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FAMILIAL PLACEMENT AND RELATIONS OF *REHMANNIA* AND *TRIAENOPHORA* (SCROPHULARIACEAE S.L.) INFERRED FROM FIVE GENE REGIONS[1]

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Accurate classification systems based on evolution are imperative for biological investigations. The recent explosion of molecular phylogenetics has resulted in a much improved classification of angiosperms. More than five phylogenetic lineages have been recognized from Scrophulariaceae sensu lato since the family was determined to be polyphyletic; however, questions remain about the genera that have not been assigned to one of the segregate families of Scrophulariaceae s.l. *Rehmannia* Liboschitz and *Triaenophora* Solereder are such genera with uncertain familial placement. There also is debate whether *Triaenophora* should be segregated from *Rehmannia*. To evaluate the phylogenetic relations between *Rehmannia* and *Triaenophora*, to find their closest relatives, and to verify their familial placement, we conducted phylogenetic analyses of the sequences of one nuclear DNA (ITS) region and four chloroplast DNA gene regions (*trnL-F*, *rps16*, *rbcL*, and *rps2*) individually and combined. The analyses showed that *Rehmannia* and *Triaenophora* are each strongly supported as monophyletic and together are sister to Orobanchaceae. This relation was corroborated by phytochemical and morphological data. Based on these data, we suggest that *Rehmannia* and *Triaenophora* represent the second nonparasitic branch sister to the remainder of Orobanchaceae (including *Lindenbergia*).

Key words: DNA sequence; familial placement; Orobanchaceae; phylogenetic relations; *Rehmannia*; Scrophulariaceae sensu lato; *Triaenophora*.

Nothing in biology makes sense except in the light of evolution. —Theodosius Dobzhansky (1964)

As a corollary to Theodosius Dobzhansky's famous quote, understanding the evolutionary history of organisms can improve our understanding of biology. Recent molecular phylogenetic analyses have resulted in a major rearrangement of angiosperm classification that now better reflects the evolutionary history of these plants. However, many species remain unsampled and thus unplaced in the new classification scheme. A series of molecular systematic studies of Scrophulariaceae s.l. have revealed that the traditionally circumscribed Scrophulariaceae is polyphyletic (Olmstead and Reeves, 1995; dePamphilis et al., 1997; Olmstead et al., 2001; Oxelman et al., 2005). These studies have resulted in recircumscriptions and new descriptions of families to encompass the monophyletic lineages that were recovered. However, questions remain about the genera that have not been assigned to one of the segregate families of Scrophulariaceae s.l., such as *Rehmannia* and *Triaenophora*.

The genus *Rehmannia* Liboschitz consists of six species endemic to China (Chin, 1979), in which *R. glutinosa* is widely distributed in central China and cultivated in Japan and Korea (Rix, 1987) and is an important species in traditional Chinese medicine. *Rehmannia* has longstanding controversies surrounding its systematic placement at both the generic and familial levels. It was originally included within *Digitalis* (Gaertner, 1770) and was established by Liboschitz in 1835 because of its corolla shape and fruit dehiscence (Fischer and Meyer, 1835). Since then, *Rehmannia* has usually been placed in the tribe Digitaleae in Scrophulariaceae s.l. (Bentham, 1876; Solereder, 1909; Li, 1948; Chin, 1979). Others have suggested *Rehmannia* to be part of subfamily Cyrtandroideae in Gesneriaceae based on reports of its unilocular ovary (de Candolle, 1845; Hemsley, 1895; Solereder, 1909; Li, 1948; Burtt, 1954).

Some species initially described as *Rehmannia* have been segregated as monotypic, i.e., *Triaenophora* and *Titanotrichum* (Solereder, 1909). *Titanotrichum* was transferred to Gesneriaceae (Solereder, 1909), a move that is supported by both morphological and molecular data (Burtt, 1954; Wang et al., 1990, 1992, 2002, 2004; Smith et al., 1997a, b; Pan et al., 2002). On the contrary, *Triaenophora*, which now contains three species (Solereder, 1909; Chin, 1979; Li et al., 2005), has received almost no attention besides Li (1948) who returned it to *Rehmannia*, and Chin (1979) who later segregated it.

As for the phylogenetic relations of *Rehmannia* and *Triaenophora*, one issue is whether there is any phylogenetic affinity with *Digitalis* (Gaertner, 1770). *Digitalis*, and *Rehmannia*/*Triaenophora* are remarkably different from each other in their corolla shapes and a series of morphological characters such as inflorescence morphology and fruit dehiscence, as well as geographic distribution (Chin, 1979; Wang and Wang, 2005). All phylogenetic analyses of Scrophulariaceae s.l. have placed *Digitalis* in Plantaginaceae (Olmstead et al., 2001; APG II, 2003; Albach et al., 2005; Oxelman et al., 2005; Tank et al., 2006). A second, equally likely probability is that *Rehmannia* and *Triaenophora* are members of Gesneriaceae because *Titanotrichum*, a segregate from *Rehmannia*, has been convincingly placed there (Burtt, 1954; Wang et al., 1990, 1992, 2002, 2004; Smith et al., 1997a, b; Pan et al., 2002).

Recently, *Rehmannia* was included in a cladistic analysis of DNA sequence data for the further disintegration of Scrophulariaceae, in which a single species of *Rehmannia* was sister to

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Lancea and *Mazus* (subfamily Mazoideae of Phyrmaceae sensu Beardsley and Olmstead, 2002) and in a clade with *Paulownia*, Orobanchaceae, and Phrymaceae (Oxelman et al., 2005). Sampling all six species of *Rehmannia*, Albach et al. (2007), using nuclear ITS and chloroplast *trnL-F* and *rps16* sequences, placed *Lindenbergia* as sister to *Rehmannia*. However, the low sampling of species other than *Rehmannia* weakens their result (only four species including outgroups are sampled outside *Rehmannia*).

As we have outlined, the familial placement of *Rehmannia* and *Triaenophora* has not been well resolved either from a morphological or a molecular perspective. The debate regarding the familial placement of *Rehmannia* and *Triaenophora* in morphology is mainly based on the selection of characters used for classification (Solereder, 1909; Burtt, 1954), many of which have been revealed to be convergent (Olmstead et al., 2001) and thus provide little insight regarding the evolutionary relations for the classification system. Meanwhile, the controversy in molecular data lies in the lack of sufficient sampling among the putative relatives of *Rehmannia* and *Triaenophora* and the relation between these two genera.

Greater sampling of taxa putatively close to *Rehmannia* and *Triaenophora* and the analysis of additional DNA regions are necessary to determine the familial placement of these two genera and their closest relatives in Lamiales s.l. This study is thus conducted with a comprehensive sampling of putative relatives of *Rehmannia* and *Triaenophora* in Lamiales s.l. and the use of five DNA regions (ITS, *trnL-F*, *rps16*, *rbcL*, and *rps2*) that have been shown to be particularly informative in the Lamiales s.l. (dePamphilis et al., 1997; Smith et al., 1997a, b; Nickrent et al., 1998; Young et al., 1999; Olmstead et al., 2001; Beardsley and Olmstead, 2002; Albach et al., 2005; Oxelman et al., 2005; Wolfe et al., 2005; Tank et al., 2006). The goal of this study was to (1) evaluate the phylogenetic relation between *Rehmannia* and *Triaenophora*, (2) find their closest relatives, and thereby (3) verify their familial placement.

MATERIALS AND METHODS

Taxon sampling—We sampled all six species of *Rehmannia* and two of three species of *Triaenophora*. To fully examine the putative relatives of *Rehmannia* and *Triaenophora*, we selected one species of *Paulownia*, two genera of Gesneriaceae, six genera of Plantaginaceae (including *Digitalis*), seven genera of Scrophulariaceae sensu stricto, six genera of Phyrmaceae (four in Phrymoideae and two in Mazoideae including four species of *Mazus* in addition to the one species sampled in previous studies), 12 representative genera of four major clades in Orobanchaceae sensu lato (including the nonparasitic genus *Lindenbergia*), three genera of Acanthaceae, two genera of Bignoniaceae, one genus of Lamiaceae, and one genus of Pedaliaceae. Taxon sampling was based on recent molecular systematic studies (dePamphilis et al., 1997; Smith et al., 1997a, b; Nickrent et al., 1998; Young et al., 1999; Olmstead et al., 2001; Beardsley and Olmstead, 2002; Albach et al., 2005; Oxelman et al., 2005; Wolfe et al., 2005; Bennett and Mathews, 2006; Tank et al., 2006). Voucher specimens are deposited in the Herbarium of the Institute of Botany, Chinese Academy of Sciences (PE). The materials studied and details of voucher specimens are shown in Table 1.

DNA extraction, PCR amplification, and sequencing—Total DNA was extracted from silica-gel-dried leaf material using the CTAB method following the protocol of Rogers and Bendich (1988) and used as the template in the polymerase chain reaction (PCR). The entire ITS region, comprising ITS1, 5.8S rDNA, and ITS2, was amplified with primers ITS1 and ITS4 (Wendel et al., 1995). The *trnL-F* region was amplified with primers c and f of Taberlet et al. (1991). The *rps16* intron was amplified with primers *rps16*-2F and *rps16*-R3 (Bremer et al., 2002). The *rbcL* and *rps2* gene regions were amplified with primers RH1 and Z1352R (Olmstead and Reeves, 1995; Wolfe and dePamphilis, 1997; Olmstead et al., 2001), and *rps2*-18F and *rps2*-661R (dePamphilis et al., 1997), respectively. PCR products were purified with the UNIQ-10 PCR purification kit (Sangon, Shanghai, China). Sequencing primers were the same as amplification primers. Automated sequencing was performed on a MegaBACE 1000 automatic sequencer (Amersham Biosciences, Sunnyvale, California, USA) using manufacturer's protocols. The DNA sequences reported in the paper have been deposited in GenBank with accession numbers shown in Table 1. For some genera, sequences of the five regions sampled here were available only for different species. Rather than limit our sampling of either genera or sequences, we combined sequences from different species into a single genus in our analyses provided that there was evidence that the genus was monophyletic and the genus was not the primary focus of our study.

Sequence alignment and phylogenetic analysis—Sequence alignments were made with the program CLUSTAL_X (Thompson et al., 1997) and refined manually for the maximization of sequence homology using the program BioEdit 5.0.9 (Hall, 1999).

Parsimony analysis for each matrix was carried out using maximum parsimony (MP) methods in the program PAUP* version 4.0b10 (Swofford, 2002). All characters and character-state changes were specified as unordered and weighted equally, and gaps were coded as missing data. Heuristic searches were performed with 1000 replicates of random addition, one tree held at each step during stepwise addition, tree-bisection-reconnection (TBR) branch swapping, Multrees in effect, and steepest descent off. To examine the robustness of various clades, we ran a bootstrap analysis (Felsenstein, 1985) with 500 replicates using a heuristic search with 1000 replicates of random sequence addition and TBR branch swapping.

Bayesian inference (BI) was conducted using the program MrBayes version 3.0b4 (Ronquist and Huelsenbeck, 2003). The program Modeltest 3.06 (Posada and Crandall, 1998) was employed to determine the appropriate model of sequence evolution for each DNA data set. Four chains of Markov chain Monte Carlo (MCMC) were each run for 10 000 000 generations and were sampled every 100 000 generations, starting with a random tree. For each run, the first 50 samples before the chains reached stationarity were discarded as burn-in. Posterior probability (PP) was used to estimate robustness.

For combined sequence data, the incongruence-length-difference (ILD) test (Farris et al., 1994) was conducted, using the partition homogeneity test in PAUP* 4.0b10 (Swofford, 2002), to examine the congruence between nuclear ITS and chloroplast data sets. Test settings were 100 random stepwise additions and 1000 replicates of heuristic search with TBR branch swapping (Farris et al., 1994). The resulting P value was used to determine whether the two data sets had significant incongruence ($P < 0.05$). We excluded species from the combined data set if they only had ITS or cpDNA sequences available.

Topological congruence between the trees constraining Phrymaceae as monophyletic and no constraint was evaluated with the Templeton (1983) test using PAUP* 4.0b10 (Swofford, 2002). The phylogenetic analysis herein was divided into two steps as follows. We first conducted cladistic analyses of combined cpDNA (*trnL-F*, *rps16*, *rbcL*, and *rps2*) from all sampled taxa. The four chloroplast regions (cpDNA) included in this study formed a single linkage group as part of the chloroplast genome, so conflicts that might arise between data partitions from different sources subject to different evolutionary histories should not exist (Olmstead et al., 2001). The genus *Calceolaria* was selected as the outgroup based on Olmstead et al. (2001). Attempts to use ITS sequences at this level resulted in numerous ambiguities, and analyses of these sequences resulted in spurious relations among some taxa. Second, based on the described analyses, together with results of previous molecular systematic studies (Beardsley and Olmstead, 2002; Oxelman et al., 2005), we selected different taxa for separate and combined analyses of nrDNA ITS and combined cpDNA (e.g., excluding Scrophulariaceae s.s., Plantaginaceae, Gesneriaceae), focusing on clades presumably closest to *Rehmannia/Triaenophora* and Orobanchaceae. The representative species of Acanthaceae, Bignoniaceae, Lamiaceae, and Pedaliaceae were selected as the outgroups.

RESULTS

A comparison of the sequences we generated with those published from Albach et al. (2007) indicates that the ITS sequences are identical to each other, the *rps16* sequences were 99.76–100% similar, and the *trnL-F* sequences were 99.29–100% similar. These comparisons confirm the accuracy of both the sequences generated herein and those of Albach et al. (2007) for *Rehmannia*.

TABLE 1. Species, voucher with collection locality and GeneBank accession number for taxa included in this study (new sequences are in boldface). NA: not applicable; PE: Herbarium, Institute of Botany, Chinese Academy of Sciences where the voucher specimens were deposited.

Taxon	Voucher, collection locality and citation	GenBank accession number				
		ITS	*trnL-F*	*rps16*	*rbcL*	*rps2*
Ingroups						
Rehmannia						
R. chingii H. L. Li	XZ-2004–04–004; Zhejiang, China. (PE)	**EF363673**	**EF363679**	**FJ172696**	**FJ172724**	**FJ172710**
R. elata N. E. Brown	Albach et al., 2007	DQ069315	DQ856496	DQ856490	—	—
R. glutinosa (Gaert.) Libosch. ex Fisch. et Mey.	XZ-2004–04–005; Beijing, China. (PE)	**EF363674**	**EF363680**	**FJ172697**	**FJ172725**	**FJ172711**
R. henryi N. E. Brown	XZ-2004–04–002; Hubei, China. (PE)	**EF363671**	**EF363677**	**FJ172694**	**FJ172722**	**FJ172708**
R. piasezkii Maximowicz	XZ-2004–04–001; Hubei, China. (PE)	**EF363670**	**EF363676**	**FJ172693**	**FJ172721**	**FJ172707**
Rehmannia solanifolia Tsoong et Chin	XZ-2004–04–003; Chongqing, China. (PE)	**EF363672**	**EF363678**	**FJ172695**	**FJ172723**	**FJ172709**
Triaenophora						
T. rupestris (Hemsl.) Solereder	XZ-2004–04–009; Hubei, China. (PE)	**EF363675**	**EF363681**	**FJ172698**	**FJ172726**	**FJ172712**
T. shennongjiaensis X. D. Li	XZ-2007–0522; Hubei, China. (PE)	**FJ172741**	**FJ172690**	**FJ172704**	**FJ172732**	**FJ172717**
Acanthaceae						
Barleria lupulina Lindl.	McDade et al., 2000	AF169751	—	—	—	—
B. prionitis Lindl.	Olmstead et al., 2001	—	—	—	L01886	AF248247
Elytraria crenata Vahl.	Olmstead et al., 2001	—	—	—	AF188127	—
E. imbricata Vahl.	McDade et al., 2000	AF169852	AF061819	—	—	—
Thunbergia alata Bojer ex Sims	McDade et al., 2000; Olmstead et al., 2001; Oxelman et al., 2005	AF169850	AJ608564	AJ609131	—	AF248248
T. usumbarica Lindau	Olmstead et al., 2001	—	—	—	L12596	—
Bignoniaceae						
Catalpa speciosa Warder ex Engelm	Oxelman et al., 2005; Li, 2008	AY486307	AJ608599	AJ609197	—	—
Catalpa sp.	Olmstead et al., 2001	—	—	—	L11679	AF248256
Kigelia africana Benth.	Olmstead et al., 2001; Gutierrez and Freeman, unpublished data	AY178638	—	—	AF102648	U48764
Gesneriaceae						
Streptocarpus caulescens Vatke	Oxelman et al., 2005	NA	AJ608601	AJ609135	—	—
Streptocarpus holstii Engl.	Olmstead et al., 2001	NA	—	—	L14409	—
Titanotrichum oldhamii (Hemsl) Solereder.	Wang et al., 2004	NA	AY423129	—	AF206829	—
Lamiaceae						
Lamium purpureum L.	Olmstead et al., 2001; Oxelman et al., 2005; Sudarmono and Okada, unpublished data	AB266244	AJ608588	AJ609175	U75702	AF248259
Orobanchaceae						
Alectra sessiliflora Benth.	Olmstead et al., 2001; Wolfe et al., 2005	AY911210	—	—	AF026820	U48742
Boschniakia strobilacea A. Gray	Olmstead et al., 2001; Wolfe et al., 2005	AY911215	—	—	AF26818	U48758
Buchnera glabrata Benth	Wolfe et al., 2005	AY911216	NA	NA	NA	NA
Castilleja linariifolia Benth	Olmstead et al., 2001; Tank and Olmstead, 2008	—	—	EF103788	AF026823	U48739
Castilleja sulphurea Rydberg	Beardsley and Olmstead, 2002	AF478944	AF479008	—	—	—
Lindenbergia philippensis Benth.	Olmstead et al., 2001; Oxelman et al., 2005; Wolfe et al., 2005	AY911231	AJ608586	AJ609169	AF123664	AF055151
Melampyrum lineare Lam.	Olmstead et al., 2001; Jobson and Albert, 2002	—	AF482608	—	AF026834	—
M. sylvaticum L.	Olmstead et al., 2001; Wolfe et al., 2005	AY911232	—	—	—	AF055148
Melasma scabrum Berg.	Olmstead et al., 2001; Wolfe et al., 2005	AY911233	—	—	AF190904	U48743
Orobanche corymbosa (Rydb.) Ferris	Olmstead et al., 2001; Wolfe et al., 2005	AY911236	—	—	U73969	U48760
Orobanche hederae Duby	Bremer et al., 2002	—	—	AJ431050	—	—
O. minor Sm.	Lohan and Wolfe, 1998	—	AJ007724	—	—	—
Pedicularis attollens (A.) Gray	Tank and Olmstead, 2008	—	EF103899	EF103821	—	—
P. foliosa L.	Olmstead et al., 2001; Ree, 2005	AY949679	—	—	AF026836	U48740
Schwalbea americana L.	Wolfe et al., 2005	AY911252	NA	NA	NA	NA
Seymeria laciniata Standl.	Tank and Olmstead, 2008	—	EF103898	EF103820.	—	—
S. pectinata Pursh	Olmstead et al., 2001; Wolfe et al., 2005	AY911253	—	—	AF026837	AF055141
Tozzia alpina L.	Olmstead et al., 2001; Wolfe et al., 2005	AY911258	—	—	AF026843	U48754
Paulownia						
P. tomentosa (Thunb.) Steud	Beardsley and Olmstead, 2002; Oxelman et al., 2005	AF478941	AF479005	AJ609153	L36447	AF055155
Pedaliaceae						
Sesamum indicum L.	Olmstead et al, 2001; Beardsley and Olmstead, 2002; Oxelman et al., 2005	AF478946	AF479010	AJ609226	L14408	AF248261

TABLE 1. Continued.

Taxon	Voucher, collection locality and citation	GenBank accession number				
		ITS	*trnL-F*	*rps16*	*rbcL*	*rps2*
Phrymaceae						
Berendtia laevigata B. L. Rob et Greenm.	Oxelman et al., 2005	—	AJ608615	AJ609208	—	—
B. rugosa (Benth.) Gray	Beardsley et al., 2004	AY575398	—	—	—	—
Hemichaena fruticosa Benth.	Beardsley and Olmstead, 2002; Oxelman et al., 2005	AF478921	AJ608632	AJ609179	—	—
Lancea tibetica Hook. f. et Thoms.	XZ-2007–0525; Sichuan, China. (PE)	**FJ172736**	**FJ172685**	**FJ172699**	**FJ172727**	**FJ172713**
Mazus gracilis Hemsl.	XZ-2007–058; Henan, China. (PE)	**FJ172738**	**FJ172687**	**FJ172701**	**FJ172729**	**FJ172715**
M. japonicus (Thunb.) O. Kuntze.	XZ-2007–051; Beijing, China. (PE)	**FJ172737**	**FJ172686**	**FJ172700**	**FJ172728**	**FJ172714**
M. omeiensis Li.	XZ-2007–0515; Sichuan, China. (PE)	**FJ172739**	**FJ172688**	**FJ172702**	**FJ172731**	—
M. reptans N. E. Br.	Beardsley and Olmstead, 2002	AF478940	NA	NA	NA	NA
M. spicatus Vaniot.	XZ-2007–0514; Henan, China. (PE)	**FJ172740**	**FJ172689**	**FJ172703**	**FJ172730**	**FJ172716**
M. stachydifolius (Turcz.) Maxim	Oxelman et al., 2005	—	AJ607432 AJ607433	AJ609167	—	—
Mimulus aurantiacus Curtis	Olmstead et al., 2001; Beardsley and Olmstead, 2002; Oxelman et al., 2005	AF478917	AF478982	AJ609163	AF026835	AF055154
M. tenellus var. tenellus Bunge	XZ-2007–053; Henan, China. (PE)	**FJ172742**	**FJ172691**	**FJ172705**	**FJ172733**	**FJ172718**
Mimulus szechuanensis Pai	XZ-2007–0523; Sichuan, China. (PE)	**FJ172743**	**FJ172692**	**FJ172706**	**FJ172734**	**FJ172719**
Phryma leptostachya L.	Oxelman et al., 2005	—	AJ430928	AJ609150	—	—
P. leptostachya L.*var asiatica* Hara	Beardsley and Olmstead, 2002; XZ-2007–061; Henan, China. (PE)	AF478924	—	—	**FJ172735**	**FJ172720**
Plantaginaceae						
Antirrhinum majus L.	Olmstead et al., 2001; Oxelman et al., 2005	NA	AJ608634	AJ609218	L11688	U48766
Chelone obliqua L.	Olmstead et al., 2001; Oxelman et al., 2005; Wolfe et al., 2006	NA	DQ531203	AJ609220	AF026824	U48770
Collinsia grandiflora Lindley	Olmstead et al., 2001	NA	—	—	AF026825	AF248252
C. heterophylla R.Grah	Wolfe et al., 2006	NA	DQ531198	—	—	—
C. tinctoria Hartw. ex. Benth	Albach et al., 2005	NA	—	AY492200	—	—
Digitalis obscura L.	Albach et al., 2004; Albach and Chase, 2004	NA	AF486418	AY218799	—	—
D.s purpurea L.	Olmstead et al., 2001	NA	—	—	L01902	U48767
Plantago coronopus L.	Albach and Chase, 2004	NA	—	AY218801	—	—
P. lanceolata L.	Olmstead et al., 2001; Rønsted et al., 2002	NA	AY101952	—	L36454	—
P. major L.	Olmstead et al., 2001	NA	—	—	—	AF248254
Veronica arvensis L.	Olmstead et al., 2001	NA	—	—	—	U48768
V. persica Poir.	Olmstead et al., 2001; Albach et al., 2004	NA	AF513336	—	L36453	—
V. campylopoda Boiss.	Albach and Chase, 2004	NA	—	AY218811	—	—
Scrophulariaceae s.s.						
Alonsoa unilabiata (L. f.) Steud.	Olmstead et al., 2001; Oxelman et al., 2005	NA	AJ608620	AJ609217	AF026821	AF248262
Buddleja davidii Franchet	Olmstead et al., 2001; Oxelman et al., 2005	NA	AJ608612	AJ609204	L14392	AF248264
Leucophyllum frutescens I. M. Johnston	Olmstead et al., 2001; Oxelman et al., 2005	NA	AF380873	AJ609171	AF123665	AF055156
Myoporum mauritianum A. DC.	Olmstead et al., 2001; Oxelman et al., 2005	NA	AJ608582	AJ609161	L36445	—
M. parvifolium R.Br.	Olmstead et al., 2001	NA	—	—	—	AF055157
Nemesia strumosa Benth.	Olmstead et al., 2001; Oxelman et al., 2005	NA	AJ608631	AJ609159	AF123663	AF248265
Scrophularia californica Cham.& Schldl.	Olmstead et al., 2001; Oxelman et al., 2005	NA	—	AJ609224	L36449	U48762
Scrophularia ningpoensis Hemsl.	Chen et al., 2005	NA	AY695886	—	—	—
Verbascum arcturus L.	Oxelman et al., 2005	NA	—	AJ609128	—	—
V. blattaria L.	Olmstead et al., 2001	NA	—	—	—	U48763
V. thapsus L.	Olmstead et al., 2001	NA	—	—	L36452	—
V. speciosum Schrad.	Mayer et al., 2003	NA	AJ492271	—	—	—
Outgroup						
Calceolariaceae						
Calceolaria mexicana Benth	Olmstead et al., 2001; Oxelman et al., 2005	NA	AJ608611	AJ609202	AF123669	AF055162

Analyses with all sampled taxa—Data for one or two of the four chloroplast regions are missing for 15 genera, but no genus was missing more than two sequences of the four genes. The entire cpDNA data set consists of 4125 bp, of which 2554 (61.9%) were constant, 721 (17.5%) were variable but uninformative, and 850 (20.6%) were parsimony informative. Parsimony analyses resulted in nine trees of 3511 steps each (consistency index [CI] = 0.623; retention index [RI] = 0.608). One most parsimonious (MP) tree of cpDNA data (Fig. 1) was congruent with the Bayesian tree (the best-fit model GTR + I + G) in topology. The MP tree comprised six main clades labeled A–G. *Titanotrichum oldhamii* was sister to *Streptocarpus* in Gesneriaceae (clade A) with high support (bootstrap support [BS] = 94%; posterior probability [PP] = 100%). Clades B and C contained the species of Plantaginaceae (BS = 91%; PP = 100%) and Scrophulariaceae sensu stricto (BS = 87%; PP = 100%), respectively. Clade D was Acanthaceae, Pedaliaceae, Lamiaceae, and Bignoniaceae (BS = 73%, PP = 100%) and clade E was Mazoideae (BS = PP = 100%). Clade F comprised Phrymoideae with BS = PP = 100%. Clade G included *Rehmannia*, *Triaenophora*, and Orobanchaceae with BS = 62% and PP = 99%. *Rehmannia* and *Triaenophora* formed one strongly supported lineage (BS = 100%; PP = 100%) and was sister to Orobanchaceae, that was likewise strongly supported as monophyletic (BS = 88%; PP = 100%). *Paulownia* was sister to clade G with low support.

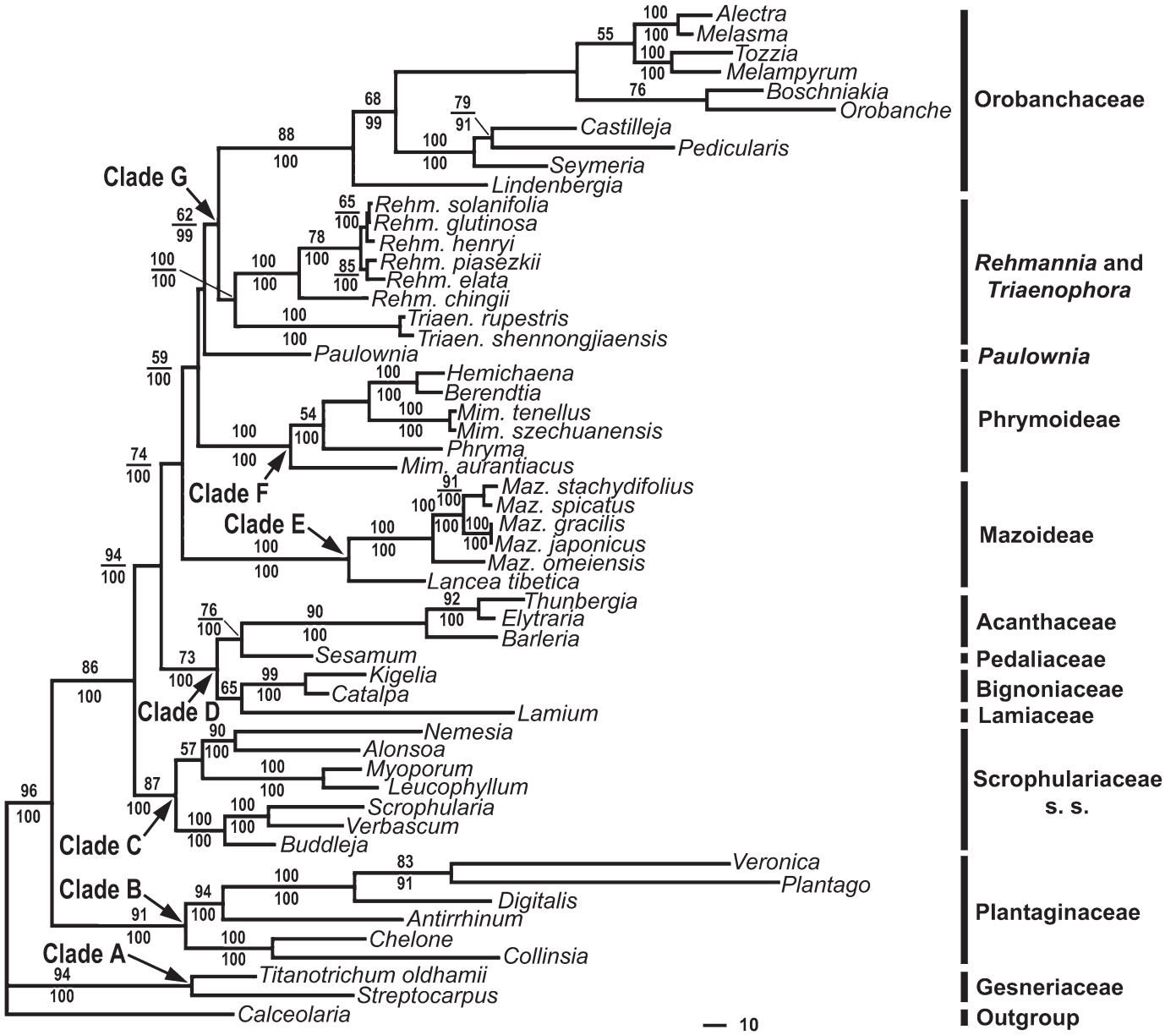

Fig. 1. One of nine most parsimonious trees generated from analysis of combined chloroplast for all sampled taxa. Branch lengths are proportional to number of nucleotide substitutions (scales represent 10 substitutions). Bootstrap (BS) values (≥50%) are above the branches; Bayesian posterior probabilities (PP) (≥90%) are below the branches. *Maz* = *Mazus*, *Mim* = *Mimulus*, *Rehm* = *Rehmannia*, *Triaen* = *Triaenophora*.

Analyses with selected taxa—*ITS analysis*—The aligned sequences of ITS had 656 bp, of which 209 (31.9%) were constant, 94 (14.3%) were variable but uninformative, and 353 (53.8%) were parsimony informative. Parsimony analysis resulted in 10 trees of 2004 steps each, CI of 0.445, and RI of 0.591. The MP tree (Fig. 2) was congruent with the Bayesian tree (the best-fit model GTR + I + G) in topology except for the position of *Paulownia*. *Paulownia* was sister to Phrymoideae (clade F) in the MP tree, but sister to the group that includes clades E, F, and G in the Bayesian tree. The ITS MP tree comprised three main clades labeled as E, F, and G, that correspond to the clades recovered in Fig. 1. *Rehmannia*, *Triaenophora*,

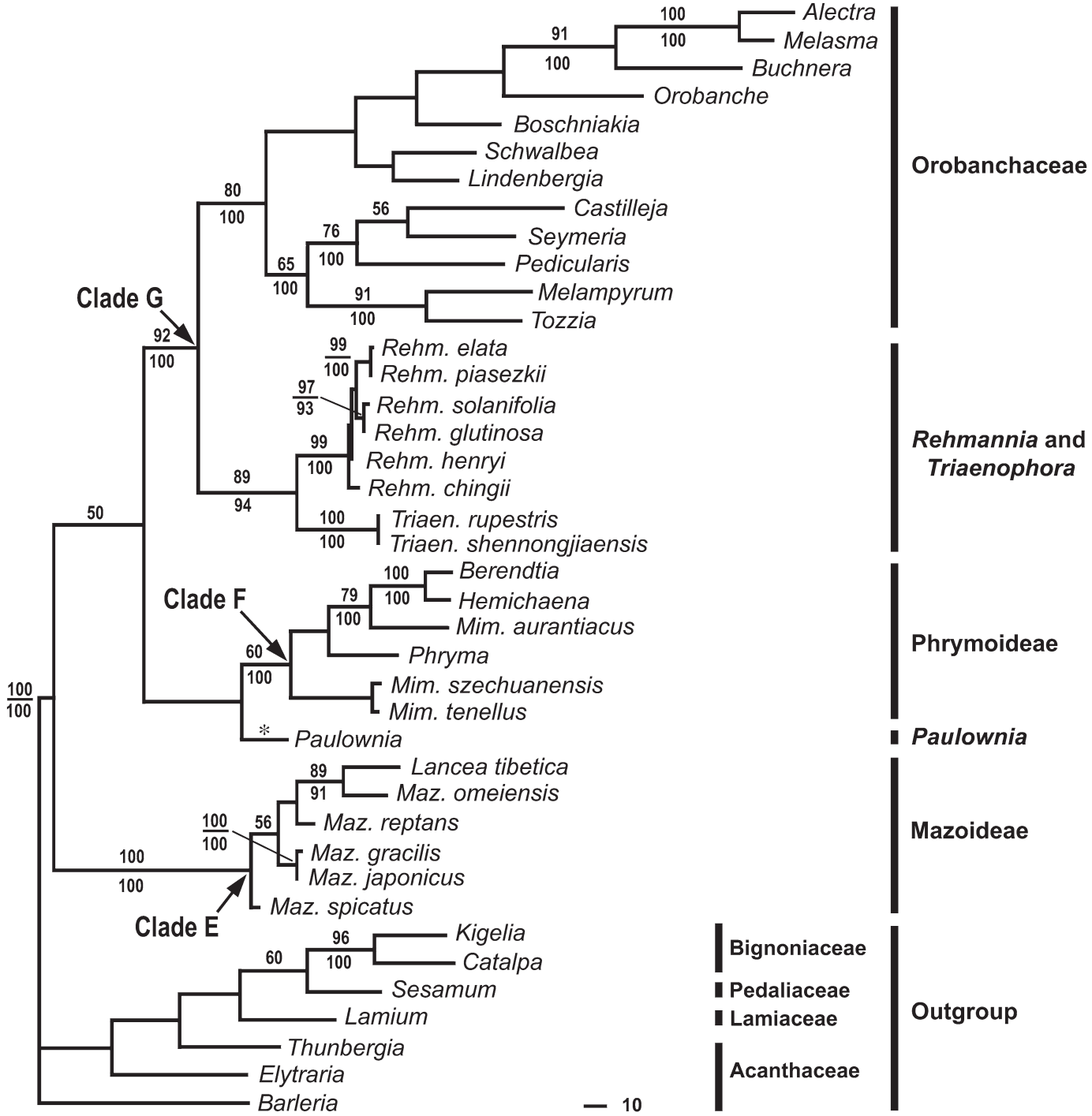

Fig. 2. One of 10 most parsimonious trees generated from the ITS data for selected taxa. Branch lengths are proportional to number of nucleotide substitutions (scales represent 10 substitutions). Branches marked by an asterisk indicate the topological discordance between most parsimonious (MP) and Bayesian trees. Bootstrap (BS) values (≥50%) are above the branches; Bayesian posterior probabilities (PP) (≥90%) are below the branches. *Maz* = *Mazus*, *Mim* = *Mimulus*, *Rehm* = *Rehmannia*, *Triaen* = *Triaenophora*.

Paulownia, Mazoideae, Phrymoideae, and Orobanchaceae formed a monophyletic group with maximum support. Clade E was Mazoideae, and clade F comprised all sampled species of Phrymoideae with BS = 60% and PP = 100%. Clade G was also recovered as monophyletic including *Rehmannia*, *Triaenophora*, and Orobanchaceae with BS = 92% and PP = 100%. *Rehmannia* and *Triaenophora*, each as a monophyletic group, formed one strongly supported lineage (BS = 89%; PP = 94%), which was sister to Orobanchaceae. Orobanchaceae was likewise strongly supported as monophyletic (BS = 80%; PP = 100%). The low supports for the relations within Orobanchaceae were probably due to the sparse taxon sampling within the family. Templeton's test indicated incongruence between the trees constraining Phrymaceae (Phrymoideae and Mazoideae) as monophyletic and that no constraint was insignificant ($P = 0.6103$).

Analysis of combined chloroplast data—The combined chloroplast data set consisted of 3880 positions, of which 2783 (71.7%) were constant, 556 (14.4%) were variable but uninformative, and 541 (13.9%) were parsimony informative. Parsimony analyses resulted in six trees of 1845 steps each (CI = 0.73; RI = 0.737). The MP tree of cpDNA data (Fig. 3) was congruent with the Bayesian tree (the best-fit model GTR + I + G) in topology. Clades E, F, and G, found in the ITS tree (Fig. 2), were also recovered in the cpDNA tree with high support (Fig. 3). The topology of the cpDNA MP tree (Fig. 3) differed from the ITS topology (Fig. 2) mainly in the position of *Paulownia*. *Paulownia* was sister to Phrymoideae (Clade F) with low support in the ITS tree (Fig. 2), but was sister to clade G with low support in the cpDNA tree (Fig. 3). Clade G, which includes *Rehmannia*, *Triaenophora*, and Orobanchaceae, was recovered as monophyletic with BS = 59% and PP = 100%. *Rehmannia* and *Triaenophora*, each as a monophyletic group, formed one maximum supported lineage, which was sister to Orobanchaceae. Orobanchaceae was strongly supported as monophyletic (BS = 78%; PP = 100%) in which *Lindenbergia* was sister to the remainder of Orobanchaceae. Clade G and *Paulownia* were further clustered with Phrymoideae (clade F) with moderate support. Templeton's test indicated incongruence between the trees constraining Phrymaceae as monophyletic and that no constraint was insignificant ($P = 0.5316$).

Analysis of combined chloroplast and nuclear ITS data—The ILD test gave a value of $P = 0.21$, indicating that the data sets were not significantly different from random partitions of the combined chloroplast and ITS data. The combined chloroplast and ITS data sets consisted of 4536 positions, of which 2998 (66.1%) were constant, 653 (14.4%) were variable but uninformative, and 885 (19.5%) were parsimony informative. Parsimony analyses resulted in one tree of 3735 steps (CI = 0.592, RI = 0.652). The MP tree (Fig. 4) was congruent with the Bayesian tree (the best-fit model GTR + I + G) in topology. The topology of the MP tree (Fig. 4) from combined cpDNA and nuclear ITS data were completely congruent with the ITS MP tree in the major clades (Fig. 2) and the cpDNA MP tree except for the position of *Paulownia* in the cpDNA tree (Fig. 3). The ingroup nodes in the topology of the combined chloroplast and ITS data received higher support than those in the separate analyses of either cpDNA or nuclear ITS data alone. Templeton's test indicated incongruence between the trees constraining Phrymaceae as monophyletic and that no constraint was insignificant ($P = 0.4054$).

DISCUSSION

Phylogenetic relation between Rehmannia and Triaenophora—*Triaenophora* has been considered closely related to *Rehmannia* in traditional systematics (Forbes and Hemsley, 1890; Solereder, 1909; Li, 1948; Chin, 1979). Our molecular data show that *Rehmannia* and *Triaenophora* form a strongly supported clade, in which *Triaenophora* is sister to *Rehmannia*. The sister relation between *Rehmannia* and *Triaenophora* is also corroborated by allozymic variability (Li et al., 2007) and numerical analysis of morphological data (Li et al., 2008). This sister relation is not surprising because *Rehmannia* and *Triaenophora* share a series of uniform synapomorphies, such as two lateral bracteoles at the base of the pedicel just above the subtending bract (they are aborted early in development in *R. chingii*, *R. solanifolia*, and *R. glutinosa*), four stamens with a gap at the expected site of the adaxial staminode, and the unidirectional initiation of corolla lobes and stamens from the abaxial to the adaxial side (Wang and Wang, 2005). *Triaenophora* has a series of unique traits distinctive from those of *Rehmannia*, i.e., five trifid calyx lobes; dense, white, spreading, lanose-villous hairs on the stems, leaves, and pedicels; and a bilocular ovary (Chin, 1979; Wang and Wang, 2005). *Rehmannia,* as a strongly supported monophyletic group distinct from *Triaenophora,* is characterized by five revolute and undivided calyx lobes; brown or white glandular hairs on stems, leaves, and pedicels; and one ovarian locule (Chin, 1979; Wang and Wang, 2005).

Our results regarding the relations among species within *Rehmannia* are in agreement with Albach et al. (2007) and Li et al. (2007, 2008). Albach (D. C. Albach, Johannes Gutenberg-Universität Mainz, Germany, unpublished data) conducted a study similar to ours with the exception that a single species of *Triaenophora* was included (*T. rupestris*) and in place of *rps2*, analyzed *ndhF* sequences. The results of these two independently conducted studies provide mutual confirmation that *Triaenophora* and *Rehmannia* are sister to each other and together are sister to Orobanchaceae. Likewise, both studies find evidence against the monophyly of Phyrmaceae (discussed later). Our results further show that the monotypic genus *Titanotrichum* initially described as a species of *Rehmannia,* is not closely related, but better included in Gesneriaceae (Burtt, 1954; Wang et al., 1990, 1992, 2002, 2004; Smith et al., 1997a, b; Pan et al., 2002).

Familial placement of Rehmannia and Triaenophora—*Rehmannia* and *Triaenophora* were traditionally placed in the Digitaleae (Scrophulariaceae s.l.) with close affinity to *Digitalis* (Bentham, 1846, 1876; Forbes and Hemsley, 1890; Wettstein, 1891; Li, 1948; Chin, 1979). However, the inclusion of *Rehmannia* within Digitaleae was questioned when Oxelman et al. (2005) placed one species of *Rehmannia* as sister to *Mazus* and *Lancea* (Scrophulariaceae s.l. or Mazoideae sensu Beardsley and Olmstead, 2002). In all trees herein, *Rehmannia* and *Triaenophora* are shown to not have a close affinity with any Digitaleae. Our results also show no close relation between *Rehmannia* and Gesneriaceae including *Titanotrichum,* a relation that has been traditionally suggested (de Candolle, 1845; Hemsley, 1895; Solereder, 1909; Li, 1948; Burtt, 1954).

Rehmannia and *Triaenophora*, as a monophyletic group, are shown herein to be sister to Orobanchaceae (including *Lindenbergia*) with moderate to high support BS = 92 for ITS, 59–62 for cpDNA, 94 for combined and high to maximum PP (99–100) from all analyses. *Lindenbergia* is sister to other parasitic genera

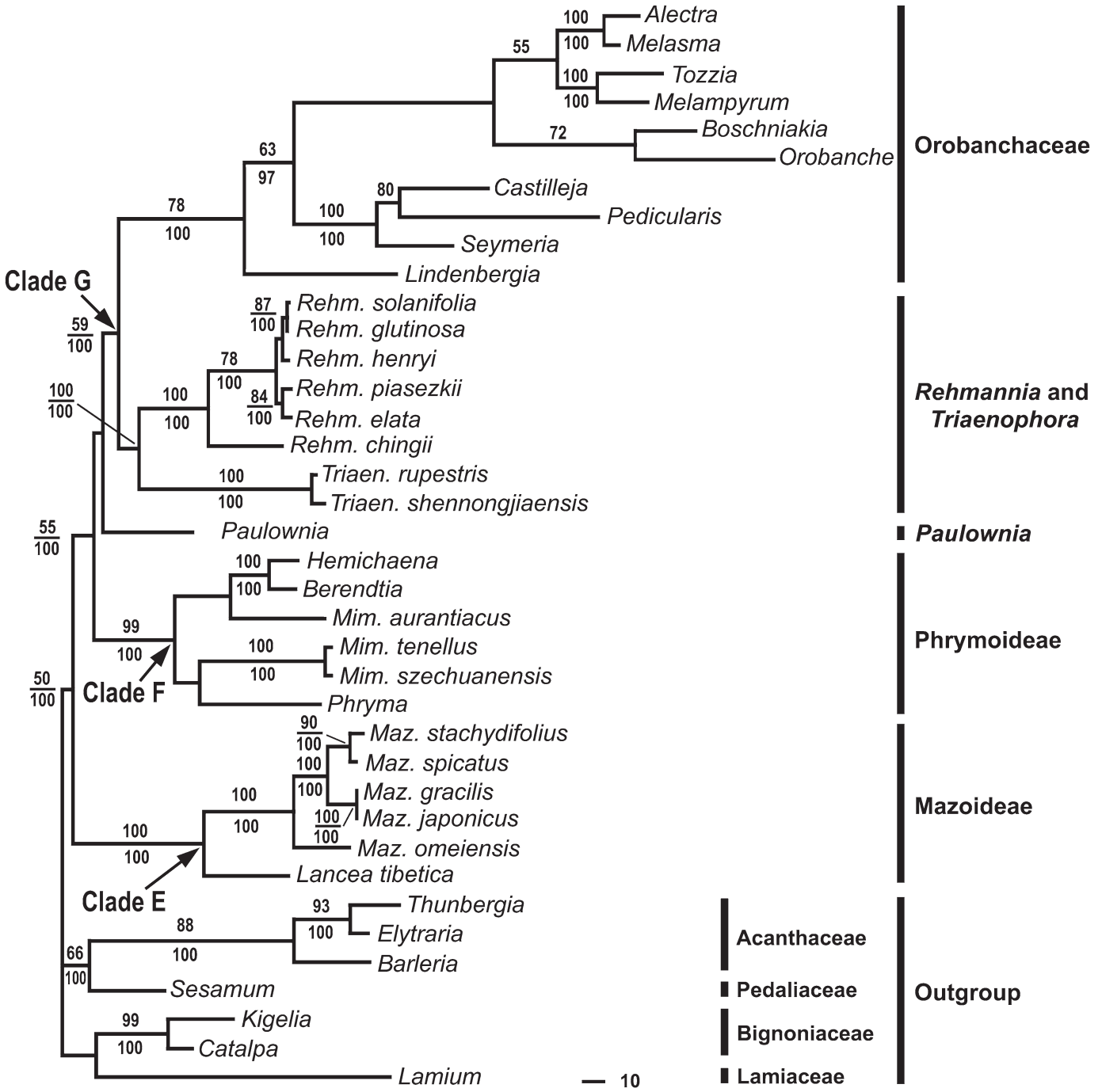

Fig. 3. One of six most parsimonious trees generated from combined chloroplast data for selected taxa. Branch lengths are proportional to number of nucleotide substitutions (scales represent 10 substitutions). Bootstrap (BS) values (≥50%) are shown above the branches, and Bayesian posterior probabilities (PP) values (≥90%) are indicated below the branches. *Maz* = *Mazus*, *Mim* = *Mimulus*, *Rehm* = *Rehmannia*, *Triaen* = *Triaenophora*.

of Orobanchaceae with high support in trees of cpDNA and combined ITS and cpDNA data as seen in previous molecular phylogenies (Young et al., 1999; Olmstead et al., 2001; Wolfe et al., 2005; Bennett and Mathews, 2006; Tank et al., 2006) rather than sister to *Rehmannia* and *Triaenophora* (Albach et al., 2007). Our results further confirm that Orobanchaceae is a well-supported lineage that includes the holoparasitic members traditionally treated in Orobanchaceae, the hemiparasitic taxa previously treated under Scrophulariaceae s.l. and the nonparasitic genus *Lindenbergia* (dePamphilis et al., 1997; Nickrent et al., 1998; Wolfe and dePamphilis, 1998; Young et al., 1999; Olmstead et al., 2001; Wolfe et al., 2005; Bennett and Mathews, 2006; Tank et al., 2006). The sister relationship between *Rehmannia* and Mazoideae recognized in Oxelman et al. (2005) is not supported by our molecular data with greater sampling both in *Rehmannia* and Mazoideae. *Rehmannia* and *Triaenophora* together with Orobanchaceae are sister to *Paulownia* and Phrymoideae.

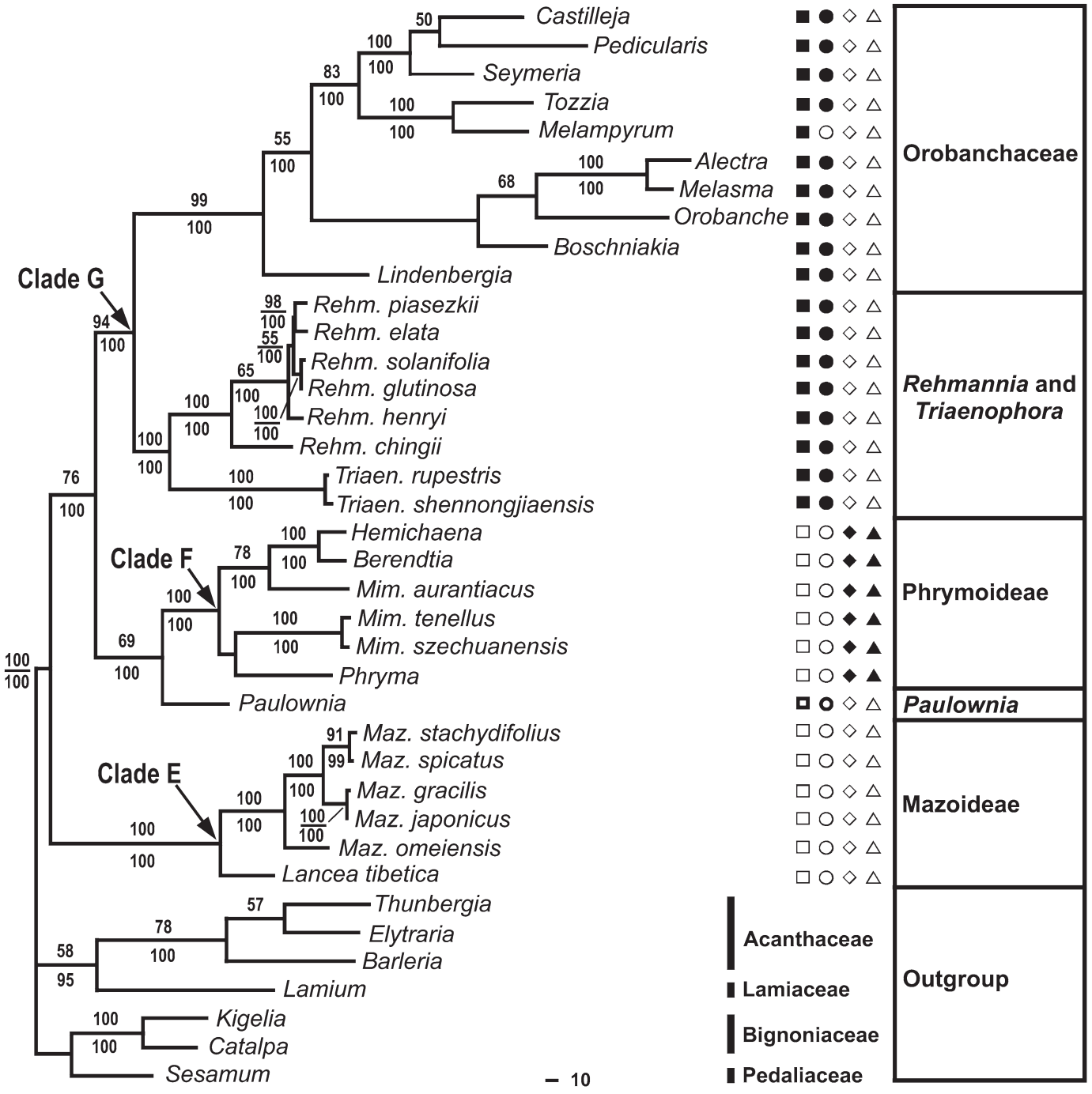

Fig. 4. Single most parsimonious tree generated from combined chloroplast and ITS data for selected taxa. Bootstrap (BS) values (≥50%) are above the branches; Bayesian posterior probabilities (PP) (≥90%) are below the branches. *Maz* = *Mazus*, *Mim* = *Mimulus*, *Rehm* = *Rehmannia*, *Triaen* = *Triaenophora*. ■ part of capsule exserted from the persistent calyx tube; □ capsule included in persistent calyx tube; ◘ capsule almost completely exserted from the persistent calyx tube; ● seeds with alveolate and pitted testa; ○ seeds with smooth or anomalous reticulate surfaces; ◘ seeds with membranous wings; ▲ calyx of connate sepals with spinescent apices; △ calyx with long triangular and lanceolate calyx lobes; ◆ corolla with two highly reduced and triangular upper lobes; ◇ corolla with two oblong and orbicular upper lobes.

On the basis of observations of morphological characters, together with previous reported data (Chin, 1979; Zhang, 1990a), we conclude that *Rehmannia*, *Triaenophora*, and Orobanchaceae often have capsules that are half or partly exserted from the persistent calyx tubes, whereas in Phrymaceae, the capsules are completely included in the persistent calyx tube (Fig. 4). Seed coat characters are seldom used for systematic analyses at higher taxonomic levels. However, seed coat characters may provide synapomorphies for some of the relations recovered in our molecular based trees. *Rehmannia* and *Triaenophora* are characterized by numerous minute seeds with alveolate and reticulate testa similar to seeds of Orobanchaceae with alveolate,

pitted and reticulate testa (except for seeds of *Melampyrum* with smooth testa; Fig. 4) (Musselman and Mann, 1976; Zhang, 1990b; An and Hong, 2003; Plaza et al., 2004). Meanwhile, Phrymaceae possess minute seeds with smooth testa, and *Paulownia* is characterized by seeds with membranous wings (Tsoong, 1979; Yang, 1979; Fig. 4). Along with *Lindenbergia*, *Rehmannia*, and *Triaenophora* have tricolporate pollen with reticulate sculpting of the exine (Ying et al., 1993; Hjertson, 1995; Wang et al., 1997), which differs from the characteristic tricolpate pollen (and its variants) with retipilate sculpting of the exine in other parasitic genera in Orobanchaceae (Tsoong and Chang, 1965; Minkin and Eshbaugh, 1989; Bolliger and Wick, 1990; Abu Sbaih et al., 1994; Zhang, 1990b; Lu et al., 2007). The evolutionary trends of pollen exine ornamentation from reticulate to retipilate sculpting have been seen in Dichapetalaceae and in many different groups (Punt, 1976; Minkin and Eshbaugh, 1989), as well as from tricolporate to tricolpate pollen in some taxa (Ferguson and Skvarla, 1981; Hong, 1984; Minkin and Eshbaugh, 1989; Martínez-Ortega et al., 2000). The tricolpate pollen with retipilate sculpting of the exine in parasitic genera of Orobanchaceae may be derived from tricolporate pollen with reticulate sculpting in the nonparasitic genera *Lindenbergia*, *Rehmannia*, and *Triaenophora*.

In addition, phytochemical characters are often used to complement or improve molecular trees (Grayer et al., 1999). Iridoids have been found as natural constituents in most taxa of Lamiales s.l. (Jensen, 1992). Comparison of iridoid glycosides distributed among *Rehmannia/Triaenophora*, Orobanchaceae, Scrophulariaceae s.s and Plantaginaceae shows that catalpol and aucubin are widely present in these taxa (Kitagawa et al., 1971; Oshio and Inouye, 1982; Grayer et al., 1999; Albach et al., 2007). However, *Rehmannia/Triaenophora* and most Orobanchaceae (including *Lindenbergia*) lack harpagide and 6-rhamnopyranosyl-catalpol and their esters, which are characteristic for many Scrophulariaceae s.s. (Jensen et al., 2008). This distribution pattern supports the sister relationship between *Rehmannia/Triaenophora* and Orobanchaceae. Meanwhile, the lack of sorbitol as the reserve carbohydrate in *Rehmannia* and its presence among members of Digitaleae (Kitagawa et al., 1971; Taskova et al., 2005; Albach et al., 2007) is further evidence against a close affinity between *Rehmannia* and Digitaleae.

Lastly, *Rehmannia* and *Triaenophora* are characterized by two lateral bracteoles borne at the flower pedicel just above the leaf-like subtending bract (Wang and Wang, 2005). In some species of *Rehmannia*, they are aborted early in development and cannot be detected at anthesis (Wang and Wang, 2005). Similarly, two lateral bracteoles frequently occur in Orobanchaceae, where they are borne at the pedicel above the leaf-like subtending bract or just below the flower due to a much shortened pedicel (Zhang, 1990a). *Rehmannia/Triaenophora* and Orobanchaceae (s.s.) are characterized by simple racemes, while *Lindenbergia* has compound racemes with each branch composed of flowers and several pairs of bracts, all of which are subtended by a large leaf-like bract (Yang, 1979; Zhang, 1990a). The reduction from inflorescence branch to a single or double flower frequently occurs in several major clades of angiosperms, such as *Silene* in Caryophyllaceae, *Salvia* in Lamiaceae, and *Rhynchoglossum* in Gesneriaceae (Weber, 1978; Weberling, 1989; Wang and Li, 2002). The two lateral bracteoles in *Rehmannia/Triaenophora* and Orobanchaceae (s.s.) might be the result of a transformation from compound racemes to simple racemes; however, further developmental analyses will be necessary to resolve this.

In comparison to other groups in Lamiales s.l. with respect to the distribution of related phytochemical and morphological characters, the combination of the aforementioned features are synapomorphies for *Rehmannia/Triaenophora* and Orobanchaceae. Based on the molecular results, corroborated by phytochemical and morphological data, we suggest that *Rehmannia* and *Triaenophora* represent the second nonparasitic branch sister to the remainder of Orobanchaceae s.l. (including *Lindenbergia*) or a clade at the rank of family sister to Orobanchaceae. Our results recognizing this sister relationship represent the first step toward better understanding the relations of *Rehmannia* and *Triaenophora* with other segregate families of Scrophulariaceae s.l. Further detailed studies are needed to better understand morphological and anatomical synapomorphies among these species.

The familial status of *Paulownia* and *Mazus*/*Lancea* (Mazoideae) remain uncertain in the results presented here. *Paulownia* has been placed alternately in the Scrophulariaceae s.l., Bignoniaceae, or assigned to a family of its own (Nakai, 1949; Beardsley and Olmstead, 2002). It is distinctively different from Orobanchaceae, Scrophulariaceae s.s. and Phrymaceae in its woody habit, capsule with a persistent woody calyx tube, and seeds with membranous wings (Fig. 4). The phylogenetic analyses herein, with increased sampling of *Mazus,* indicate that *Mazus* and *Lancea* (Mazoideae) may not be included in Phrymaceae as previously suggested by Oxelman et al. (2005). However, Templeton's tests (see Results, *Analyses with selected taxa*) do not reject the inclusion of Mazoideae in Phrymaceae. The systematic position of *Paulownia* and Mazoideae deserves further detailed studies with greater taxon sampling among their putative relatives and new DNA regions together with genetic or evolutionary developmental methods to gain a comprehensive understanding about their phylogenetic history.

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