

## Phylogenetic Relationships of the Monotypic Genus *Amphianthus* (Plantaginaceae Tribe Gratioleae) Inferred from Chloroplast DNA Sequences

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**Abstract**—Within the past decade, Scrophulariaceae sensu lato has been shown to be polyphyletic and, as a result, is currently undergoing major systematic revision. The traditionally recognized family is now generally considered to comprise several smaller families including the newly expanded Plantaginaceae, a family of 12 tribes, 92 genera, and approximately 2000 species. Recent evidence from molecular phylogenetics supports the inclusion of the tribe Gratioleae within the Plantaginaceae. Gratioleae includes 16–40 genera, depending on generic circumscription, many of which have yet to be assessed phylogenetically. *Amphianthus* is a monotypic genus whose systematic affinities have long been poorly known. We included *Amphianthus*, 10 additional Gratioleae genera, and several outgroup genera from Plantaginaceae in a phylogenetic investigation to examine the relationships of *Amphianthus*. We present the most complete phylogeny of the Gratioleae to date and provide evidence from chloroplast DNA sequences of the *ndhF* gene and the *trnS-trnG-trnG* intergenic spacer and intron that unequivocally place *Amphianthus* within *Gratiola*, and discuss the morphological evidence supporting our findings. Based on this evidence, we transfer the sole species of *Amphianthus* (*Amphianthus pusillus*) to *Gratiola*, establishing the new name *Gratiola amphiantha* and placing *Amphianthus* in synonymy with *Gratiola*.

**Keywords**—*Gratiola*, Gratiolaceae, *ndhF*, Scrophulariaceae s.l., *trnS-trnG-trnG*.

Within the past decade, Scrophulariaceae sensu lato (s.l.) has been shown to be polyphyletic and, as a result, is currently undergoing major systematic revision (Olmstead and Reeves 1995; Olmstead et al. 2001; Beardsley and Olmstead 2002; Albach et al. 2005; Oxelman et al. 2005; Rahmzadeh et al. 2005). Scrophulariaceae s.l. is now generally treated as several smaller families including the newly expanded Plantaginaceae (Veronicaceae sensu Olmstead et al. 2001; Olmstead et al. 2001; Fischer 2004; Albach et al. 2005). According to the circumscription of Albach et al. (2005), Plantaginaceae contains 12 tribes with 92 genera and approximately 2000 species. Olmstead et al. (2001) and more recently, Albach et al. (2005) and Oxelman et al. (2005), provided molecular evidence supporting the inclusion of tribe Gratioleae, the focus of the current study, within the Plantaginaceae.

Bentham and Hooker (1876) reported 37 genera and ca. 306 species for Gratioleae. Taxonomic modifications to the tribe were subsequently made by Wettstein (1891), Ruoy (1909), Pennell (1935), and Thieret (1954, 1967). Not until the recent application of molecular phylogenetic analysis, however, were these traditional taxonomic treatments rigorously evaluated. Many genera once included within Gratioleae (Bentham and Hooker 1876; Wettstein 1891) have recently been shown to be distantly related (Beardsley and Olmstead 2002; Albach et al. 2005; Oxelman et al. 2005; Rahmzadeh et al. 2005). With many genera now excluded from Gratioleae, the tribe is left with ca. 16–40 genera (depending on generic circumscription) and ca. 320 species (Fischer 2004; Albach et al. 2005; Estes et al. unpubl. data). Morphologically, the tribe is characterized by leaves simple, opposite or whorled, and frequently glandular-punctate; trichomes often with a pluri-cellular head; inflorescences of solitary axillary flowers or bracteate racemes; pedicels ± bibracteolate; corollas usually slightly to markedly zygomorphic, mostly tubular or campanulate, and more or less bilabiate; abaxial stamens without appendages; locules distinct and mostly with 2 anther thecae; ovules with 1–3 intermediate layers of integument; stigmas mostly distinct or 2-lobed and flattened; capsules 2–4 valved and primarily septically (sometimes primarily loculicidally) dehiscent; seeds small and numerous, generally simply reticulate with testa cells that have hook-

like wall thickenings; and endosperm mostly terete (Wettstein 1891; Pennell 1935; Thieret 1967; Fischer 2004; Rahmzadeh et al. 2005). The genera of Gratioleae are distributed throughout the world but are best represented in the Neotropics and closely adjacent temperate regions. Several of the New World genera are monotypic including *Amphianthus* Torr., *Benjaminia* Mart. ex Benj., *Boelckea* Rossow, *Braunblanquetia* Eschsch., *Geochorda* Cham. et Schlecht, *Ildefonsia* Gardn., *Maeviella* Rossow, *Schizosepala* G.M.Barroso, and *Tetraulacium* Turcz. In addition, *Sophronanthe* Benth. and *Tragiola* Small & Pennell have each been regarded as monotypic (Pennell 1935) or as members of *Gratiola* L. sect. *Sophronanthe* Benth. (Bentham 1846).

Four molecular phylogenetic studies (Olmstead et al. 2001; Albach et al. 2005; Oxelman et al. 2005; Rahmzadeh et al. 2005) have helped clarify the position of Gratioleae relative to the other major clades of Plantaginaceae. Due to limited sampling of Gratioleae genera, however, the circumscription of the tribe and relationships among its genera remain unclear. Combined, these studies included seven genera (here termed core Gratioleae): *Amphianthus*, *Bacopa* Aubl., *Gratiola*, *Mecardonia* Ruiz & Pav., *Otacanthus* Lindl., *Scoparia* L., and *Stemodia* L. Some additional genera, including *Angelonia* Humb. & Bonpl., *Basistemon* Turcz., *Melosperma* Benth., *Monopera* K. Barringer, *Monttea* Gay, and *Ourisia* Comm. ex Juss. (here informally referred to as the “Angelonieae clade”), have been assigned to Gratioleae by some authors (e.g. Bentham 1846, in part; Oxelman et al. 2005), but excluded from Gratioleae by others (e.g. Pennell 1920; Thieret 1954, 1967; Rossow 1985; Olmstead et al. 2001; Fischer 2004; Albach et al. 2005). All but *Monopera* have been included in the recent molecular studies cited above. In the three studies that have included representatives of both Gratioleae and the “Angelonieae” (Olmstead et al. 2001; Albach et al. 2005; Oxelman et al. 2005), the “Angelonieae” generally form a clade sister to Gratioleae although support for their sister relationship is low or, in some cases, nonexistent. In this paper, we do not consider the genera of the “Angelonieae” to be part of the Gratioleae, but further investigation of their relationships are underway (Estes et al. unpubl. data).

*Amphianthus*, a monotypic genus represented by the species *Amphianthus pusillus* Torrey, is a rare aquatic annual en-

demic to southeastern United States. The species is listed as federally threatened by the United States Fish and Wildlife Service (1993) and is known only from ca. 60 populations, all of which are restricted to ephemeral pools associated with granite outcrops on the Piedmont Plateau of Alabama, Georgia, and South Carolina. *Amphianthus* has been separated from other Gratioleae genera because of its unusual morphology characterized by dimorphic leaves, presence of both cleistogamous and chasmogamous flowers, and laterally compressed, obcordate capsules (Pennell 1935).

The systematic position of *Amphianthus* has not been well understood. Torrey (1837), in describing the genus, placed it within the order Scrophularineae (~ Scrophulariaceae) and considered *Amphianthus* to be closely allied to *Veronica* L. Pennell (1935), citing the presence of distinct stigmas, the external position of the posterior corolla lobes, and the glandular-punctate foliage, assigned *Amphianthus* to tribe Gratioleae noting "it is certainly of only remote affinity to any other existing genus." Kral (1983, p. 1031) addressed the systematic affinity of *Amphianthus* noting that it "is perhaps in floral character most similar to the genus *Gratiola*" and that "in *Gratiola* there are species which show reduction to two viable stamens and no staminodes, and which have bilobed, laminal stigmas and similar (though larger) corollas." Further, Kral (1983) noted that the seeds of the two genera are similar. Olmstead et al. (2001) demonstrated that *Amphianthus* was sister to *Gratiola*. However, their findings were based on a sampling of only three Gratioleae taxa, representing one species each of *Amphianthus*, *Bacopa*, and *Gratiola*. Subsequent phylogenetic studies that included Gratioleae genera did not include *Amphianthus* and found either *Otanthus* (Albach et al. 2005; Rahmzadeh et al. 2005) or *Stemodia* (Oxelman et al. 2005) to be sister to *Gratiola*.

Given the longstanding confusion regarding the relationship of *Amphianthus* to other genera of Gratioleae, the objectives of this study were to: (1) provide a preliminary assessment of phylogenetic relationships among genera within Gratioleae; (2) determine the specific phylogenetic placement of *Amphianthus* within the Gratioleae using chloroplast *ndhF* sequences; (3) investigate the relationships of *Amphianthus* and *Gratiola* using noncoding chloroplast *trnS<sup>GCU</sup>-trnG<sup>UUC</sup>-trnG<sup>UUC</sup>* sequences; and (4) address the morphological characters used by previous authors to segregate *Amphianthus* from other Gratioleae genera, particularly *Gratiola*.

#### MATERIALS AND METHODS

**Taxon Sampling**—Specimens used for this study were collected from wild populations, greenhouse-grown material, or herbarium specimens, and 21 previously published *ndhF* sequences from GenBank were included in our analysis (Appendix 1). For the *ndhF* analysis, all seven genera of Gratioleae previously sampled in published studies (Olmstead et al. 2001; Albach et al. 2005; Oxelman et al. 2005; Rahmzadeh et al. 2005) were included in addition to four genera not included in any published phylogeny of Gratioleae: *Achetaria*, *Hydrotriche* Zucc., *Leucospora* Nutt., and *Limnophila* R. Br. To test Kral's (1983) remarks on the similarity of *Amphianthus* and *Gratiola*, we also sampled six species from each of the major clades within *Gratiola* (Estes and Small, unpubl. data). Fifteen genera representing the major clades of Plantaginaceae s.l. (sensu Albach et al. 2005) and *Lindernia* of the Linderniaceae (sensu Rahmzadeh et al. 2005) were included to test the placement of Gratioleae within Plantaginaceae s.l. *Scrophularia* was selected as the outgroup based on previous studies (Olmstead et al. 2001; Albach et al. 2005; Oxelman et al. 2005; Rahmzadeh et al. 2005). For the comparison of *Amphianthus* and *Gratiola* using *trnS-trnG-trnG* sequences, *Hydrotriche* was used as the outgroup based on the results of the *ndhF* analysis, which identified *Hydrotriche* as belonging to a clade sister to *Gratiola* + *Amphianthus*.

**DNA Extraction, Amplification, and Sequencing**—DNA was extracted from freshly collected or silica dried leaves and herbarium material using the DNeasy Plant Mini Kit (Qiagen, Valencia, California). Sequence data were obtained from two chloroplast regions: the gene *ndhF*, and the *trnS-trnG* intergenic spacer plus *trnG* intron (*trnS<sup>GCU</sup>-trnG<sup>UUC</sup>-trnG<sup>UUC</sup>*). The *ndhF* region was selected based on its previously demonstrated utility in resolving generic relationships in Scrophulariaceae s.l. (Olmstead et al. 2001; Oxelman et al. 2005). The *trnS-trnG-trnG* region was used based on the study of Shaw et al. (2005) that demonstrated that it was one of the most phylogenetically informative of 21 noncoding cpDNA regions surveyed in *Gratiola*. PCR and sequencing primers for *ndhF* are described in Olmstead and Sweere (1994) and those for *trnS-trnG-trnG* are described in Shaw et al. (2005). PCR reaction volumes (25  $\mu$ L) consisted of the following components: 1  $\mu$ L template DNA (~10–100 ng), 1 $\times$  buffer (TaKaRa, Madison, Wisconsin), 200  $\mu$ mol/L each dNTP, 3.0 mmol/L MgCl<sub>2</sub> (1.5mmol/L for *trnS-trnG-trnG*), 0.1  $\mu$ mol/L each primer, 0.2  $\mu$ g/ $\mu$ L bovine serum albumin, and 1.25 units of *rTaq* or *ExTaq* (TaKaRa). PCR cycling parameters for *ndhF*: 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 50°C for 30 sec, primer extension at 72°C for 2 min. For some taxa, we had difficulty amplifying the *ndhF* region using the preceding conditions; therefore, we used the following PCR cycling parameters for these taxa: 30 cycles of denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, primer extension at 65°C for 4 min. PCR cycling conditions for *trnS-trnG-trnG*: 30 cycles of denaturation at 95°C for 1 min, primer annealing and extension at 66°C for 4 min. All PCR and sequencing reactions were performed in Eppendorf Mastercycler thermal cyclers. Prior to sequencing, PCR products were purified using ExoSAP-IT (USB, Cleveland, Ohio). DNA sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, v. 3.1 and the sequencing products were electrophoresed and detected on an ABI Prism 3100 automated sequencer (University of Tennessee Molecular Biology Resource Facility). DNA sequences generated for this study have been deposited in GenBank (Appendix 1).

**Sequence Editing and Alignment**—The sequences were assembled into contigs and edited in Sequencher 4.2.1 (Gene Codes Corporation, Ann Arbor, Michigan), aligned using Clustal X (Thompson et al. 2001), and adjusted by eye in MacClade 4.0 (Maddison and Maddison 2001). For the *ndhF* sequences, coding of the indels as presence/absence characters was not undertaken. For the *trnS-trnG-trnG* sequences, nonoverlapping parsimony informative indels were coded as binary characters and added to the end of the data matrix.

**Phylogenetic Analyses**—Phylogenetic analysis of the *ndhF* dataset was performed using Bayesian inference in MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). DNA substitution models implemented in the Bayesian analysis were determined using the Akaike Information Criterion (AIC; Posada and Buckley 2004) in MrModeltest v2.2 (Nylander 2004). The model chosen by MrModeltest was the GTR + I +  $\Gamma$  model. Bayesian analysis was run for 1 million generations, with trees sampled every 100 generations. The number of trees to discard as "burn-in" was assessed by plotting likelihoods of trees sampled throughout the run and discarding all trees prior to the stable likelihood plateau (in each case the first 1,000 out of 10,000 trees were discarded). The remaining trees were then used to construct a 50% majority rule consensus tree, which was used to estimate posterior probabilities (PP) of clades.

Phylogenetic analysis of the *trnS-trnG-trnG* dataset was performed under the optimality criterion of maximum parsimony using PAUP\* v. 4.0 b10 (Swofford 2002) with the following options: heuristic search with 1,000 random-addition-sequence replicates; tree bisection-reconnection (TBR) branch swapping; "collapse zero length branches;" saving all most parsimonious trees. Character state changes were treated as equally weighted. Nonoverlapping parsimony informative indels were coded as binary characters and added to the end of the data matrix. Relative clade support was estimated using 1,000 bootstrap (Felsenstein 1985) replicates in PAUP\* via "full heuristic" searches and simple taxon addition. The consistency index (CI) and retention index (RI) were used to assess the amount of homoplasy present in the data. The data and phylogenetic trees generated during this project have been deposited in TreeBASE (study number S1776).

#### RESULTS

***ndhF***—This data matrix contained 47 taxa and 2,091 characters, and was aligned with little difficulty. The data matrix contained 7.4% missing data. The Bayesian majority rule consensus tree is shown in Fig. 1. This tree was well-resolved and most nodes were strongly supported with posterior

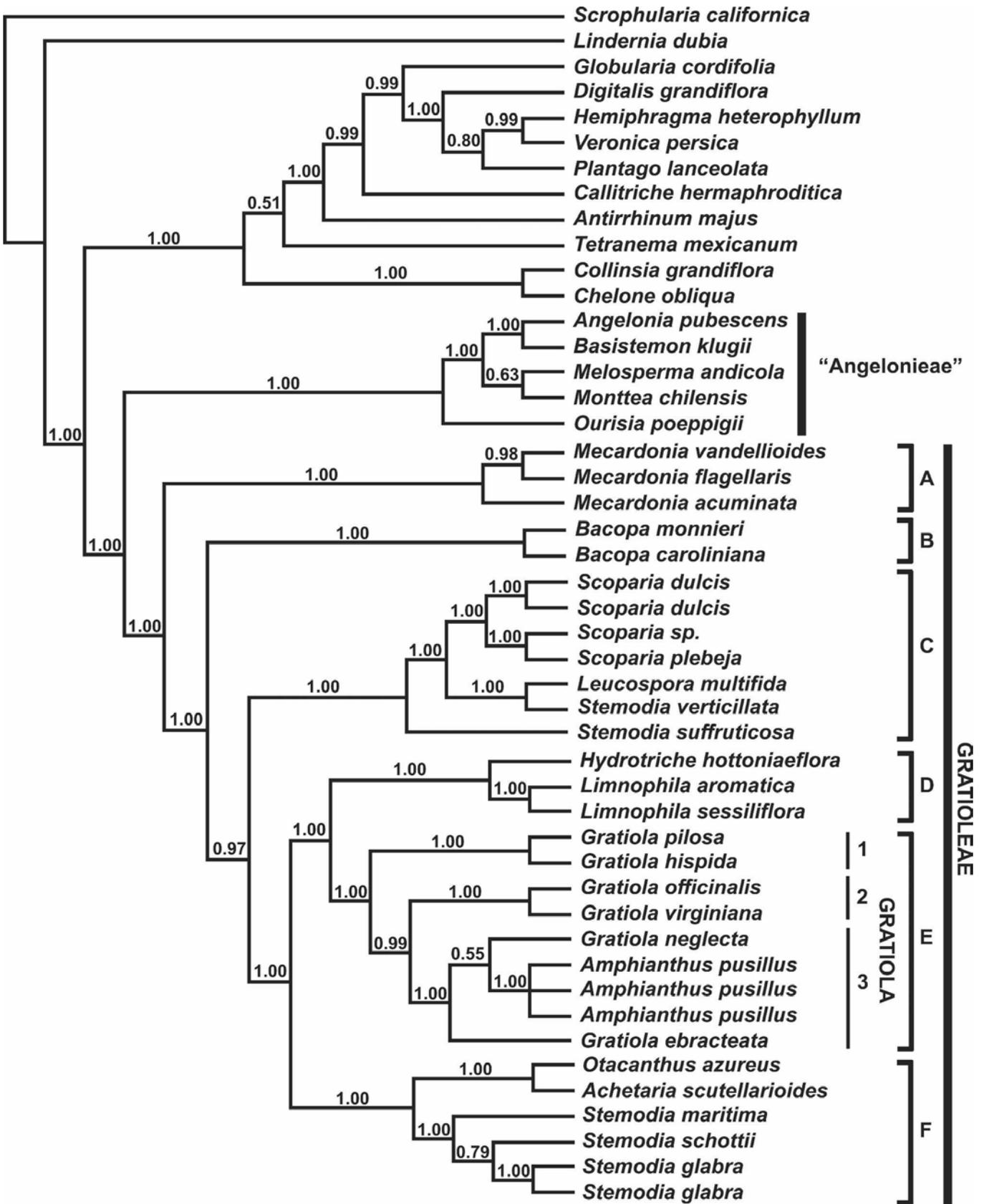


FIG. 1. Bayesian majority rule consensus tree based on analysis of *ndhF* cpDNA sequences showing relationship of *Amphanthus* to *Gratiola* and the rest of tribe Gratioleae and the Plantaginaceae. Numbers above branches indicate posterior probabilities.

probability (PP) values  $\geq 0.95$ . Genera from the Plantaginaceae representing tribes Antirrhineae, Cheloneae, Digitalideae, Globularieae, Veroniceae, and other genera (*Callitriche*, *Tetranema*) formed a strongly supported clade (1.00 PP) sister to a strongly supported (1.00 PP) clade of Gratioleae + "Angelonieae." Within the Gratioleae six major clades were identified (Fig. 1, clades A–F). The three species of *Mecardonia* (clade A) formed a strongly supported clade (1.00 PP) sister to the rest of the tribe. The two species of *Bacopa* (clade B) formed a strongly supported clade (1.00 PP) and resolved as sister to all remaining Gratioleae. Clade C was strongly supported (1.00 PP) and included three species of *Scoparia*, some New World *Stemodia* (*S. suffruticosa* and *S. verticillata*), and *Leucospora multifida*. Clade D contained the Old World genera *Limnophila* and *Hydrotriche* (1.00 PP); clade E *Gratiola* and *Amphianthus* (1.00 PP). Clade F was strongly supported (1.00 PP) and contained some New World *Stemodia* (*S. maritima*, *S. schottii*, and *S. glabra*) plus the Neotropical genera *Achetaria* and *Otacanthus*.

Within the clade containing *Gratiola* + *Amphianthus* (Fig. 1, clade E, the *Gratiola* clade), three groups were identified. Group 1 consisted of *G. hispida* and *G. pilosa*, both members of *Gratiola* sect. *Sophranthe* Benth.; this group was sister to the rest of *Gratiola* + *Amphianthus* and was supported by strong PP values (1.00). Group 2 (1.00 PP) was sister to Group 3 and was represented by *G. virginiana* and *G. officinalis*. The first is a member of sect. *Nibora* (Raf.) Pennell while the latter is the type of the genus and a member of sect. *Gratiola*. Group 3 (1.00 PP) included *G. neglecta*, *G. ebracteata*, and *Amphianthus*; the relationships within this clade were not well supported.

***trnS-trnG-trnG***—This dataset contained eight taxa and 1,821 characters, 94 of which were parsimony informative. Ten parsimony-informative indels were coded as binary characters and appended to the data set yielding a total of 104 parsimony informative characters. Most of the matrix was aligned with little difficulty except for the portion between bp 503–537 and bp 640–670. Approximately 0.2% of the dataset consisted of missing data. Phylogenetic analysis of the *trnS-trnG-trnG* region produced a single most parsimonious tree ( $L = 359$ ,  $CI = 0.916$ ,  $RI = 0.783$ ). This tree (Fig. 2) was topologically consistent with clade E from the *ndhF* analysis (Fig. 1). As in the *ndhF* analysis, three major groups were recovered; the only difference was that the relationships among *Amphianthus*, *G. ebracteata*, and *G. neglecta* were resolved and strongly supported in the *trnS-trnG-trnG* analysis. Overall the tree was highly supported with four of the five nodes supported by  $BS \geq 95\%$ .

#### DISCUSSION

**Phylogeny of the Gratioleae**—Recent molecular phylogenetic studies have shown that *Amphianthus*, *Bacopa*, *Gratiola*, *Mecardonia*, *Otacanthus*, *Scoparia*, and *Stemodia* belong to a strongly supported tribe Gratioleae (Olmstead et al. 2001; Albach et al. 2005; Oxelman et al. 2005; Rahmzadeh et al. 2005). The results of our analysis are consistent with these previous phylogenetic analyses of Gratioleae, but offer expanded taxon sampling. Further work on the circumscription of, and relationships within, Gratioleae are ongoing (Estes et al. unpubl. data). Rahmzadeh et al. (2005) proposed that the Gratioleae be segregated from Plantaginaceae and recognized as its own family, Gratiolaceae Martynov. However, we feel that more evidence (morphological, anatomical, cy-

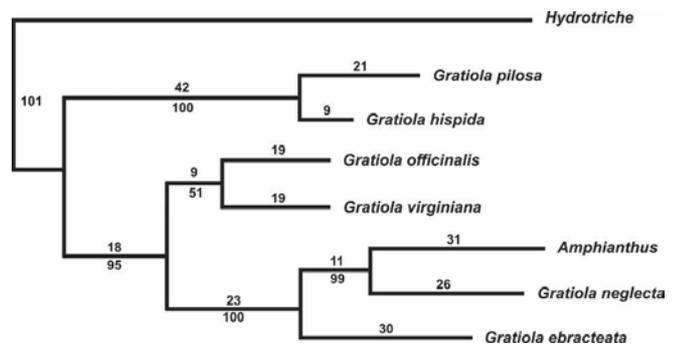


FIG. 2. Single most parsimonious tree showing phylogenetic relationships of *Amphianthus* and selected species of *Gratiola* using *trnS-trnG-trnG* cpDNA sequences. Numbers above branches are branch lengths; numbers below branches are bootstrap values.

to logical, and molecular phylogenetic) needs to be accumulated before recognizing this clade at the family level. Regardless of the taxonomic rank of the clade (Gratioleae or Gratiolaceae), our evidence supports the inclusion of *Achetaria*, *Hydrotriche*, *Limnophila*, and *Leucospora* in the Gratioleae in addition to *Bacopa*, *Gratiola*, *Mecardonia*, *Scoparia*, *Stemodia*, *Amphianthus*, and *Otacanthus* (Albach et al. 2005, Olmstead et al. 2001; Oxelman et al. 2005). Further, we show that *Amphianthus* is phylogenetically embedded within *Gratiola*.

**Relationships of *Amphianthus***—Most taxonomists have included *Amphianthus* within tribe Gratioleae (Bentham and Hooker 1876; Wettstein 1891; Pennell 1935; Fischer 2004). Olmstead et al. (2001), using a limited sampling of Gratioleae taxa, demonstrated that *Amphianthus* was sister to a single representative of *Gratiola*. Our phylogeny of Gratioleae (Fig. 1) corroborates the traditional morphology-based assessments as well as the recent molecular phylogenetic study of Olmstead et al. (2001), demonstrating that *Amphianthus* is strongly supported as a member of Gratioleae. Phylogenetic analysis of the *ndhF* region (Fig. 1) unequivocally places *Amphianthus* in a clade with six species of *Gratiola*. Within this clade, *Amphianthus* occupies a derived position along with *G. ebracteata* and *G. neglecta*. The relationships among these three taxa are not resolved with the *ndhF* data.

To further resolve relationships within the *Gratiola* + *Amphianthus* clade we analyzed sequences from the *trnS-trnG-trnG* region. The topology of the tree (Fig. 2) is consistent with clade E of the *ndhF* tree (Fig. 1), but now fully resolved, clearly demonstrating that *Amphianthus* is embedded within *Gratiola* with *G. ebracteata* sister to a highly supported (99% BS) clade comprised of *G. neglecta* + *Amphianthus*. Both *G. neglecta* and *G. ebracteata* belong to *Gratiola* sect. *Nibora* (Raf.) Pennell, a North American section containing six annual species.

**Characters Used to Separate *Amphianthus* from Other Genera**—The molecular evidence presented here clearly shows that *Amphianthus* is phylogenetically embedded within *Gratiola*. With this in mind, we evaluated the morphological characters traditionally used by previous taxonomists to separate *Amphianthus* from other Gratioleae genera. Pennell (1935) separated *Amphianthus* from other North American Gratioleae based on three main characters: (1) presence of dimorphic leaves, (2) two types of flowers (chasmogamous and cleistogamous), and (3) capsule shape.

As in many aquatic plant species, *Amphianthus* exhibits dimorphic leaves (see Lunsford 1939 for a detailed discussion

on the anatomy, morphology, and development of leaves and leaf-like structures in *Amphianthus*). The cauline leaves are usually submerged and are narrowly oblong. From the axils of these stem leaves there are usually a few slender branches that extend upward reaching the surface of the water, bearing at their apices two opposite and broadly ovate floating leaves. The fact that *Amphianthus* has strongly dimorphic leaves does not make it unique within Gratiolaceae; some species in *Dopatrium* (Fischer 1997), *Hydrotriche* (Raynal-Roques 1979), and *Limnophila* (Philcox 1970; Wannan and Waterhouse 1985) also exhibit dimorphic leaves. In *Gratiola*, leaf dimorphism had not been reported previously; however, it is interesting to note that *G. heterosepala* Mason & Bacigal., a species restricted to northern California and southern Oregon, has somewhat dimorphic leaves although not as extreme as in *Amphianthus* (D. Estes, pers. obs.). As noted by Lunsford (1939), the size of the floating leaves in *Amphianthus* "varies greatly, probably due to changes in environmental conditions" because "when the entire plant is exposed upon the evaporation of the aquatic medium, the bracts are much smaller in size than those developed under aquatic conditions."

Another character used to distinguish *Amphianthus* from other Gratiolaceae genera is the presence of two flower types (Pennell 1935; Hilton and Boyd 1996; Fischer 2004). In *Amphianthus*, the flowers borne in the axils of submerged leaves are pseudo-cleistogamous (Lunsford 1939); they remain closed until the water level recedes at which point they often open and become chasmogamous (Lunsford 1939; Hilton and Boyd 1996; United States Fish and Wildlife Service 1993). Those flowers that occur between the floating leaves are typically chasmogamous (Lunsford 1939; Hilton and Boyd 1996; United States Fish and Wildlife Service 1993; D. Estes, pers. obs.). However, other Gratiolaceae genera (*Deinostema* T. Yamaz., *Limnophila*, *Dopatrium* Buch.-Ham. ex Benth., *Hydrotriche*, *Gratiola*) may also have cleistogamous flowers (Philcox 1970; Fischer 1997, 2004). *Amphianthus* is also not alone in the Gratiolaceae in its simultaneous production of cleistogamous and chasmogamous flowers; *Limnophila australis* B.S. Wannan & J.T. Waterhouse, an Australian endemic, sometimes produces cleistogamous flowers on submerged nodes and chasmogamous flowers on exposed nodes (Wannan and Waterhouse 1985). In *G. neglecta* and *G. virginiana*, submerged individuals or plants occurring late in the growing season often bear cleistogamous flowers (Pennell 1935; D. Estes, pers. obs.) and sometimes both cleistogamous and chasmogamous flowers occur at the same time on a plant (D. Estes, pers. obs.). In short, within the Gratiolaceae, cleistogamy is not unique to *Amphianthus*.

Some taxonomists (Small 1933; Pennell 1935) have distinguished *Amphianthus* from other Gratiolaceae genera on the basis of its distinctive capsules that are laterally compressed and obcordate. Although the capsules of *Amphianthus* are relatively unique in appearance compared to other genera in the Gratiolaceae, capsule types vary widely within the tribe. In *Gratiola*, capsule shape is quite variable and includes a number of different types: conic (*G. flava* Leavenw.), ovoid (*G. neglecta*), globose (*G. virginiana*), or rarely, as in the Japanese endemic, *G. fluviatilis* Koidz., obcordate (Koidzumi 1925). It appears that the capsules of *Amphianthus* represent an extreme form that is within the range of capsule variation exhibited within *Gratiola* and that this feature, like leaf dimor-

phism and flower type, does not support the separation of *Amphianthus* from *Gratiola*.

**Characters Shared Between *Gratiola* and *Amphianthus***—Kral (1983) was the first to suggest a possible close affinity between *Amphianthus* and *Gratiola*. In light of the molecular phylogenetic evidence presented here, we investigated potential morphological characters supporting the union of *Amphianthus* and *Gratiola*.

Like the species of *Gratiola* sect. *Nibora* (clade 3, Fig. 1), *Amphianthus* is an annual. Pennell (1935) regarded this feature important for distinguishing sect. *Nibora* from sect. *Gratiola*, which contains mostly perennial species. The whitish-translucent roots of *Amphianthus* strongly resemble those of the annual species of *Gratiola*. The stem and leaves in *Amphianthus* are also very similar to those of annual *Gratiola*, particularly *G. heterosepala*. Both species have short, narrowly oblong, round-tipped, and entire-margined cauline leaves. Fischer (2004) described the leaves of *Amphianthus* as petiolate, but this characterization only loosely applies to the floating leaves terminating axillary branches that have slightly subpetiolate leaf bases. The submerged cauline leaves are sessile as in *Gratiola*. Both *Amphianthus* and *G. heterosepala* exhibit leaf dimorphism although it is more pronounced in *Amphianthus* because the distal leaves are larger and positioned at the ends of long, slender branches.

Kral (1983) commented on the similarity of the flowers of *Amphianthus* and *Gratiola*, noting that the main difference between the two is that the latter has larger flowers. Aside from the size difference, both have similar tubular-funnelform corollas, and there appear to be no substantial morphological differences between those of *Amphianthus* and *Gratiola*. For example, whereas the flowers of *Amphianthus* are sessile or subsessile, several species of *Gratiola* also have sessile or subsessile flowers (e.g. *G. virginiana*). However, sister species (*G. neglecta* and *G. ebracteata*) to *Amphianthus* (Fig. 2) have evidently pedicellate flowers. The flowers of *Amphianthus* also lack a pair of bracteoles at the base of the calyx (Lunsford 1939; D. Estes, pers. obs.). Four other species of *Gratiola*, including three species of sect. *Nibora* (*G. ebracteata*, *G. heterosepala*, *G. oresbia* B.L. Robins.), have ebracteate flowers. In most species of *Gratiola*, the calyx is equally divided to the base into five subequal lobes, the major exception being *G. heterosepala*, which has unequally divided calyces (Mason and Bacigalupi 1954). *Amphianthus* is similar to *G. heterosepala* in that it also has unequally divided calyces with the lobes "united for the lower third of their length" (Lunsford 1939). One of the morphological characteristics that separate *Gratiola* from most other genera in the Gratiolaceae is the presence of two fertile posterior stamens and the absence of the anterior pair, a feature also shared by *Amphianthus* (Lunsford 1939; D. Estes, pers. obs.).

Our examination of the seeds of *Amphianthus* and numerous species of *Gratiola* with the scanning electron microscope reveals that the seeds of *Amphianthus* strongly resemble those of species of *Gratiola* sect. *Nibora*, particularly *G. ebracteata* and *G. heterosepala*. These three taxa have oblong and slightly curvate seeds (0.7–1.2 mm long) while all other members of *Gratiola* sect. *Nibora* (except *G. virginiana* and *G. japonica*) have mostly ovoid seeds that are usually less than 0.9 mm long (D. Estes, unpubl. data). The importance of seed morphology as a taxonomic character in the Gratiolaceae has been noted by Thieret (1954, 1967) and is currently being explored further in a separate study (Estes et al. unpubl. data).

In addition to sharing several morphological characteristics with *Gratiola*, *Amphianthus* shares the same chromosome number,  $2n = 18$  (Lunsford 1939; Konda 1972), with the closely related *G. neglecta* (Gervais et al. 1999). This is significant given the fact that of the 10 other *Gratiola* with available chromosome counts, none has the same number as *Amphianthus* and *G. neglecta*: *G. ramosa* ( $2n = 14$ ; Lewis et al. 1962), *G. virginiana* ( $2n = 16$ ; Lewis et al. 1962), *G. viscidula* ( $2n = 16$ ; Konda 1972), *G. pilosa* ( $2n = 22$ ; Lewis et al. 1962), *G. aurea* ( $2n = 28$ ; Kapoor et al. 1987), *G. brevifolia* ( $2n = 28$ ; Lewis et al. 1962), *G. nana* ( $2n = 30$ ; Hair et al. 1967), *G. officinalis* ( $2n = 32$ ; Fernandes et al. 1977), *G. pedunculata* ( $2n = 32$ ; Murray and De Lange 1999), and *G. sexdentata* ( $2n = 90$ ; Hair et al. 1967).

**Justification for Transferring *Amphianthus* to *Gratiola***—*Amphianthus* is well supported as embedded within *Gratiola* sect. *Nibora* (Fig. 1, clade 3E) along with *G. neglecta* and *G. ebracteata*, two annual North American species. This placement is supported by DNA sequence data from two chloroplast loci as well as morphological and cytological evidence. In short, there is no justification for maintaining *Amphianthus* as a distinct genus; we propose the transfer of *Amphianthus pusillus* to *Gratiola*.

According to the International Code of Botanical Nomenclature (McNeill et al. 2006), it is recommended that when a species is transferred from one genus to another that the specific epithet be retained. In this case, *Amphianthus pusillus* would become *Gratiola pusilla*; this name would be illegitimate because the name *G. pusilla* is a later homonym of *G. pusilla* Willd. (1797) and *G. pusilla* Torr. (1846). Consequently, a new name is proposed below to replace *Amphianthus pusillus*.

#### TAXONOMIC TREATMENT

***Gratiola amphiantha*** D. Estes & R. L. Small, nom. nov. *Amphianthus pusillus* Torrey, Ann. Lyc. New York 4: 82. 1837.—TYPE: U.S.A. Georgia: [No locality data associated with the type but according to the original description by Torrey (1837) "In small excavations on flat rocks, where the soil is wet during the flowering season; Newton (now part of Rockdale) County, Georgia"], 1836, *Leavenworth s.n.* (holotype: NY!).

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#### LITERATURE CITED

Albach, D. C., H. M. Meudt, and B. Oxelman. 2005. Piecing together the "new" Plantaginaceae. *American Journal of Botany* 92: 297–315.

Beardsley, P. M. and R. G. Olmstead. 2002. Redefining Phrymaceae: the placement of *Mimulus*, tribe Mimuleae, and *Phryma*. *American Journal of Botany* 89: 1093–1102.

Bentham, G. 1846. Scrophulariaceae. Pp. 180–586 in *Prodromus* vol. 10, ed. A.P. de Candolle. Paris: Victoris Masson.

Bentham, G. and J. D. Hooker. 1876. Scrophulariaceae. Pp. 913–980 in *Genera Plantarum* vol. 2. London: Reeve and Co.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.

Fernandes, A., M. Queirós, and M. Fátima Santos. 1977. Contribution à la connaissance cytotoxonomique des spermatophyte du Portugal, XV Scrophulariaceae. *Boletim da Sociedade Broteriana* 51: 37–90.

Fischer, E. 1997. A revision of the genus *Dopatrium* (Scrophulariaceae-Gratioloideae). *Nordic Journal of Botany* 17: 527–555.

Fischer, E. 2004. Scrophulariaceae. Pp. 333–390 in *The families and genera of vascular plants* vol. 7, ed. K. Kubitzki. Berlin: Springer-Verlag.

Gervais, C., R. Trahan, and J. Gagnon. 1999. IOPB chromosome data, 14. *International Organization of Plant Biosystematists Newsletter* 30: 10–15.

Hair, J. B., E. J. Beuzenberg, and B. Pearson. 1967. Contributions to a chromosome atlas of the New Zealand flora—9. Miscellaneous families. *New Zealand Journal of Botany* 5: 185–196.

Hilton, J. L. and R. S. Boyd. 1996. Microhabitat requirements and seed/microsite limitation of the rare granite outcrop endemic *Amphianthus pusillus* (Scrophulariaceae). *Bulletin of the Torrey Botanical Club* 123: 189–196.

Huelsenbeck, J. P. and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics (Oxford, England)* 17: 754–755.

Kapoor, B. M., S. Ramcharitar, and C. Gervais. 1987. Liste annotée de nombres chromosomiques de la flore vasculaire du nord-est de L'Amérique. V. *Le Naturaliste Canadien* 114: 105–116.

Koidzumi, G. 1925. Contributiones ad cognitionem florum Asiae Orientalis, XII. *The Botanical Magazine (Tokyo)* 39: 1–30.

Konda, K. 1972. Chromosome numbers of some angiosperms in the United States. II. *Phyton* 30: 47–51.

Kral, R. 1983. *A report on some rare, threatened, or endangered forest-related vascular plants of the South* Vol. 2. Atlanta: USDA Forest Service, Southern Region, Technical Publication R8-TP2.

Lewis, W. H., H. L. Stripling, and R. G. Ross. 1962. Chromosome numbers for some angiosperms of the southern United States and Mexico. *Rhodora* 64: 147–161.

Lunsford, D. E. 1939. *Studies in the life cycle of Amphianthus pusillus* Torrey. M.S. thesis. Atlanta: Emory University.

Maddison, D. R. and W. P. Maddison. 2001. MacClade v. 4. Sunderland: Sinauer Associates.

Mason, H. L. and R. Bacigalupi. 1954. A new *Gratiola* from Boggs Lake, Lake County, California. *Madrono* 12: 150–152.

McNeill, J., F. R. Barrie, H. M. Burdet, V. Demoulin, D. L. Hawksworth, K. Marhold, D. H. Nicolson, J. Prado, P. C. Silva, J. E. Skog, J. H. Wiersema, and N. J. Turland. EDS. 2006. *International code of botanical nomenclature* (Vienna code). *Regnum Vegetabile* vol. 146. Ruggell: Gantner.

Murray, B. G. and P. J. De Lange. 1999. Contributions to a chromosome atlas of the New Zealand flora—35. Miscellaneous families. *New Zealand Journal of Botany* 37: 511–521.

Nylander, J. A. 2004. MrModeltest—version 2. Available from the author: <http://www.ebc.uu.se/systzoo/staff/nylander.html>

Olmstead, R. G. and J. A. Sweere. 1994. Combining data in phylogenetic systematics: an empirical approach using three molecular data sets in the Solanaceae. *Systematic Biology* 43: 467–481.

Olmstead, R. G. and P. A. Reeves. 1995. Evidence for the polyphyly of the Scrophulariaceae based on chloroplast *rbcL* and *ndhF* sequences. *Annals of the Missouri Botanical Garden* 82: 176–193.

Olmstead, R. G., H. J. Michaels, K. Scott, and J. D. Palmer. 1992. Monophyly of the Asteridae and identification of their major lineages inferred from DNA sequences of *rbcL*. *Annals of the Missouri Botanical Garden* 79: 249–265.

Olmstead, R. G., C. W. DePamphilis, A. D. Wolfe, N. D. Young, W. J. Elisons, and P. A. Reeves. 2001. Disintegration of the Scrophulariaceae. *American Journal of Botany* 88: 348–361.

Oxelman, B., P. Kornhall, R. G. Olmstead, and B. Bremer. 2005. Further disintegration of Scrophulariaceae. *Taxon* 54: 411–425.

Pennell, F. W. 1920. Scrophulariaceae of Colombia—1. *Proceedings. Academy of Natural Sciences of Philadelphia* 72: 136–188.

Pennell, F. W. 1935. The Scrophulariaceae of eastern temperate North America. *Academy of Natural Sciences of Philadelphia Monographs* 1: 1–650.

Philcox, D. 1970. A taxonomic revision of the genus *Limnophila* R. Br. (Scrophulariaceae). *Kew Bulletin* 24: 101–170.

Posada, D. and T. R. Buckley. 2004. Model selection and model averaging

- in phylogenetics: advantages of akaike information criterion and Bayesian approaches over likelihood ratio tests. *Systematic Biology* 53: 793–808.
- Rahmanzadeh, R., K. Müller, E. Fischer, D. Bartels, and T. Borsch. 2005. The Linderniaceae and Gratiolaceae are further lineages distinct from the Scrophulariaceae (Lamiales). *Plant Biology* 7: 67–78.
- Raynal-Roques, A. 1979. Le genre *Hydrotriche* (Scrophulariaceae). *Adansonia* 19: 145–173.
- Rossow, R. A. 1985. Melospermeae, nueva tribu de Scrophulariaceae. *Parodiana* 3: 365–396.
- Ruoy, G. 1909. Conspectus des tribus et des genres de la famille des Scrophulariacées. *Revue Generale de Botanique* 21: 194–207.
- Shaw, J., E. B. Lickey, J. T. Beck, S. B. Farmer, W. Liu, J. Miller, K. C. Siripun, C. T. Winder, E. E. Schilling, and R. L. Small. 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92: 142–166.
- Small, J. K. 1933. *Manual of the southeastern flora*. Chapel Hill, North Carolina: University of North Carolina Press.
- Swofford, D. L. 2002. PAUP\*: phylogenetic analysis using parsimony (\*and other methods), v. 4b10. Sunderland: Sinauer Associates.
- Thieret, J. W. 1954. The tribes and genera of Central American Scrophulariaceae. *Ceiba* 4: 164–184.
- Thieret, J. W. 1967. Supraspecific classification in the Scrophulariaceae: a review. *Sida* 3: 87–106.
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 2001. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876–4882.
- Torrey, J. 1837. New genera and species of North American plants. *Annals of the Lyceum of Natural History of New York* 4: 80–94.
- United States Fish and Wildlife Service. 1993. Recovery plan for three granite outcrop plant species. Jackson, Mississippi.
- Wannan, B. S. and J. T. Waterhouse. 1985. A taxonomic revision of the Australian species of *Limnophila* R. Br. (Scrophulariaceae). *Australian Journal of Botany* 33: 367–380.
- Wettstein, R. 1891. Scrophulariaceae. Pp. 39–107 in *Die natürlichen Pflanzenfamilien*, vol. 4 (3b), eds. A. Engler and K. Prantl. Leipzig: Wilhelm Englemann.
- Wolfe, A. D. and C. W. dePamphilis. 1997. Alternate paths of evolution for the photosynthetic gene *rbcL* in four nonphotosynthetic species of *Orobanchae*. *Plant Molecular Biology* 33: 965–977.
- Young, N. D., K. E. Steiner, and C. W. dePamphilis. 1999. The evolution of parasitism in the Scrophulariaceae/Orobanchaceae: plastid gene sequences refute an evolutionary transition series. *Annals of the Missouri Botanical Garden* 86: 876–893.
- Achetaria scutellarioides* Wettst., Brazil, Bahia, Souza et al. 14483 (MO), [EF527469]. *Amphianthus pusillus* Torr., USA, Georgia, DeKalb Co., Wofford et al. s.n. (TENN), [EF527465; EF536075]. DeKalb Co., Estes et al. 06951 (TENN), [EF527466]. Olmstead et al. 2001, AF123674. *Angelonia pubescens* Benth., Olmstead et al. 2001, AF123675. *Antirrhinum majus* L., Olmstead et al. 1992, L36392. *Bacopa caroliniana* (Walt.) B.L. Robins., Olmstead et al. 2001, AF123677. *Bacopa monnieri* (L.) Pennell, USA, Tennessee, cultivated at University of Tennessee, Estes s.n. (TENN), [EF527447]. *Basistemon klugii* Barringer, Oxelman et al. 2005, AJ619554 & AJ619555. *Callitriche hermaphroditica* L., Olmstead & Reeves 1995, L36396. *Chelone obliqua* L., Young, Steiner, & dePamphilis 1999, AF123680. *Collinsia grandiflora* Lindley, Wolfe & dePamphilis 1997, AF188182. *Digitalis grandiflora* Mill, Olmstead & Reeves 1995, L36399. *Globularia cordifolia* L., Olmstead et al. 2001, AF124557. *Gratiola ebracteata* Benth., USA, California, Shasta Co., Estes 06046 (TENN), [EF527464; EF536077]. *Gratiola hispida* (Benth. ex Lindl.) Pollard, USA, Florida, Putnam Co., Beck s.n. (TENN), [EF527460; EF536072]. *Gratiola neglecta* Torr., USA, Tennessee, Rutherford Co., Estes 06214 (TENN), [EF527463; EF536076]. *Gratiola officinalis* L., Bulgaria, Rhodopi, Frost-Olsen 4356 (MO), [EF527461; EF536073]. *Gratiola pilosa* Michx., USA, Tennessee, Moore Co., Estes 03800 (TENN), [EF527459; EF536071]. *Gratiola virginiana* L., USA, North Carolina, Stokes Co., Estes 06875 (TENN), [EF527462; EF536074]. *Hemiphragma heterophyllum* Wall., Young, Steiner, & dePamphilis 1999, AF123683. *Hydrotriche hottoniae-flora* Zucc., USA, Tennessee, cultivated at University of Tennessee, Estes s.n. (TENN), [EF527456; EF536070]. *Leucospora multifida* Nutt., USA, Texas, Williamson Co., Estes 06143 (TENN), [EF527453]. *Limnophila aromatica* (Lamarck) Merrill, Taiwan, Huang 357 (MO), [EF527457]. *Limnophila sessiliflora* Blume, Japan, Tsugaru et al. 31968 (MO), [EF527458]. *Lindernia dubia* (L.) Pennell, USA, Tennessee, Giles Co., Estes 02768 (TENN), [EF527446]. *Mecardonia acuminata* (Walt.) Small, USA, Tennessee, Perry Co., Estes 04215 (TENN), [EF527449]. *Mecardonia flagellaris* (Cham. & Schlecht.) Rossow, Oxelman et al. 2005, AJ617601. *Mecardonia vandellioides* (Kunth) Pennell, USA, Texas, Jeff Davis Co., Estes et al. 08215 (TENN), [EF527448]. *Melosperma andicola* Benth., Oxelman et al. 2005, AJ617602. *Monttea chilensis* Gay, Oxelman et al. 2005, AJ617604. *Otacanthus azureus* (Linden) Ronse, USA, Florida, cultivated at Durko Nursery, Durko s.n. (TENN), [EF527468]. *Oursia poeppigii* Benth., Oxelman et al. 2005, AJ619560, AJ619561, & AJ619562. *Plantago lanceolata* L., Olmstead & Reeves 1995, L36408. *Scoparia* sp. (cultivar=Mellongolly Blue), USA, California, cultivated at Proven Winners North America, Estes s.n. (TENN), [EF527451]. *Scoparia dulcis* L., Oxelman et al. 2005, AJ619569 & AJ619568. *Scoparia dulcis* L., USA, Florida, Putnam Co., Beck s.n. (TENN), [EF527450]. *Scoparia plebeja* Cham. & Schldtl., Bolivia, Carretero 1099 (NY), [EF527452]. *Scrophularia californica* Cham. & Schldtl., Olmstead & Reeves 1995, L36411. *Stemodia glabra* Oerst., Kornhall & Bremer (unpubl. data), AJ617584 & AJ550574. *Stemodia maritima* L., Bahamas, Vincent 13326 (TENN), [EF527467]. *Stemodia schottii* Holz., USA, Texas, Val Verde Co., Johnston 12449 (TEX), [EF527470]. *Stemodia suffruticosa* Kunth, Ecuador, Madsen 85727 (MO), [EF527455]. *Stemodia verticillata* (Mill.) Hassler, Costa Rica, Rodriguez 3091 (MO), [EF527454]. *Tetranema mexicana* Benth., Olmstead et al. 2001, AF123692. *Veronica persica* L., Olmstead & Reeves 1995, L36419.

APPENDIX 1. Taxa included in phylogenetic analyses, with voucher information (specimen origin, collector, collection number, and herbarium where specimen is deposited) and GenBank accession number(s) [*ndhF*; *trnS-trnG-trnG*]. For *ndhF* sequences published previously a citation is provided indicating the source of the sequences as well as the GenBank accession number.