using primers *matK-8F* (Johnson and Soltis, 1994) and *trnK-2R* (Steele and Vilgays, 1994). PCR products were run on a 1.0% TBE-agarose gel, cut from the gel, and cleaned using a QIAquick PCR purification and gel extraction kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocols. Alternatively, the PCR product was cleaned with Centrisep columns (Princeton Separations Inc., Adelphia, New Jersey, USA) repacked with Sephadex G-50 fine beads (Amersham Biosciences Corp., Piscataway, New Jersey, USA), or QIA-Quick columns (QIAGEN Inc., Valencia, California, USA) following manufacturer's instructions. Sequencing reactions (10 μl) were carried out using 1–2 μl of the *taq* DyeDeoxy Terminator Cycle Sequencing mix (Applied Biosystems), and unincorporated dye terminators were removed either by cleaning with 4% Sodium acetate in ethanol followed by two rounds of cleaning in 70% ethanol or using Centrisep columns repacked with Sephadex before automated sequencing. Reactions were run out on a Prism 377 automated sequencer (Applied Biosystems) or a Basestation (MJ Research, Watertown, Massachusetts, USA), and both strands were sequenced. Sequences were assembled and edited using Sequence Navigator or Editview 1.0.1 (both by Applied Biosystems) or Sequencer versions 3.0 and 4.1 (Gene codes corporation, Ann Arbor, USA). Assembled sequences were manually aligned prior to analysis. Aligned sequence matrices are available from D.C.A. and H.M.M. by request. Parts of the ITS region are highly variable (corresponding to positions 135–207 (ITS 1), 568–603, and 787–end (both ITS2) and are only ambiguously alignable across Plantaginaceae. Analyses of the ITS alone were therefore conducted once excluding and once including these 174 characters. Combined analyses were conducted excluding those characters. A combined analysis of the three plastid DNA regions without the ITS data set was also conducted. Results of the analyses of all four DNA regions combined, however, were so similar that we do not show the results of the combined plastid data set.

Sequence analysis—Phylogenetic analyses were run on dual 800 MHz or 1.2 GHz PowerPC G4 computers, or the “Phylocluster”, an NPACI Rocks Linux cluster (<http://rocks.npaci.edu/Rocks/>) containing 13 dual-processor nodes using AMD Athlon processors (at The University of Texas Center for Computational Biology and Bioinformatics). Heuristic searches were performed in PAUP* version 4.0b10 (Swofford, 2002) with 10 random addition replicates and no MAXTREES limit under the parsimony criterion. Parsimony

TABLE 1. Synopsis of Plantaginaceae circumscription. A comparison of the hypothesized circumscription as proposed here and the placement of plantaginaceous genera as included in Wettstein's (1895) classification in his Scrophulariaceae sensu lato. Numbers following tribal names refer to number of genera in the tribe according to the present study or to the present study/Wettstein (1895). Taxa in bold are those included in our molecular phylogenies; underlined taxa are those that have been included in other molecular analyses.

Present study		Wettstein (1895)	
Antirrhineae (28)	Gratiroleae (25)	Antirrhineae (9/14)	Gratiroleae (37/38)
<i>Acanthorrhinum</i>	<i>Achetaria</i>	<u>Antirrhinum</u>	<i>Achetaria</i>
<i>Albraunia</i>	<i>Adenosma</i>	<u>Chaenorrhinum</u>	<i>Adenosma</i>
<u>Anarrhinum</u>	<i>Ambulia</i>	<u>Cymbalaria</u>	<i>Ambulia</i>
<u>Antirrhinum</u>	<u>Amphianthus</u>	<u>Galvezia</u>	<u>Amphianthus</u>
<i>Asarina</i>	<i>Artanema</i>	<u>Linaria</u>	<i>Artanema</i>
<u>Chaenorrhinum</u>	<u>Bacopa</u>	<u>Maurandya</u>	<u>Bacopa</u>
<u>Cymbalaria</u>	<i>Bryodes</i>	<i>Mohavea</i>	<i>Bryodes</i>
<i>Ephixiphium</i>	<i>Bythophyton</i>	<i>Rhodochiton</i>	<i>Bythophyton</i>
<u>Galvezia</u>	<i>Dizygostemon</i>	<u>Schweinfurthia</u>	<i>Conobea</i>
<i>Gambelia</i>	<i>Dodartia</i>	Digitalideae (14/20)	<u>Craterostigma</u>
<i>Holmgrenanthe</i>	<i>Dopatrium</i>	<u>Aragoa</u>	<i>Curanga</i>
<i>Holzneria</i>	<i>Encopella</i>	<i>Camptoloma</i>	<i>Dizygostemon</i>
<u>Howelliella</u>	<i>Geochorda</i>	<u>Campylanthus</u>	<i>Dodartia</i>
<u>Kickxia</u>	<u>Gratiola</u>	<u>Digitalis</u>	<i>Dopatrium</i>
<u>Lafuentea</u>	<i>Hydrantheium</i>	<u>Erinus</u>	<i>Encopa (=Encopella)</i>
<u>Linaria</u>	<i>Hydrotriche</i>	<u>Hemiphragma</u>	<i>Geochorda</i>
<i>Lophospermum</i>	<i>Ildefonsia</i>	<u>Lafuentea</u>	<u>Glossostigma</u>
<i>Mabrya</i>	<i>Limnophila</i>	<i>Lagotis</i>	<u>Gratiola</u>
<u>Maurandella</u>	<i>Limosella</i>	<u>Ourisia</u>	<i>Hydrantheium</i>
<u>Maurandya</u>	<u>Mecardonia</u>	<u>Picrorhiza</u>	<i>Hydrotriche</i>
<i>Misopates</i>	<i>Morgania</i>	<i>Scoparia</i>	<i>Ildefonsia</i>
<i>Mohavea</i>	<u>Otacanthus</u>	<u>Sibthorpia</u>	<i>Ilysanthes</i>
<i>Neogaerrhinum</i>	<u>Scoparia</u>	<u>Veronica</u>	<u>Lancea</u>
<i>Nuttallanthus</i>	<u>Stemodia</u>	<u>Wulfenia</u>	<i>Limosella</i>
<i>Pseudorontium</i>	<i>Tetraulacium</i>	Cheloneae (9/25)	<u>Lindenbergia</u>
<i>Rhodochiton</i>		<i>Brookea</i>	<u>Lindernia</u>
<i>Sairocarpus</i>		<u>Chelone</u>	<i>Mazus</i>
<u>Schweinfurthia</u>		<i>Chionophila</i>	<u>Micranthemum</u>
Veroniceae (9)	Callitricheae (2)	<u>Collinsia</u>	<i>Mimetanthe</i>
<i>Kashimira</i>	<u>Hippuris</u>	<u>Penstemon</u>	<i>Morgania</i>
<i>Lagotis</i>	<u>Callitriche</u>	<u>Russelia</u>	<u>Otacanthus</u>
<i>Paederota</i>	Sibthorpieae (2)	<u>Tetranema</u>	<u>Peplidium</u>
<u>Picrorhiza</u>	<u>Ellisiophyllum</u>	<i>Tonella</i>	<u>Stemodia</u>
<i>Scrofula</i>	<u>Sibthorpia</u>	<i>Uroskimmera</i>	<i>Tetraulacium</i>
<u>Veronica</u>	Globularieae (3)	Hemimerideae (1/4)	<u>Torenia</u>
<u>Veronicastrum</u>	<u>Campylanthus</u>	<u>Angelonia</u>	<u>Monttea</u>
<u>Wulfenia</u>	<u>Globularia</u>		<u>Melosperma</u>
<i>Wulfenopsis</i>	<u>Poskea</u>		
Digitalideae (2)	Hemiphragmeae (1)	Not included in Scrophulariaceae (4)	
<u>Digitalis</u> (incl. <u>Isoplexis</u>)	<u>Hemiphragma</u>	<u>Callitriche</u>	
<u>Erinus</u>	Angelonieae (6)	<u>Collinsia</u>	
Plantagineae (2)	<u>Angelonia</u>	<u>Globularia</u>	
<u>Aragoa</u>	<u>Basistemon</u>	<u>Hippuris</u>	
<u>Plantago</u> (incl. <u>Littorella</u>)	<u>Melosperma</u>	<i>Holmgrenanthe</i>	
Cheloneae (10)	<u>Monttea</u>	<i>Limnophila</i>	
<i>Brookea</i>	<i>Monopera</i>	<u>Plantago</u>	
<u>Chelone</u>	<u>Ourisia</u>	<u>Poskea</u>	
<i>Chionophila</i>			
<u>Collinsia</u>	not Plantaginaceae (12)		
<u>Keckiella</u>	<i>Conobea</i>		
<i>Nothochelone</i>	<u>Craterostigma</u>		
<u>Penellianthus</u>	<i>Curanga</i>		
<u>Penstemon</u>	<u>Glossostigma</u>		
<i>Tonella</i>	<u>Lancea</u>		
<i>Uroskimmera</i>	<u>Lindenbergia</u>		
Russellieae (2)	<u>Lindernia</u> (incl. <i>Ilysanthes</i>)		
<u>Russelia</u>	<i>Mazus</i>		
<u>Tetranema</u>	<u>Micranthemum</u>		
	<i>Mimetanthe</i>		
	<u>Peplidium</u>		
	<u>Torenia</u>		

bootstrap support for branches was also estimated in PAUP* using 1000 replicates with 10 replicates of random taxon addition, tree bisection-reconnection (TBR) branch swapping, and MAXTREES set to 1000. Parsimony bootstrap (PB) values $\geq 70\%$ were considered moderately to highly supported (Hillis and Bull, 1993), while PB values $< 50\%$ were considered to be unsupported (even though we recognize that some characters may support these clades). Bayesian analyses were conducted for all data sets using MrBayes version 3.0B4 (Huelsenbeck and Ronquist, 2001) using the Hasegawa-Kishino-Yano model (HKY; Hasegawa et al., 1985) and optimal starting values for (α) and (I) chosen according to Modeltest using likelihood ratio tests (Posada and Crandall, 1998; see Results). Because Modeltest always chose a model that MrBayes could not implement (i.e., the Tamura Nei model [TrN]; Tamura and Nei, 1993, in the case of the ITS and combined data sets and the transversion model (TVM) in the case of the cpDNA data sets) we chose the next less complex model for Bayesian analyses, which in all cases was HKY. Default parameters implemented in MrBayes for the model were used. Single cpDNA regions (*matK-trnK* intron, *rps16* intron, *trnL-F*) were run twice up to 1.4 million generations, whereas the ITS, combined cpDNA, and all combined data sets were run four times with four million generations each. Prior to each full run, we ran short consecutive analyses of 20 000 to 50 000 generations each to determine the proposals ("props") that placed most or all chain acceptance rates roughly between 20–60%. This was done to improve chain convergence rates. Four parallel Markov chain Monte Carlo chains were used for each replicate run, and one tree for every 100 generations was saved. For both runs with combined data sets, separate models were allowed for each DNA region. After graphing all free parameters (except topology and branch lengths), we verified that they had reached stationarity around the 100 000th generation for all analyses, and then conservatively discarded the first 250 000 generations as burn-in. A 50% majority rule consensus tree of the remaining saved trees was reconstructed in PAUP* version 4b10 for each run, however only relationships supported by posterior probability (PP) values ≥ 0.95 are considered statistically significant and thus highly supported, while all values < 0.95 were considered to be nonsignificant. Because posterior probability values are consistently higher than PB values, a higher cutoff PP value for highly supported clades is considered to be reasonable; additionally, PP has been shown to be prone to much higher type I error rates (e.g., Erixon et al., 2003). We compared the mean, variance, and 95% credible interval for all meristic parameters and compared posterior probabilities of clades and tree topologies to verify that the replicate runs of each analysis had reached convergence. We also attempted maximum likelihood analyses of the combined data set (excluding 174 ITS characters) and the combined cpDNA data set using the appropriate model of evolution for all sites combined (general time reversible model assuming rate heterogeneity among sites following a gamma distribution plus invariant sites), one random addition replicate, no MAXTREES, and subtree pruning-regrafting (SPR) branch swapping, but the runs did not finish after 30 days and were aborted. Maximum likelihood analysis of the ITS data set (excluding 174 characters), using the appropriate model of evolution and the same settings as described earlier, ran to completion in 15 days, but the results did not differ significantly from parsimony and Bayes analysis (results not shown). MacClade 4.0 (Maddison and Maddison, 2001) was used for mapping (ACCTRAN optimization) the biogeographical origin on one arbitrarily chosen most parsimonious tree. *Plantago* and *Antirrhinum* are considered Old World taxa, which subsequently spread to the New World again based on results by Hoggard et al. (2003) and Ghebrehiwet et al. (2000).

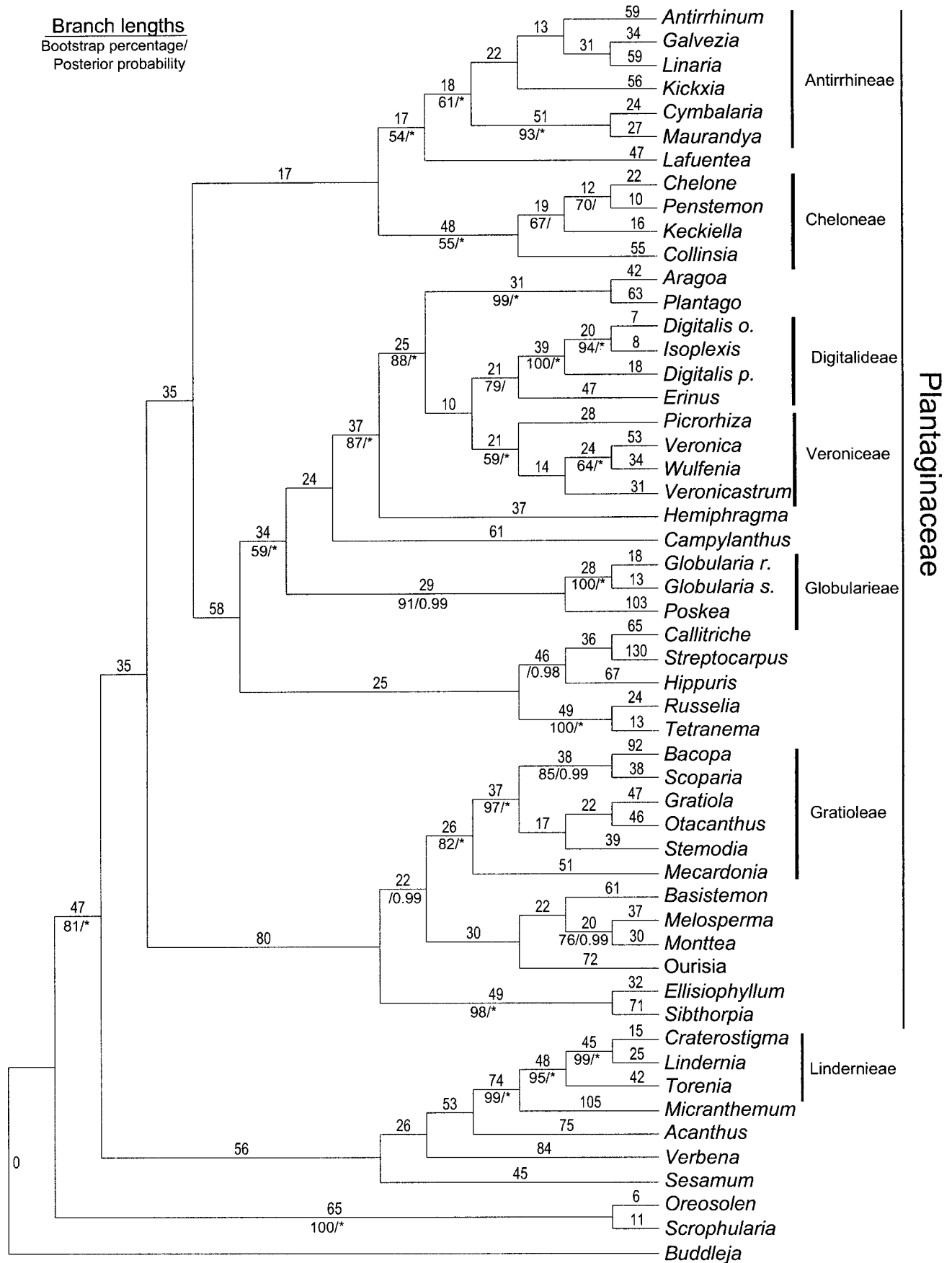
RESULTS

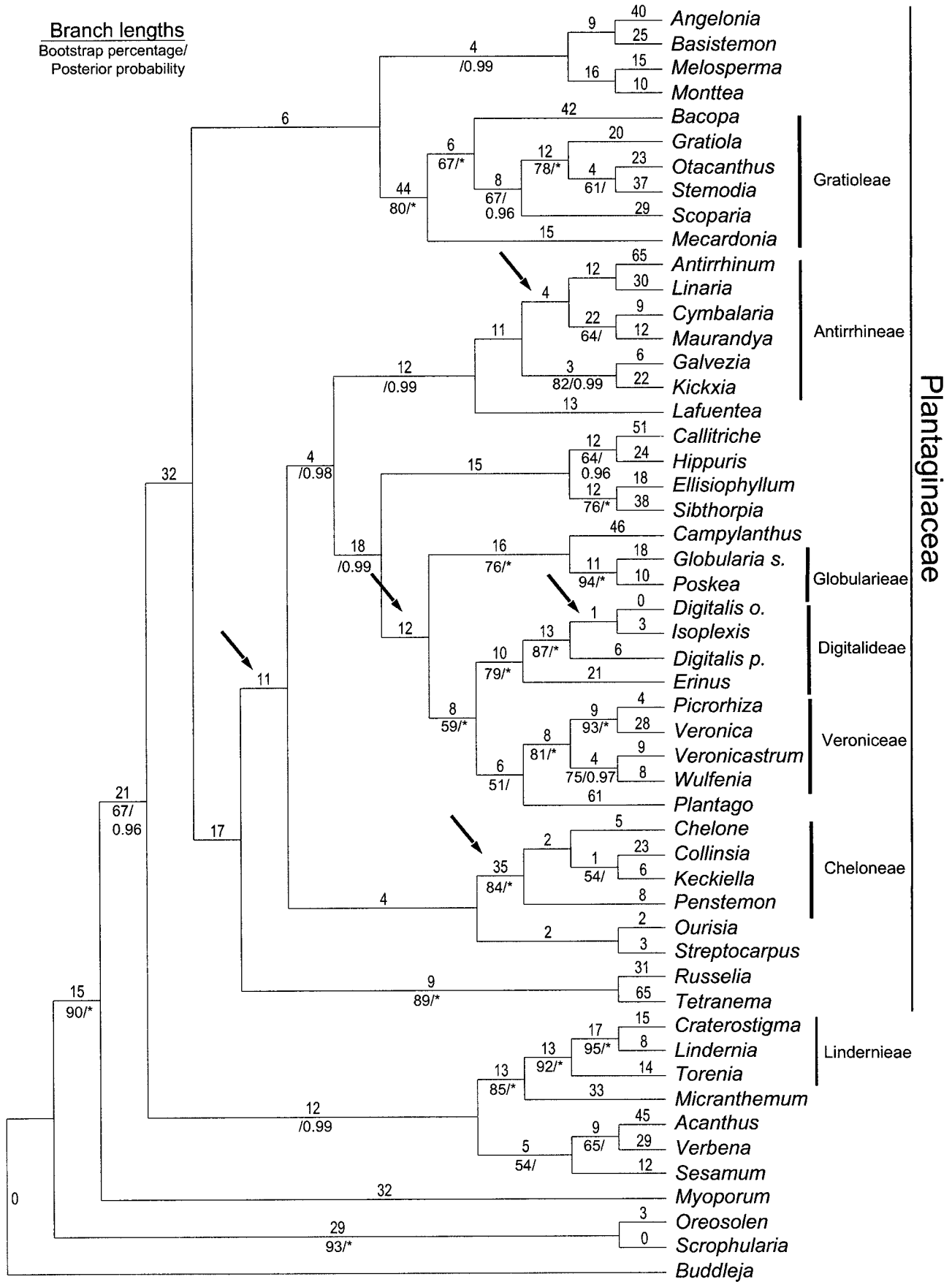
ITS—Results of analyses including and excluding the 174 characters that are only ambiguously alignable were remarkably similar. We therefore show only those trees based on anal-

yses using all ITS characters. The ITS data set includes 851 characters, 421 of them potentially parsimony informative. The single most parsimonious tree has 3742 steps (Fig. 1; consistency index, CI = 0.33, retention index, RI = 0.44). The most parsimonious tree differs from those of the combined analysis most notably in that *Ellisiophyllum* and *Sibthorpia* are sister to the New World Plantaginaceae, Cheloneae is sister to Antirrhineae (including *Lafuentea*), and *Russelia* and *Tetranema* are sister to *Callitriche* and *Hippuris*. However, none of these relationships are supported by bootstrap or posterior probabilities (all < 50 PB, < 0.50 PP). The position of *Streptocarpus* as sister to *Callitriche* is likely due to spurious long branch attraction. This relationship is also seen in the results of the analysis that excluded the ambiguously aligned characters and therefore is not due to an alignment problem. Results from the Bayesian analyses (with estimated starting values for proportion of invariant sites (I) = 0.1451 and gamma distribution shape (α) = 0.6944) differ from the parsimony analysis in several respects, although none of them have a posterior probability of more than 0.95. These results include the sister-group relationships of *Hippuris* and *Callitriche* (0.56 PP) and of *Campylanthus* and Globularieae (0.65 PP), both of which are also seen in the results of the combined analysis (discussed later). The Bayesian analysis also shows *Kickxia* as sister to the rest of Antirrhineae (0.72 PP) and Digitalideae as sister to *Aragoa* and *Plantago* (0.53 PP). Further differences can be found within Gratiolae with support (although not significant) for a clade of *Bacopa-Scoparia-Gratiola* (0.72 PP), the previous clade plus *Otacanthus* (0.82 PP), and all of Gratiolae plus *Ourisia* (0.78 PP).

trnL-F—The data set of the *trnL-F* region includes 1160 characters, 335 of them potentially parsimony-informative. The analysis resulted in 48 most parsimonious trees that are 1577 steps long (CI = 0.614, RI = 0.531). One of the most parsimonious trees is shown in Fig. 2. They are very similar to the most parsimonious trees of the combined analysis. The only major differences are the sister-group relationship of *Ourisia* and *Streptocarpus*, which are in turn sister to Cheloneae, but neither of these two relationships receives significant support. In contrast, the results from the Bayesian analysis (with estimated starting values I = 0.1660 and α = 1.5734) support the relationship of *Ourisia* with Gratiolae and the *Angelonia* clade (1.00 PP; 0.85 PP for all Plantaginaceae). This placement of *Ourisia* was also found in the most parsimonious tree from the combined analysis but requires two more steps on the most parsimonious tree of the *trnL-F* data set. Without *Ourisia* and *Streptocarpus*, the clade of Cheloneae-*Russelia-Tetranema* receives a posterior probability of 0.71 and together with the clade that contains mostly Old World genera from Antirrhineae, Veroniceae, Digitalideae, Globularieae, and relatives (hereafter, the Old World clade) has a posterior probability of 0.96 (results not shown). A further difference between the Bayesian and parsimony analyses is found within Antirrhineae; the Bayesian analysis shows sister-group relationships of *Antirrhinum* with the rest of Antirrhineae (0.66 PP) and *Linaria* to *Cymbalaria* and *Maurandya* (0.79 PP).

Fig. 1. The most parsimonious tree of the analysis of the ITS region. Asterisks indicate posterior probabilities of 1.00. Only PB > 50 and PP > 95 are shown. Abbreviations: PB = percentage bootstrap support, PP = posterior probability, *Digitalis o.* = *Digitalis obscura*, *Digitalis p.* = *Digitalis purpurea*, *Globularia r.* = *Globularia repens*, *Globularia s.* = *Globularia salicina*.





***rps16* intron**—The data set of the *rps16* intron includes 1183 characters, 438 of them potentially parsimony-informative. We found two most parsimonious trees of 2190 steps (one most parsimonious tree is shown in Fig. 3; CI = 0.55, RI = 0.51). They differ from those of the combined analysis in that the first branch of Plantaginaceae is a clade of Cheloneae plus *Russelia* + *Tetranema* and Gratioleae, and the second branch is the *Angelonia* clade plus *Ourisia*; none of these branches receives support from either PB or Bayesian analysis, however. There is some indication in the Bayesian analysis for the sister-group relationship of *Ellisiophyllum* and *Sibthorpia* to Globularieae and *Campylanthus* (<50 PB, 0.68 PP) and stronger support for that of *Hemiphragma* to Veroniceae (82 PB, 1.00 PP). The strangest result in the parsimony analysis of the *rps16* intron data set is the unresolved position of *Antirrhinum* among the outgroup taxa. This *rps16* intron sequence of *Antirrhinum* had previously been used by Jobson and Albert (2002) as an outgroup sequence for their parsimony analyses of Lentibulariaceae. Its position here among members of the outgroup is suspicious. Bayesian analysis with estimated starting values $I = 0.1006$ and $\alpha = 1.4914$, however, shows it to be sister to *Lafuentea* (0.56 PP), a position similar to that in other DNA regions. Such a position would require only four more steps in the parsimony analysis. We have, therefore, not excluded that sequence from subsequent analyses. Finally, in the Bayesian analysis, Antirrhineae + *Lafuentea* is strongly supported (1.00 PP), as is the Old World clade (0.97 PP) and Plantaginaceae (0.97 PP).

***matK-trnK* intron**—The data set for the *matK-trnK* intron includes 541 characters, 199 of them potentially parsimony-informative. We found 366 most parsimonious trees of 985 steps (CI = 0.52, RI = 0.55). One of these trees is shown in Fig. 4. Due to the lack of resolution, the strict consensus tree shows few incongruent results compared with the most parsimonious trees of the combined analysis; one exception to this is the position of *Lafuentea* within Antirrhineae. Results from the Bayesian analysis (with estimated starting values $I = 0$ and $\alpha = 0.6974$) differ from those of the parsimony analysis mainly in the position of *Streptocarpus*, whose position within Plantaginaceae, found in most of the most parsimonious trees, is not supported. Instead, the Old World clade (0.73 PP), Old World clade plus Cheloneae and *Russelia* + *Tetranema* (0.82 PP), and the sister-group relationship of Gratioleae and *Ourisia* (0.59 PP) are found. Furthermore, a sister-group relationship of *Veronicastrum* and *Wulfenia* is shown (0.73 PP) as in other cpDNA regions.

Combined data set—The combined data set includes 3561 aligned characters, 1274 of which are potentially parsimony-informative (302 from ITS, 335 from *trnL-F*, 438 from the *rps16* intron, and 199 from the *matK-trnK* intron). The resulting six most parsimonious trees (one of which is shown in Fig. 5) include 7349 steps (CI = 0.48, RI = 0.48). The branches of these trees are generally well supported by both Bayesian and PB analyses. In contrast to the parsimony analysis which weakly (65 PB) supports an *Acanthus-Streptocarpus* sister relationship, Bayesian analysis supports a sister re-

lationship of *Streptocarpus* (Gesneriaceae) and Plantaginaceae (0.99 PP). The position of Lindernieae (plus *Micranthemum*) as sister to *Acanthus*, *Verbena*, and *Sesamum* is also strongly supported (1.00 PP; not shown in Fig. 5 because parsimony shows *Streptocarpus* as sister to *Acanthus*). Consequently, this group of former Scrophulariaceae s.l. constitutes a clade outside both Scrophulariaceae s.str. and Plantaginaceae.

Within Plantaginaceae, which receive moderate (81 PB) to strong (1.00 PP) support, we find two clades, both with weak support in the PB analysis and strong support in the Bayesian analysis. One clade comprises 11 of our sampled genera, including the mainly southern North American to South American Gratioleae, *Ourisia*, and *Angelonia* and its relatives (64 PB, 1.00 PP), whereas the other has 30 of our sampled genera and includes *Russelia* and *Tetranema*, Cheloneae, and most of the genera from the Old World (73 PB, 1.00 PP). Within the first clade, we find a strongly supported Gratioleae (100 PB, 1.00 PP), *Angelonia* clade (97 PB, 1.00 PP), and *Ourisia*. The exact position of *Ourisia* is unclear. Within Gratioleae, relationships are mostly well supported with *Mecardonia* branching off first, followed by *Bacopa*, and then *Scoparia*. The exact relationships of *Gratiola*, *Otacanthus*, and *Stemodia* are not as well supported as the rest of Gratioleae. Within the South American *Angelonia* clade, *Angelonia*, and *Basistemon* are strongly supported as sister taxa (100 PB, 1.00 PP). The sister-group relationship of *Melosperma* and *Monttea* is only weakly supported by the PB analysis (62 PB) but strongly supported by the Bayesian analysis (0.98 PP).

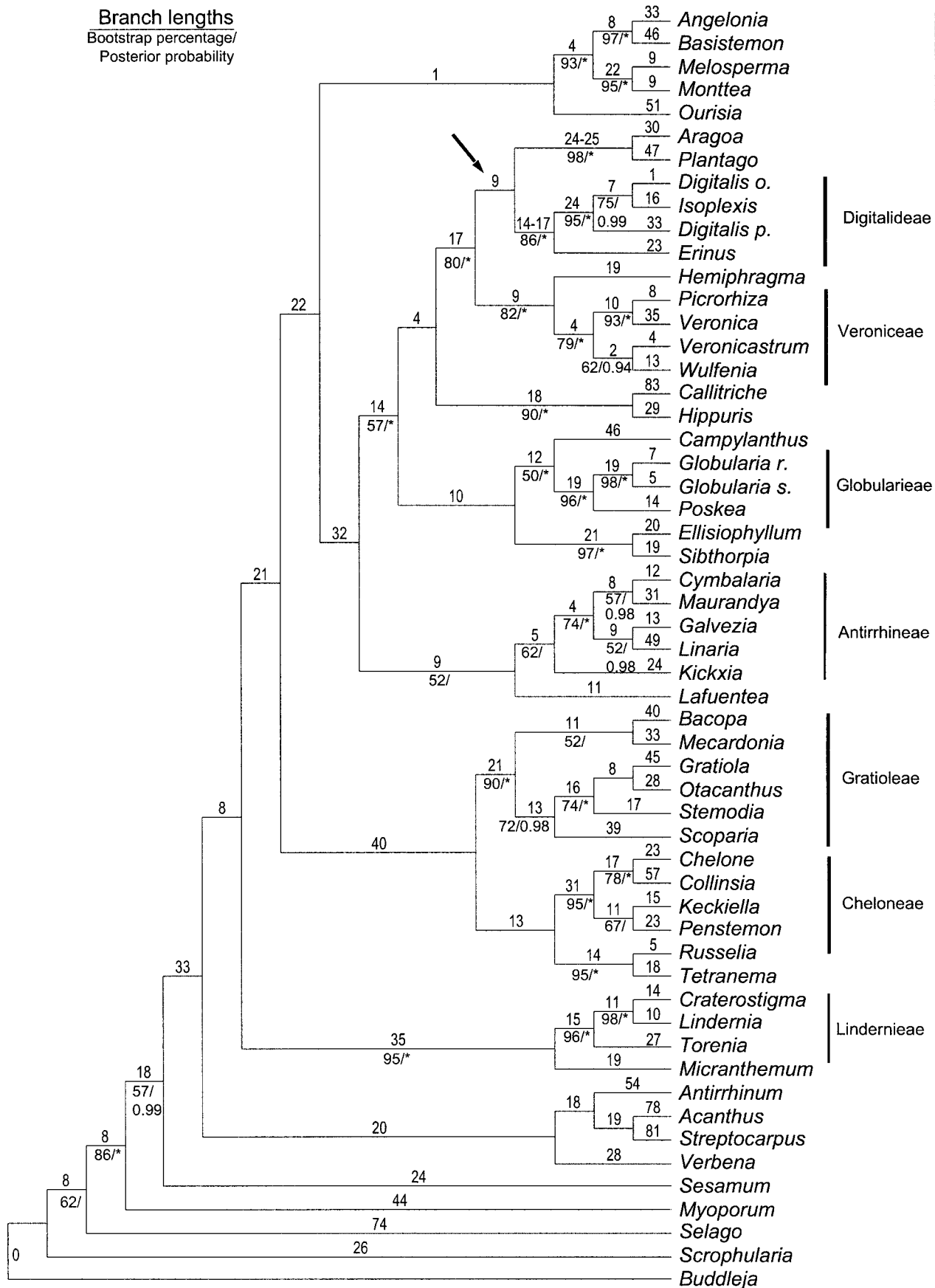
Within the second major group of Plantaginaceae, *Russelia* and *Tetranema* are strongly supported as sister genera (100 PB, 1.00 PP). They comprise the sister clade to Cheloneae, which are strongly supported as monophyletic (100 PB, 1.00 PP). The remaining genera form the strongly supported Old World clade (90 PB, 1.00 PP). It includes Antirrhineae (plus *Lafuentea*; 99 PB, 1.00 PP), which is sister to the rest of the Old World genera (97 PB, 1.00 PP). *Lafuentea* is sister to the remaining Antirrhineae (98 PB, 1.00 PP). Relationships within Antirrhineae are mostly not well supported, probably due to the sparse taxon sampling. Within the remaining group, a clade of four genera (70 PB, 0.90 PP) consisting of *Ellisiophyllum* and *Sibthorpia* (100 PB, 1.00 PP) and *Hippuris* and *Callitriche* (96 PB, 1.00 PP) branches off first. The remainder is again a strongly supported clade (86 PB, 1.00 PP), in which Globularieae (100 PB, 1.00 PP) and *Campylanthus* are strongly supported (99 PB, 1.00 PP) as sister to an equally well-supported clade (100 PB, 1.00 PP). In this group, *Hemiphragma* is sister to the clades (89 PB, 1.00 PP) of Veroniceae (100 PB, 1.00 PP), Digitalideae (99 PB, 1.00 PP), and *Plantago* + *Aragoa* (100 PB, 1.00 PP). The relationship between these three clades, however, is unclear. The parsimony analysis favors a sister-group relationship of either *Plantago* + *Aragoa* and Veroniceae or Digitalideae and Veroniceae (both with PB support of less than 50%), whereas the Bayesian analysis favors a sister-group relationship of Digitalideae and *Plantago* + *Aragoa* (0.84 PP). Also noteworthy is the paraphyly of *Digitalis* with respect to *Isoplexis* (79 PB, 0.98 PP).

The results from the four data sets are similar: most rela-

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Fig. 2. One of the 48 most parsimonious trees of the analysis of the *trnL-F* region. Arrows indicate branches that collapse in the strict consensus tree of all most parsimonious trees. Asterisks indicate posterior probabilities of 1.00. Only PB > 50 and PP > 95 are shown.

Branch lengths
Bootstrap percentage/
Posterior probability



tionships that are strongly supported with either >90 PB or >0.95 PP in one data set are also found in the most parsimonious trees of the combined analysis. However, some exceptions should be noted. One example in Veroniceae has been studied in detail by Albach and Chase (2004). Other cases are found within Antirrhineae, Cheloneae, or Gratioleae (Figs. 2, 4), in which the limited taxon sampling may have led to erroneous results. The spurious position of *Streptocarpus* (Gesneriaceae) in the ingroup is another example (Figs. 2, 3). The only other well-supported relationship in a single data set is the sister-group relationship of *Hemiphragma* and Veroniceae in the *rps16* intron data set (Fig. 3).

DISCUSSION

Support for Plantaginaceae is unambiguous (81 PB, 1.00 PP) in our combined analysis even though it is weak or unsupported in analyses of single DNA regions. This result further stresses the importance of combining data to improve resolution and support in molecular systematic results (Soltis et al., 1998). Similar high PB support for Plantaginaceae was only retrieved in other analyses combining several molecular data sets (88 PB: Oxelman et al., in press, using *ndhF*, *rps16*, *trnL-F*; 91 PB: Albach et al., 2001, using 18S rDNA, *rbcL*, *ndhF*, *atpB*). The sister group of the Plantaginaceae is unknown, and we did not sample extensively across possible sister groups. This question has been addressed in other studies (e.g., Olmstead et al., 2001; Oxelman et al., in press). Nevertheless, our results are congruent with results that show Gesneriaceae and Plantaginaceae as two of the first branching clades in Lamiales. In addition, the rest of Lamiales are supported by 0.99 PP in our analysis of the combined data set; this result is also seen in other analyses covering more diversity in Lamiales (Olmstead et al., 2001; Oxelman et al., in press).

Lindernieae, with approximately 80 species, were included in Gratioleae sensu Wettstein (1895). Therefore, they were assumed to be part of Plantaginaceae, but our results agree with Oxelman et al. (in press) who also showed that Lindernieae are well removed from this family. In support of this new finding, Lindernieae lack iridoids (Kooiman, 1970) and the kind of nuclear protein bodies (Bigazzi, 1993) that are typical for most Plantaginaceae. They also differ from most members of Plantaginaceae in their quadrangular stems (which may be found in some Gratioleae), glandular corolla trichomes (which are rare in the *Angelonia* clade), and alveolate endosperm (Fischer, 1992). The pitted seeds may be another character distinguishing them from Plantaginaceae (Thieret, 1967). *Micranthemum*, well known by aquarium gardeners, was also included in Gratioleae by Wettstein (1895), but in our analyses, it is an unsuspected sister to Lindernieae. Synapomorphies linking *Micranthemum* and Lindernieae are difficult to find. Whereas Lindernieae are African and South-East Asian with a few probably secondary migrations to North America (Lewis, 2000), *Micranthemum* occurs in America from Argentina to the southern United States. They also differ in other characters such as number of stamens (two in *Micranthemum*, four or two plus two

staminodes in Lindernieae), flower merosity (tetramerous in *Micranthemum*, pentamerous in Lindernieae), and flower shape (rotate in *Micranthemum*, zygomorphic in Lindernieae). Given their divergent characteristics and our sparse sampling among Lamiales, further investigation will be necessary to verify the strongly supported relationship found here.

In our circumscription, the family Plantaginaceae is highly heterogeneous, which is understandable considering the diverse evolutionary trends occurring in the family. It includes highly specialized aquatic plants (*Hippuris*, *Callitriche*), small annual species (e.g., *Collinsia*, *Linaria*, *Veronica*), tall shrubs (*Veronica* subg. *Hebe*, *Aragoa*), rainforest herbs (*Tetranema*), weeds (e.g., *Plantago*, *Veronica*, *Cymbalaria*), and alpine chasmophytic species (*Penstemon*, *Veronica*, *Erinus*, *Ourisia*). Species are anemophilic (*Plantago*), hydrophilic (*Hippuris*), melittophilic (most), ornithophilic (e.g., *Galvezia*, *Russelia*), or autogamous (many). Therefore, Plantaginaceae can only be vaguely described morphologically. The following description is based on Judd et al. (2002) with some extensions and updates. Plantaginaceae includes annual or perennial herbs, sometimes shrubs or aquatics, but no parasites or semi-parasites. Hairs are usually simple, often glandular, but sometimes stellate (e.g., *Callitriche*) or peltate (*Hippuris*). The lack of vertical partitions in the hairs is considered a likely synapomorphy by Judd et al. (2002). Leaves are either opposite, or alternate and spiral, rarely whorled. They are mostly simple, rarely compound, entire to pinnatifid with pinnate venation but more or less parallel in *Plantago*. Leaf heteromorphy occurs in *Hemiphragma*. Stipules are lacking. Inflorescence types include various kinds of determinate or indeterminate inflorescences, but sometimes flowers are solitary. The flowers are usually bisexual and bilateral but are polysymmetric in several genera (e.g., *Bacopa*, *Sibthorpia*) or reduced in *Callitriche*. *Hippuris* is monoecious and some species of *Plantago* and *Veronica* subg. *Hebe* are dioecious or gynodioecious. Sepals are usually four or five and may be free to connate. Petals are mostly five but occasionally four due to fusion of the two upper lobes; fusion can proceed to two lobes (*Lagotis*). *Sibthorpia* has eight petal lobes. The corolla tube can be either inconspicuous or very long relative to the petal lobes. The corolla is lacking in some species of *Veronica* formerly assigned to *Besseya*. Flowers can be up to several centimeters and are pollinated by wind, bees, flies, or birds. Some flowers have a basal nectar spur, or the lower lip can have a bulge obscuring the throat. Some species have the entrance to the tube obscured by hairs. A possible synapomorphy may be the early development of the androecium relative to the corolla (Judd et al., 2002). Stamens are usually four, didynamous to equal, sometimes reduced to two or one (*Hippuris*, *Callitriche*), but reports of a single stamen in *Plantago* subg. *Bougueria* are erroneous (R. Hoggard, University of Oklahoma, personal communication). The fifth stamen can sometimes be present as a staminode. Filaments are adnate to the corolla; anthers are two-locular, with distinct locules opening by two longitudinal slits or the apical portion of the anther sacs are adnate and open by a single inverted slit. The pollen sacs are divergent, the anthers sagittate. Pollen is mostly isopolar, tricolpate to tricolporate with

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Fig. 3. One of the two most parsimonious trees of the analysis of the *rps16* intron. The arrow indicates the branch that collapses in the strict consensus tree of all most parsimonious trees. Asterisks indicate posterior probabilities of 1.00. Only PB > 50 and PP > 95 are shown.

reticulate exine but cannot be utilized as a taxonomic marker because this character is neither restricted to this family nor stable in it (e.g., *Aragoa* and *Plantago*; see later). Styles are reduced in *Hemiphragma* and *Erinus*. Carpels are two (one in *Plantago* subg. *Littorella*) and connate with a single style. The stigma is either two-lobed or capitate. The ovary is superior with axile placentation and large undivided placentas. Ovules are usually numerous in each locule but can be just one per locule (e.g., *Lagotis*). Ovules are anatropous or hemitropous and have one integument and a thin-walled megasporangium. The nectar disc is usually present but lacking in some genera. The fruit is usually a septicidal or loculicidal capsule, occasionally poricidal or circumscissile. Seeds are one to many, ovoid, and angular or winged. The exotestal cells have inner walls that are more or less thickened when they are winged; cells have reticulate thickenings. Chromosome base number varies between six and 11.

Even the phytochemistry of Plantaginaceae is diverse; iridoids predominate but are sometimes lacking and seemingly replaced by diverse glycosides (e.g., Gratiroleae, *Sibthorpia*, *Ellisiophyllum*, *Digitalis*). Antirrhineae is characterized by the presence of antirrhinoside (Nicoletti et al., 1988), but *Monttea* also contains this compound (S. R. Jensen, Danish Technical University, personal communication). *Bacopa*, *Gratiola*, and *Stemodia* do not contain iridoids but instead have di- and triterpene glucosides (von Poser et al., 1996); *Scoparia dulcis* has methylester-iridoids (von Poser et al., 1996). One could claim that this group of genera has lost the ability to make iridoids, and *Scoparia* has regained it to make carboxylated iridoids. Apart from iridoids, flavonoid compounds may be useful chemotaxonomic characters. For example, 6-hydroxyflavones may be a synapomorphy within Plantaginaceae for the clade including *Globularia*, *Erinus*, *Veronica*, and *Plantago* (Tomás-Barberán et al., 1988).

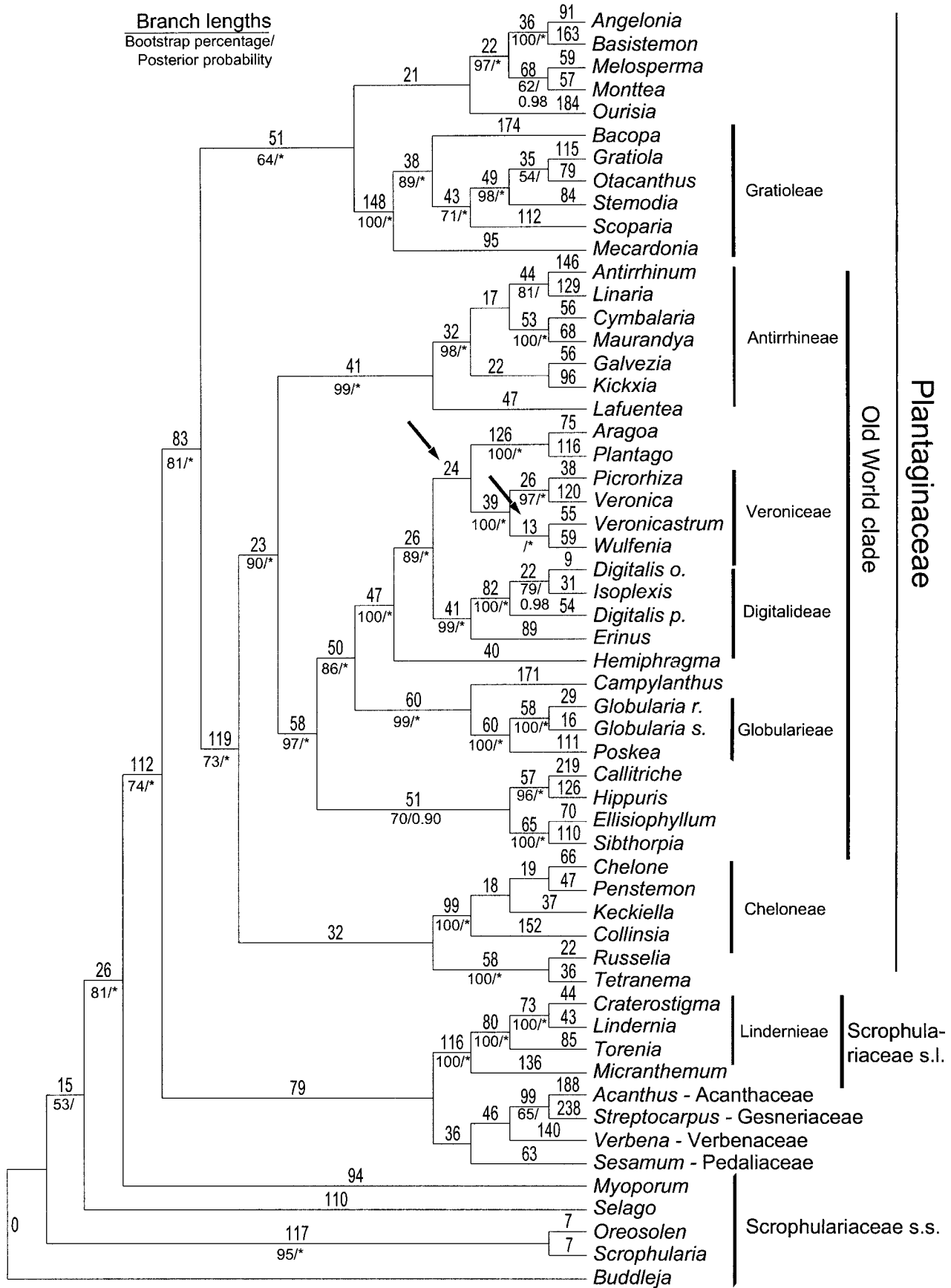
Nuclear protein inclusions have been studied intensively by Speta (1972, 1977, 1979) and Bigazzi (1993). The splitting of Scrophulariaceae s.l. into smaller monophyletic groups is fully supported by their results, and Plantaginaceae may therefore be a model for studying the evolution of these protein bodies. Most genera in Plantaginaceae have amorphous or globular protein bodies, while the majority of the remaining species formerly classified in Scrophulariaceae s.l. are characterized by lamellar inclusions (Speta, 1972, 1977, 1979; Bigazzi, 1993). Whereas some types of inclusions are diagnostic at the generic level (e.g., *Linaria*; Bigazzi, 1989), there is an overall trend detectable in Plantaginaceae. Based on data from Speta (*Gratiola*; 1972) and Bigazzi (1984, 1993), Gratiroleae and Angelonieae have either lamellar or no inclusions, except for *Monttea*, which has globular plus lamellar inclusions (Speta, 1979) and *Angelonia*, with its globular inclusions (Speta, 1979). Lamellar inclusions are uncommon in the Cheloneae and the Old World clade, in which amorphous inclusions predominate. Unfortunately, nothing is known about the biological significance of nuclear protein inclusions and the chemical basis for the different types of inclusions. Plantaginaceae may therefore be an ideal model group in which to study this.

Discussion of the intrafamilial groups—The mainly South and Central American Gratiroleae and *Angelonia* clades form the first major group of Plantaginaceae. Wettstein (1895) treated Gratiroleae in a much wider circumscription (including Lindernieae, *Mimulus*, etc.) based on two thecae in the anthers and racemose inflorescences. Rouy (1909) included *Stemodia*, *Bacopa*, *Otacanthus*, and *Gratiola* in Dodartieae but all in different subtribes. *Scoparia* was included in Digitalideae by Wettstein (1895), Veroniceae by Bellini (1907), and Hemiphragmeae by Rouy (1909). Hallier (1903), however, suggested that *Scoparia* had gratiolean affinities based on the regular four-lobed corolla, the branching pattern of the stem, and the shape of the calyx, capsule, and leaf. *Scoparia* seeds are similar to those of *Bacopa* (Thieret, 1967); seed characters may be useful for characterizing Gratiroleae in a more narrow sense. *Scoparia* pollen is, however, similar to *Sopubia* (Orobanchaceae) and different from that of other Plantaginaceae (Minkin and Eshbaugh, 1989). Thieret (1967) suggested that distinct stigmas were a synapomorphy of Gratiroleae, although this character state is not found in *Scoparia* or in some species of *Bacopa* (Thieret, 1954). Four of the gratiolean genera sampled in this analysis (i.e., *Otacanthus*, *Scoparia*, *Bacopa*, and *Mecardonia*) are neotropical, while *Gratiola* is cosmopolitan and *Stemodia* pantropical. Olmstead et al. (2001) showed that the North American *Amphianthus* is also a member of this clade. Old World members of Gratiroleae sensu Wettstein (1895), however, have been shown to have affinities with Orobanchaceae (*Lindenbergia*; Oxelman et al., 1999) or Phrymaceae (*Glossostigma*, *Lancea*, *Mazus*, *Peplidium*; Beardsley and Olmstead, 2002). Due to our limited sampling of this tribe, both in terms of geography (mainly New World members) and number (only 6 of 30 genera), it will be especially important to determine whether the other Old World members of Gratiroleae such as *Adenosma* and *Limnophila* or the cosmopolitan *Limosella* belong in this clade. Further analyses are therefore necessary to give a clear circumscription of this tribe, including specifically which genera belong in it and the synapomorphies that define it.

Angelonia, a genus of about 25 species, was placed in Hemimerideae by Bentham and Hooker (1876) and Wettstein (1895) based on its lack of a corolla tube and saccate flowers, traits shared with South African *Hemimeris* and *Diascia*. Pennell (1920), however, segregated it into its own tribe, Angelonieae. *Basistemon* includes eight shrubby species in South America, last revised by Barringer (1985). Wettstein (1895) included it near *Brandisia* in tribe Cheloneae. Chodat (1904) first noted its similarities to *Angelonia*, with which it shares saccate corolla tubes with internal oil-secreting hairs (Vogel, 1974); minute, entire stigmas; distinct anther thecae; seeds with loose, reticulate exotestae; and absence of endosperm and staminodes (Barringer, 1985). The two other members in the *Angelonia* clade, *Melosperma* and *Monttea*, are small southern Andean genera that were included in Gratiroleae-Mimulinae by Wettstein (1895) but were separated into their own tribe, Melospermeae by Rossow (1985). Thieret (1967), however, denied their relationship with *Mimulus* based on their reduced

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Fig. 4. One of the 366 most parsimonious trees of the analysis of the *matK-trnK* -intron. Arrows indicate branches that collapse in the strict consensus tree of all most parsimonious trees. Asterisks indicate posterior probabilities of 1.00. Only PB > 50 and PP > 95 are shown.



number of large seeds, united stigmas, and spatulate embryos. Barringer (1983) had already suggested a close relationship of *Monttea* and *Melosperma* with *Angelonia* and *Basistemom*, but he did not give reasons for his hypothesis. Reduced seed number and shrubby habit are the most apparent similarities between these four genera. In addition, *Angelonia*, *Monttea*, *Monopera* (a genus of two species segregated from *Angelonia*; Barringer, 1983), and some species of *Basistemom* all have flowers that produce oil instead of nectar as a pollinator reward (Vogel, 1974; Simpson et al., 1990; Vogel and Cocucci, 1995). Within Scrophulariaceae s.l., this unusual characteristic is only found in one other New World genus (*Calceolaria*) and several South African genera (Vogel, 1974). Vogel (1974) hypothesized that oil flowers evolved four times in Scrophulariaceae s.l.: twice in Scrophulariaceae s. str. (*Diascia*, *Bowkeria*) in the Old World, once in *Calceolaria*, and once in Plantaginaceae (*Angelonia*, *Basistemom*; it was not yet known that *Monttea* had oil flowers). In our analyses, one gain and two losses, two gains and one loss, or three gains of oil flowers in Plantaginaceae are all equally parsimonious, but considering that oil flowers are only found in eight angiosperm families, perhaps one gain in Plantaginaceae is the most likely.

Ourisia was considered to belong to Digitalideae by Wettstein (1895), but Rouy (1909) grouped it in Rehmanniae-Ourisieae with *Lafuentea* and *Oreosolen*. Thieret (1967) considered the united stigmas of *Ourisia* as evidence for placement in Veroniceae but its divaricate anther cells for affinity with Digitalideae. *Ourisia* comprises about 30 species, half of which are found in the mountains of New Zealand. One species is endemic to Tasmania, and the remaining species are distributed in the South American Andes. Molecular phylogenetic evidence suggests a South American origin of *Ourisia* (Meudt and Simpson, unpublished manuscript). These results make sense in light of our combined analysis (Fig. 5) in which *Ourisia* is at the base of the South American *Angelonia* clade (which in turn is sister to New World Gratiroleae), but there is no significant support for this placement (<50 BS, 0.88 PP). *Ourisia* was also found to be sister to Gratiroleae and the four genera in the *Angelonia* clade with 86 BS in the phylogenetic analyses of Oxelman et al. (in press). *Ourisia* has zygomorphic to subrotate, pentamerous flowers with four didynamous stamens, loculicidal capsules, and numerous small, reticulate, angled seeds. A staminode may or may not be present. Some species of *Ourisia* have a hypogynous disc that remains attached to the gynoecium when the corolla is shed; this structure is similar to the unique nectary described for *Monttea* and *Melosperma* (Sérsic and Cocucci, 1999). Although most species in the genus are rhizomatous herbs, three species are suffruticose, a character that may also link *Ourisia* to the *Angelonia* clade. It is hoped that detailed morphological and molecular studies of *Ourisia* currently underway (Meudt, unpublished manuscript; Meudt and Simpson) will uncover more synapomorphies and aid in elucidating the obscure relationships of this genus.

The second major lineage in Plantaginaceae comprises a group of Old World taxa and Cheloneae, *Russelia*, and *Tetranema*. *Russelia* (52 species) and *Tetranema* (two species) are both members of the Cheloneae sensu Wettstein (1895). *Rus-*

selia comprises shrubby species, whereas *Tetranema* are herbs with a basal leaf rosette. Pennell (1920) moved *Russelia* into its own tribe Russelieae based on the unique character of dense pubescence on the fruit. The relationship between *Russelia* and *Tetranema* was first found by Wolfe et al. (2002), but they could find few morphological characters supporting it except for staminode morphology. They recommended further research to explain the relationship of these two genera that seem to share only plesiomorphies and a distributional range in Central America. We can only add here that this sister relationship is not an artifact of the DNA regions used by Wolfe et al. (2002), but also holds with other molecular markers.

Sister to *Russelia* and *Tetranema* are Cheloneae, a relationship that has already been found in previous analyses (Wolfe et al., 2002; Oxelman et al., in press). Cheloneae was circumscribed by Bentham (1846) based on cymose inflorescences. In this circumscription, Cheloneae exhibited great morphological heterogeneity, and consequently 22 of the genera with cymose inflorescences have also been included in other tribes or even families (reviewed in Wolfe et al., 1997). Straw (1966) delimited Cheloneae based on the staminode form, gross pollen morphology, pubescence characteristics, and inflorescence structure; molecular studies supported this circumscription of the tribe (Wolfe et al., 1997). *Collinsia* and *Tonella* were removed from Cheloneae based on the different nectary position (Bellini, 1907), annual life history, capsule and corolla shape (Pennell, 1935), embryology, and seed characteristics (Thieret, 1967). Apparently, the evolution of these morphological characters, which may be due to a shift to an annual life history in *Collinsia* and *Tonella*, has occurred since the divergence of these two genera from the remaining species of Cheloneae because *Collinsia* and *Tonella* have been shown to be sister to Cheloneae in all molecular analyses (Wolfe et al., 1997, 2002; Fig. 5). Straw (1966) and Wolfe et al. (2002) proposed a New World ancestry of this group, consistent with our results (Fig. 6). *Pennellianthus* is the only Old World (Japan and eastern Russia) member of Cheloneae sensu Straw (1966) and Wolfe et al. (2002). We did not include it in our analyses, but subsequent inclusion of published ITS (GenBank accession AF375156) and *matK* (AF375194) sequences in our ITS and *matK* data sets support the monophyly of Cheloneae sensu Straw (1966) and Wolfe et al. (2002; results not shown).

Antirrhineae are characterized by poricidal dehiscence of the capsule and in general extreme zygomorphy of the flower; its monophyly has rarely been doubted. Linnaeus (1753) originally assigned all European members of the tribe to one genus, *Antirrhinum*, which was subsequently split several times. The tribe was last revised by Sutton (1988). It is characterized by the almost ubiquitous presence of antirrhinoside (Nicoletti et al., 1988), found outside the tribe in only rare cases (e.g., *Monttea*; S. R. Jensen, Danish Technical University, personal communication). A morphological and molecular cladistic analysis (Ghebrehwet et al., 2000) further increased knowledge of the evolution of the tribe. With the exception of the position of *Antirrhinum*, which appears in various places using different genes, our analysis is fully congruent with results from Ghebrehwet et al. (2000), who sampled more extensively in this tribe. Nevertheless, the relationships within Antir-

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Fig. 5. One of the six most parsimonious trees of the combined analysis. Arrows indicate branches that collapse in the strict consensus tree of all most parsimonious trees. Asterisks indicate posterior probabilities of 1.00. Only PB > 50 and PP > 95 are shown.



Fig. 6. Biogeographical origin of taxa mapped on one random most parsimonious tree of the combined analysis (ACCTRAN optimization). *Plantago*, *Veronica*, and *Veronicastrum* have been scored as Old World origin based on Rønsted et al. (2002), Albach and Chase (2001), and Albach (unpublished data), respectively.

rhineae are worthy of further detailed studies. The position of *Antirrhinum* is especially important because it is a model organism for molecular biologists interested in flower and inflorescence development (Martin and Gerats, 1993; Luo et al., 1995; Bradley et al., 1996). The biogeography of Antirrhineae suggests an Old World origin of the tribe with two subsequent dispersal events to the New World (Fig. 6; *Galvezia*, *Maurandya*). This is supported by the sister-group relationship of Antirrhineae with *Lafuentea*, which includes two species from Southern Spain and Morocco. It was included in Digitalideae (Wettstein, 1895) and in Rehmanniae-Ourisieae by Rouy (1909) together with *Ourisia* and *Oreosolen*. The strongly supported relationship of *Lafuentea* sister to Antirrhineae is surprising based on their divergent inflorescence and flower morphologies. *Lafuentea* has small flowers densely packed in large inflorescences. Its flowers are bilabiate as are most Antirrhineae, but the corolla is not palate, narrowly cylindrical or spurred. Furthermore, stamens of *Lafuentea* are clearly exerted as opposed to the included stamens of Antirrhineae. Future studies of Antirrhineae should therefore include *Lafuentea* to corroborate these results. It may further be a good candidate for an outgroup in studies investigating the genetic basis of flower morphology evolution in Antirrhineae.

Callitriche (up to 40 species) and *Hippuris* (one species) are anomalous, aquatic plants. Due to the modifications related to their habitat, relationships were difficult to discern prior to the inception of molecular phylogenetic techniques. Initially, the reduced flower suggested an affinity with Haloragaceae (for Callitrichaceae: Hutchinson, 1959; for Hippuridaceae: Thorne, 1983). The detection of iridoid compounds in both taxa (Hegnauer, 1966), however, led to the suggestion that they were either members of Lamiales/Scrophulariales or monogeneric orders close to the aforementioned orders (Dahlgren, 1980; Takhtajan, 1997). Callitrichaceae were believed to be close to Lamiaceae also based on their possession of similar schizocarps (Dahlgren, 1977). The lamiale affinity of both taxa was supported in a cladistic analysis based on morphology (An-Ming, 1990). Early molecular systematic analyses further supported this affinity of *Callitriche* with *Antirrhinum* (Olmstead et al., 1992). *Hippuris* was only later included in these studies and found to be sister to *Callitriche* (Reeves and Olmstead, 1998). The sister-group relationship is supported by the reduction of the androecium to a single stamen and the loss of one perianth whorl (Reeves and Olmstead, 1998).

Ellisiophyllum, which is included here for the first time in a molecular systematic analysis, shows a close relationship with *Sibthorpia* (Fig. 5). *Sibthorpia* consists of five species as revised by Hedberg (1955, 1975). It has a peculiar distribution from southern Africa through tropical, montane regions of that continent, to the Mediterranean and along the Atlantic coast to the British Isles, and also in the mountains from Central America to Argentina; this distribution was extensively discussed by Croizat (1968). Based on the optimization of biogeography on one of our most parsimonious tree (Fig. 6), *Sibthorpia* originated in the Old World and subsequently dispersed to the New World, but this hypothesis needs further testing. *Sibthorpia* was included in Digitalideae by Wettstein (1895) and Hemiphragmeae by Rouy (1909). *Ellisiophyllum pinnatum*, the only species of this genus, occurs from the Himalayas to Japan and was sometimes believed to be a member of Hydrophyllaceae (Peter, 1897). Others believed it was closely related to *Sibthorpia* (Hedberg, 1955) or even a species of *Sibthorpia* (Bentham, 1846). Several characters, such as en-

dosperm formation (Yamazaki, 1957), are markedly different from any other Scrophulariaceae s.l., but the tetramerous, slightly didynamous androecium, bilocular fruit, and seed characters show its affinity with Scrophulariaceae s.l. and Plantaginaceae as described here. *Ellisiophyllum* and *Sibthorpia* also both lack iridoids.

Globularieae (or Globulariaceae) s.str. comprise only two genera, *Globularia* and *Poskea*, and are characterized by their endospermless seeds (Wettstein, 1895; Barringer, 1993). Those who did not accept the tribal or familial status of this group have placed these two genera either in Digitalideae (Hallier, 1903) or Veroniceae (Thieret, 1967) based on the shared reduction of ovule number with *Lagotis* (Veroniceae). Our results show a close relationship between *Globularia* and *Poskea* with Digitalideae-Veroniceae (Fig. 5); however, *Campylanthus* is sister to the Globularieae. This genus of 15 species also traditionally belonged to the Digitalideae-Veroniceae (e.g., Wettstein, 1895; Hallier, 1903) but had no apparent close relative (Miller, 1980; Hjertson, 2003). There is no obvious morphological similarity of Globularieae and *Campylanthus*. Chemotaxonomy shows a relationship of *Campylanthus* with *Plantago* by the common presence of sorbitol, aucubin, gardsoside, lavandulifolioside, and melittoside (Rønsted and Jensen, 2002). However, none of the characteristic 8,9-unsaturated iridoids of *Veronica*, *Plantago*, and *Globularia* have been found in *Campylanthus* (Rønsted and Jensen, 2002). The Globularieae-*Campylanthus* clade is an important example of the Macaronesian-northeastern African disjunction (Marrero et al., 1998; Hjertson, 2003). *Campylanthus* is disjunct between these areas, *Poskea* is found only in one region (Socotra, off the northeastern African coast), and *Globularia* is found continuously from Macaronesia to northeastern Africa through southern Europe. Within its two geographic extremes, *Globularia* is found in the same habitat as the best-known example of the Macaronesian-Northeastern African type of disjunction, the dragon trees (*Dracaena*; Marrero et al., 1998). Further analysis of this group, especially with the molecular dating techniques used previously for *Globularia* (Comes and Kadereit, 2003), will therefore be important in understanding the biogeography of the region.

Hemiphragma is a monotypic genus from the Himalayas. It is sister to the clade of *Plantago* and Veroniceae in Olmstead et al. (2001) and Bello et al. (2002); in our analyses, it is sister to the clade containing these groups plus Digitalideae (Fig. 5). The genus is unusual in Scrophulariaceae s.l. and also here in Plantaginaceae due to its fleshy, septical capsule; five-lobed, actinomorphic corolla; and dimorphic leaves (stem leaves flat and orbicular, branch leaves crowded and needlelike). The tribe Hemiphragmeae was established by Rouy (1909) and also included *Sibthorpia*, *Scoparia*, and *Capraria* on the basis of a similar rotate flower with short corolla tube; our data do not support these relationships. Further studies are necessary to understand the evolution and relationships of *Hemiphragma* found here.

Digitalis encompasses 19 species native to Europe and Central Asia and is well known for the medicinal use of its cardiac glycosides. A first molecular analysis (Nebauer et al., 2000) seems to corroborate the morphology-based taxonomy (Werner, 1964), but no species of *Isoplexis* was included. *Isoplexis* is a genus of four species from Macaronesia. The first species were originally described as species of *Digitalis*, but they have most often been considered to belong to a separate genus on the basis of their upper corolla lip being larger than the lower

lip. Molecular systematic analyses using ITS and *trnL-F* sequences using all but one species of *Digitalis* and *Isoplexis* (C. Bräuchler et al., University of Munich, unpublished manuscript) further substantiate the result of this study that *Isoplexis* is derived from within *Digitalis*. Both genera are unusual in Plantaginaceae in that they lack iridoids and contain glucitol. *Erinus* comprises one or two species from the Alps, Pyrenees, and Morocco, which are probably relict occurrences of a formerly more widespread distribution in the western Mediterranean mountains. It has long been considered to be closely related to *Digitalis* based on the similar flower morphology (long corolla tube, deeply split calyx, four didynamous stamens, distinct stigmas) and alternate leaves. In contrast to *Digitalis*, *Erinus* contains aucubin, arborescosidic acid, and sugars common in related groups and has thus retained a phytochemical arsenal similar to that in related *Plantago* or Veroniceae.

Veroniceae are a tribe that has met with little doubt concerning its delimitation. Only *Lagotis* has sometimes been excluded because of its one-seeded, schizocarpic fruit (Bentham, 1846). Based on floral, vegetative (e.g., Hallier, 1903; Pennell, 1935; Thieret, 1967) and molecular characters (Albach and Chase, 2001), *Lagotis* is included in Veroniceae. In its most recent classification (Albach et al., 2004b), Veroniceae include nine genera: *Veronicastrum*, *Scrofula* (likely to be included in *Veronicastrum*), *Lagotis*, *Wulfenia*, *Kashmiria*, *Wulfeniopsis*, *Picrorhiza* (including *Neopicrorhiza*), *Paederota*, and *Veronica* (including *Synthyris*, *Besseyia*, *Pseudolysimachion*, *Paederotella*, *Hebe* and related genera, and *Detzneria*). Veroniceae is shown to be sister to *Plantago* in almost all molecular analyses (Olmstead and Reeves, 1995; Albach and Chase, 2001; Olmstead et al., 2001), a placement corroborated by chemotaxonomy (Rønsted et al., 2000). Morphological similarities between Veroniceae and *Plantago* have been discussed by Albach et al. (2004c).

Aragoa was recently shown to be sister to *Plantago* (Bello et al., 2002). The genus includes 19 species from northern parts of the South American páramo. With its shrubby habit, xeromorphic leaves, and actinomorphic flower with four corolla lobes and four stamens, *Aragoa* has had a diversity of proposed relatives. Based on flower and fruit morphology, *Aragoa* has usually been positioned in Veroniceae (or Digitaliadeae sensu Wettstein, 1895) near *Veronica* (e.g., Bentham, 1846; Hallier, 1903; Thieret, 1967) or *Picrorhiza* (Wettstein, 1895) (reviewed by Bello et al., 2002). Its position as sister to *Plantago*, although never proposed before Bello et al. (2002), is not refuted by the similarities in flower and fruit morphology because these characters may be symplesiomorphies. It shares several floral developmental characters and the (almost) actinomorphic four-lobed corolla with both Veroniceae and *Plantago*. Four stamens are plesiomorphic for this group and may have been reduced to two in parallel in *Plantago* and Veroniceae (Albach et al., 2004c). Possible synapomorphies of *Plantago* and *Aragoa* are found among pollen and seed ultrastructural characters (Bello et al., 2002), as well as phytochemical characters (Rønsted et al., 2003). Other characters of *Aragoa* represent specialized adaptations to its páramo habitat. Its occurrence in South America also provides further evidence for migration of ancestral plantaginaceous lineages from the Old World to the New World (Fig. 6). This, however, does not suggest an Old World origin of *Aragoa* because all extant species of *Aragoa* diverged within the last 170 000 years (Bello et al., 2002), the minimum age of *Aragoa*

in the New World. Older pollen fossils of the *Aragoa-Plantago* group from the northern Andes (van der Hammen and Cleef, 1986) indicate an earlier presence within the Americas. Thus, it seems likely that an ancestor of *Aragoa* migrated from the Old World to the New World and later adapted to the high Andean páramo environment.

Plantago is one of the largest genera of the family with about 200 species and is probably the most widely distributed genus. Its inclusion in one family with Scrophulariaceae s.l. has only been proposed by Hallier (1903) based on similarities in flower morphology with *Veronica* and seed morphology with *Veronica* and *Erinus*. *Plantago* is sometimes segregated into three genera (*Littorella*, *Bougueria*, *Plantago*), although *Bougueria* was recently shown to be derived from within *Plantago* (Rønsted et al., 2002). *Littorella* is sister to the rest of *Plantago* (Rønsted et al., 2002; Hoggard et al., 2003); therefore, its recognition at the genus level is ambiguous from a cladistic point of view. We follow here the suggestion of Rahn (1996) to recognize just one genus (*Plantago*) based on morphological similarity. Relationships within *Plantago* are reasonably well understood because extensive morphological (Rahn, 1996) and molecular cladistic analyses (Rønsted et al., 2002) have been published.

Based on our phylogenetic hypothesis, we infer a New World ancestry of the Old World clade (Fig. 6) because *Russelia*, *Tetranema*, and Cheloneae are sister to the rest of this clade, and together they are sister to the New World Gratioleae + *Angelonia* clade. This is in contrast to the hypothesis by Raven (1974) who considered the Scrophulariaceae of Laurasian origin and *Angelonia* and other genera in South America to be recent arrivals. This is just one example highlighting how the new family circumscription changes our biogeographic hypotheses for the origin of taxa in Plantaginaceae. Furthermore, the disjunction of Plantaginaceae taxa between the New World and Old World, especially the Mediterranean region (Fig. 6), exemplifies the biogeographic pattern of a Madro-Tethyan disjunction, best known in *Cercis* (Davis et al., 2002) and Arbutoideae (Hileman et al., 2001). North America and Europe geographically divided in the early to mid-Eocene (Axelrod, 1975; Tiffney, 1985; Manchester, 1999). Molecular clock estimates for the whole family are currently not available, but the split between sisters *Plantago* and *Aragoa* was estimated at seven million years ago (Rønsted et al., 2002). Based on this estimate, vicariance of ancestral plantaginaceous lineages during the Eocene seems plausible but would need more serious investigation. Unfortunately, fossils for molecular clock calibration do not exist for this group. Therefore, calibration with known geological events and a phylogenetic hypothesis based on better intratribal and intrageneric sampling is necessary.

In this study, we have outlined the new circumscription of Plantaginaceae, which significantly expands the family to contain approximately 92 genera and 2000 species. With the exception of several gratioleae genera that were not included in our analyses, we can now be certain of the genera that are included in this family. Furthermore, this study significantly increases our knowledge of intrafamilial relationships. Table 1 offers a synopsis of our new circumscription of Plantaginaceae, including the 92 genera we hypothesize to be included in the family, and compares it to Wettstein's (1895) placement of 70 of these genera in his classification of 152 genera of Scrophulariaceae s.l. Although we do not formally address the question, several subclades of Plantaginaceae could be rec-

ognized at the tribal level mostly in a circumscription resembling morphology-based classifications (e.g., Antirrhineae, Cheloneae, Veroniceae). Several tribes with fewer genera appear in a new expanded circumscription (Angelonieae, Globularieae, Russelieae, Sibthorpieae). Gratiolieae in particular is one tribe that needs further study to identify its correct circumscription. It should be emphasized that we are not proposing a formal classification of Plantaginaceae here; instead, our new circumscription of Plantaginaceae should be viewed as a guide with which systematists and also other botanists may comparatively study the genera and increase our understanding of the evolution of this family. This will be especially important for those extending their work on the molecular model organism *Antirrhinum majus* to other members of the family. Our study will also be significant for those who investigate evolution within Scrophulariaceae s.l. because it will allow comparison of earlier results with a new phylogenetic hypothesis. One of those areas, biogeography, has been addressed, and the results demonstrate the necessity to reevaluate results from previous studies assuming different familial circumscription. So many members of Plantaginaceae are well known among botanists that it will be exciting to see how future studies will alter our knowledge on the evolution of the Plantaginaceae in light of the new relationships that we have proposed.

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