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Systematics of *Gratiola* (Plantaginaceae)

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To the Graduate Council:

I am submitting herewith a dissertation written by Larry D. Estes entitled "Systematics of *Gratiola* (Plantaginaceae)." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology and Evolutionary Biology.

Randall L. Small, Major Professor

We have read this dissertation and recommend its acceptance:

Edward E. Schilling, Karen W. Hughes, Sally P. Horn

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Systematics of *Gratiola* (Plantaginaceae)

A Dissertation Presented for the Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Larry Dwayne Estes

May 2008

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ABSTRACT

Gratiola (Plantaginaceae tribe Gratioleae) is a genus of ca. 33 wetland-adapted herbaceous species found mostly in temperate or tropical-montane regions of the Americas, Eurasia, and Australasia. The only worldwide taxonomic treatment of *Gratiola* was published more than 160 years ago and only a few representatives of the genus have been included in published molecular phylogenetic studies. The overall aim of this investigation was to provide a modern systematic study of *Gratiola* by determining the phylogenetic placement of *Gratiola* within the Gratioleae using chloroplast DNA sequence data (Chapter 2); examining the phylogenetic relationships, morphological character evolution, and biogeographical patterns within *Gratiola* using a combination of chloroplast DNA sequence data and morphology (Chapter 3); and conducting a taxonomic study of the *Gratiola neglecta* species complex, a group of four closely related and predominantly eastern North American species (Chapter 4). This dissertation is the accumulation of information from three original research papers. The first paper (Chapter 2) has been accepted for publication in *Systematic Botany.* The second paper (Chapter 3) will be submitted to *Systematic Botany.* The third paper (Chapter 4) has been published in the *Journal of the Botanical Research Institute of Texas.* The results from this study show (1) that *Gratiola* sensu lato (including *Amphianthus*, *Fonkia*, *Sophronanthe*, and *Tragiola*) is monophyletic and firmly embedded in a monophyletic Gratioleae and is sister to the Old World genera *Hydrotriche* and *Limnophila*; (2) that the 33 species (including four undescribed taxa) of *Gratiola* group into four major clades (*Diandrae*, *Gratiola*, *Nibora*, *Sophronanthe*) that are each morphologically cohesive and largely correspond to major biogeographic regions; and (3) that the *Gratiola neglecta* Complex includes four species (*G. neglecta*, *G. floridana*, *G. graniticola*, and *G. quartermaniae*), two of which constitute previously undescribed species that are morphologically and ecologically distinct.

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Chapter 1

INTRODUCTION TO PLANTAGINACEAE TRIBE GRATIOLEAE AND *GRATIOLA*

INTRODUCTION

Overview of the recently expanded Plantaginaceae—Molecular phylogenetic analyses have drastically altered the composition and circumscription of many plant families due to the recognition that several traditionally recognized families are para- or polyphyletic. The circumscription of some families has been significantly broadened to encompass previously distinct families (e.g., Malvaceae includes former Tiliaceae, Sterculiaceae and Bombacaceae; Brassicaceae includes former Capparaceae and Cleomaceae; Sapindaceae includes former Aceraceae and Hippocastanaceae). In other cases, however, large traditionally recognized families (e.g., Liliaceae; Saxifragaceae; Scrophulariaceae) have been split into many smaller monophyletic families. Such taxonomic alterations have the positive effect of making classification better reflect phylogeny, but also introduce complications in circumscribing newly revised families and their components. This is especially true when large families have been split into many smaller families. Taxonomic sampling in molecular phylogenetic analyses is rarely complete, often leaving many genera potentially in limbo with respect to familial affiliation. Now that a strongly supported and well-resolved broad scale phylogeny for angiosperms has been achieved, we must turn our efforts to more rigorously defining those lineages whose circumscriptions have been altered by these advances.

A prime example of a family whose circumscription has been significantly altered by molecular phylogenetics is the Scrophulariaceae. As traditionally circumscribed the Scrophulariaceae had a global distribution with ca. 200 genera and 4000 species, but within the past decade Scrophulariaceae s.l. has been shown to be polyphyletic and 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, Rahmanzadeh et al. 2005). Scrophulariaceae s.l. is now generally considered to be comprised of several smaller families including the newly expanded Plantaginaceae (Veronicaceae sensu Olmstead et al. 2001) (Olmstead et al. 2001; Fischer 2004; Albach et al. 2005). While the traditionally circumscribed Plantaginaceae included only 3 genera and ca. 275 species, the newly circumscribed family includes 12 tribes with 92 genera and approximately 2000 species (Albach et al. 2005). Importantly, however, only a small fraction of these genera and species have been explicitly analyzed in a phylogenetic framework.

One of the largest tribes within this newly circumscribed Plantaginaceae is tribe Gratioleae. Molecular evidence supports the inclusion of the tribe Gratioleae within the Plantaginaceae (Olmstead et al. 2001; Albach et al. 2005; Oxelman et al. 2005); however, as noted by Oxelman et al. (2005) the Gratioleae are still probably the “least understood group within the former Scrophulariaceae.” Rahmanzadeh et al. (2005) placed *Gratiola* and its relatives in their own family, the Gratiolaceae, a move that has not been accepted by current systematists (Fritsch et al. 2007; Judd et al. 2008). Albach et al. (2005) contend that additional work is needed to clearly circumscribe the Gratioleae, including "specifically which genera belong in it and the synapomorphies that define it."

Overview of Plantaginaceae tribe Gratioleae—The genera of Gratioleae (Table 1) are widely distributed, but are best represented in the Neotropics and closely adjacent temperate regions. Bentham and Hooker (1876) reported ca. 306 species in 37 genera and five subtribes for the Gratioleae. Since Bentham and Hooker's treatment, the tribe's circumscription has been modified by Wettstein (1891), Ruoy (1909), Pennell (1935), Thieret (1954, 1967), and Fischer (2004), most notably in the shifting of certain genera between subfamilies, into or out of the tribe, or between subtribes. Additional new genera have also been described (e.g. Taylor et al. 2000). Not until the late 20th century, with the advent of molecular phylogenetics, were these traditional taxonomic treatments evaluated. Many genera (e.g. *Capraria*, *Limosella*, *Lindernia*, and *Mimulus*) once included within the Gratioleae in nearly all traditional taxonomic treatments (e.g., Bentham and Hooker 1876; Wettstein 1891, Pennell 1935), including all taxa in subtribes Mimuleae and Vandellieae, recently have been shown with molecular data (which is supported by morphology) to be quite distantly related and are now considered part of other tribes within the Plantaginaceae s.l. or other families (Beardsley and Olmstead 2002; Albach et al. 2005, Oxelman et al. 2005, Rahmanzadeh et al. 2005). With many genera now excluded from the Gratioleae the tribe is left with approximately 32 genera (Table 1) and ca. 320 species (Fischer 2004; Albach et al. 2005; Estes et al., unpubl. data). To date, only eight of the genera have been monographed (Fischer 1997; Raynal-Roques 1979; Philcox 1970; Taylor et al. 2000; Ronse 2001; Turner and Cowan 1993a,b; Rossow 1987). Treatments of other taxa within the tribe are fragmentary and are mostly confined in scope to narrow geographic regions (e.g. Pennell 1920, 1935, 1943; Barker 1986, 1992; Ohwi 1965; Seymour 1976). Many of these genera are poorly known and poorly represented in herbaria, especially monotypic genera that are restricted to isolated regions of South America. Larger genera such as *Bacopa* and *Stemodia* that are sometimes segregated into numerous smaller genera are also poorly understood. Turner (1993a,b), as part of a revision of the New World *Stemodia* noted "*Stemodia* (s.s.) and its cohorts are in need of detailed character analysis, especially those derived from comparative DNA studies, before drastic generic redispositions are made, if any." Such comments are applicable to many genera within the tribe.

Four recent broad scale molecular phylogenetic studies have included members of tribe Gratioleae (Olmstead et al. 2001; Albach et al. 2005; Oxelman et al. 2005; Rahmanzadeh et al. 2005), although no individual study has included all genera: *Amphianthus*, *Bacopa, Gratiola, Mecardonia, Otacanthus, Scoparia*, and *Stemodia*. We refer to these as 'core Gratioleae' to distinguish from additional genera including

Angelonia, *Basistemon*, *Melosperma*, *Monopera*, *Monttea*, and *Ourisia* (informally referred to in this document as the "Angeloniae"), some of which have been assigned to Gratioleae by some authors (e.g. Bentham and Hooker 1876; Oxelman et al. 2005), but excluded from Gratioleae by others (e.g. Pennell 1920; Thieret 1954, 1967; Rossow 1985; Fischer 2004; Albach et al. 2005). With the exception of *Monopera* which has not yet been included in any phylogenetic study, these taxa form a clade sister to the core Gratioleae based on molecular data (Albach et al. 2005; Olmstead et al. 2001; Oxelman et al. 2005), and also differ morphologically (Fischer 2004; Rahmanzadeh et al. 2005; Albach et al. 2005). The relationships and circumscriptions of these genera and their relationship to Gratioleae require further study (Oxelman et al. 2005).

The studies cited above establish an approximate position of the Gratioleae relative to the other major clades within Plantaginaceae, its sister relationship with Angelonieae, and show that the core Gratioleae genera formed a strongly supported clade. These data provide a framework for further evaluation of the composition and circumscription of Gratioleae.

Overview of the *Gratiola neglecta* Species Complex—Within *Gratiola* there are a number of groups or complexes that consist of similar taxa that appear, based on morphology, to be closely related. Examples of such complexes include the *G. latifolia-G. sexdentata-G. peruviana* complex of Australasia and South America (Barker 1986, 1990), the *G. officinalis-G. linifolia* complex of Eurasia, the *G. virginiana-G. japonica-G. griffithii* complex of eastern Asia and eastern North America, the *G. nana-G. concinna* complex of Australasia (Cheeseman 1925, Allan 1961, Barker 1990), and the *G. neglecta* complex of North America (Pennell 1935). At present, no attempt has been made to critically evaluate the taxonomy of any of these complexes.

The *Gratiola neglecta* complex includes two species, *G. floridana* and *G. neglecta*. *Gratiola floridana* has a restricted distribution and is found predominantly in the Coastal Plain of the southeastern United States from northwestern Florida and southeastern Mississippi north to northern Alabama, southeastern Tennessee, and northwestern Georgia (Pennell 1935). *Gratiola floridana* inhabits wet bottomland hardwood forests and shaded muddy areas in forested ravines. This species is fairly consistent morphologically, being in all aspects larger than *G. neglecta.* In comparison, *G. neglecta* has one of the largest ranges of any species in the genus and is found from Nova Scotia and British Columbia, Canada south through most of the United States to central Georgia, southeastern Texas, and northern California (Pennell 1935). It also is known to occur in Western Europe (Suominen 1984) where it was probably naturalized. In addition to having a large geographic range, *G. neglecta* also has rather wide ecological amplitude, occurring in a variety of wetland types (e.g. bottomland hardwood forest, ephemeral pools on rock outcrops, sandy riverbanks) over a considerable elevation and latitudinal range. This species is also variable in leaf morphology, flower color, capsule size, and stem pubescence. Glabrous-stemmed plants from tidal mudflats in southeastern Canada were described by Fernald (1932) as *G. neglecta* var. *glaberrima* (Fern.) Fern. Based on the examination of herbarium specimens, the glabrous-stemmed condition also occurs in populations not associated with tidal mudflats, but instead are found in some regions of eastern North America that have an abundance of limestone outcrops. These plants also have narrower leaves than typical for either *G. neglecta* var. *neglecta* or *G. neglecta* var. *glaberrima.* In addition to these glabrous-stemmed populations within *G. neglecta* s.l., other workers have identified unusual plants of *G. neglecta* from other regions of the southeastern United States.

Specimens from the Texas Coastal Plain with less pubescence than typical for *G. neglecta*, and narrower, subentire leaves were described as *G. gracilis* by Bentham (1846). Despite being known only from herbarium specimens, *G. gracilis* was maintained as a species by Small (1903) and Pennell (1921). Later, Pennell (1935) reduced *G. gracilis* to synonymy with *G. neglecta* and noted the characters Bentham used to distinguish *G. gracilis* from *G. neglecta* "are all variable features that occur without geographic correlation." Eight years after Pennell (1935) synonymized *G. gracilis* with *G. neglecta*, Rogers McVaugh (1943) collected an interesting *Gratiola* from granitic outcrops in Oglethorpe County, Georgia. McVaugh sent the specimens to Pennell, who wrote “they seem to match well my description and photograph of *G. gracilis* of Texas," and "I am inclined to take this species out of the synonymy of *G. neglecta*" (McVaugh 1943, p. 159). However, Pennell did not reinstate *G. gracilis* and it has since remained in synonymy (e.g. Correll and Johnston 1970). The issue has not been revisited and further investigation is needed to clarify the circumscription of *Gratiola neglecta* to determine whether any of these noted variants are worthy of taxonomic recognition.

Overview of *Gratiola*—*Gratiola* is the fourth largest and most widely distributed genus of the Gratioleae following *Bacopa* (~60 spp.), *Stemodia* (ca. 56 spp.), and *Limnophila* (36 spp.) (Table 1). Its approximately 33 species (Table 2) are wetland-adapted perennial or annual herbs distributed throughout temperate, tropical-montane, and, rarely, subtropical regions of the Americas, Eurasia, Australasia, and extreme northwestern Africa. The major centers of diversity for *Gratiola* are in North America (16 spp.) and Australasia (11 spp., 4 of which are undescribed).

From the time of Linnaeus’ (1753) *Species Plantarum* to well into the early 19th century, dozens of species from all parts of the world had been assigned to *Gratiola*. However, Bentham (1846), in what remains the only worldwide treatment of *Gratiola*, excluded 58 species from the genus. Many of these species are now placed in *Lindernia* All. (Linderniaceae) or in other gratiolean genera such as *Limnophila*, *Stemodia*, and *Bacopa*. After significant pruning, Bentham (1846) recognized just 20 species for

Gratiola and assigned them to two sections, sect. *Gratiolaria* (=sect. *Gratiola*) and sect. *Sophronanthe* (Table 2). Ten years earlier, Bentham (in Lindley 1836) had described the monotypic *Sophronanthe hispida* Benth., a narrowly distributed species of southeastern North America, but in his 1846 synopsis he reduced this genus to *Gratiola* sect. *Sophronanthe*. He placed two species in sect. *Sophronanthe*, *G. subulata* Baldwin (= *Sophronanthe hispida* and *Gratiola hispida*) and *G. pilosa* Michx. Since Bentham's (1846) treatment, there has been little consensus regarding the circumscription of *Gratiola*, this mostly hinging on the status of *G. hispida* and *G. pilosa.* Small (1903) placed both of these species in the genus *Sophronanthe*. Thirty years later, Small and Pennell (in Small 1933) retained *Sophronanthe* as a distinct genus but with a single species, *S. hispida*; they assigned *S. pilosa* to the newly erected monotypic genus *Tragiola* Small and Pennell (as *Tragiola pilosa*). Pennell (1935), in his comprehensive taxonomic treatment of eastern North American Scrophulariaceae, maintained both *Sophronanthe* and *Tragiola* as distinct from *Gratiola*, noting these genera "stand sharply distinct from *Gratiola*" and that "with the removal of *Sophronanthe* and *Tragiola*, *Gratiola* remains a quite natural genus." Interestingly, in spite of the numerous morphological, cytological, and ecological differences that separate *Sophronanthe* and *Tragiola* from *Gratiola*, most subsequent floristic botanists have not followed the concepts of Small and Pennell (in Small 1933) or Pennell (1935) but instead have recognized *Gratiola* sensu lato (s.l.) as including *Sophronanthe* and *Tragiola* (Fernald 1950, Radford et al. 1968, Gleason and Cronquist 1991, Wunderlin and Hansen 2003). Fischer (2004) treated *Sophronanthe* as distinct from *Gratiola* but, interestingly, he placed *Tragiola* in synonymy with *Gratiola.* Currently, the relationships of *Sophronanthe*, *Tragiola*, and *Gratiola* remain unresolved and are in need of clarification especially from a molecular phylogenetic context. This clarification is essential to accurately circumscribing *Gratiola.* Some taxonomists (Eskuche 1974; Kral 1983) have recognized that at least two other genera, *Amphianthus* and *Braunblanquetia*, bear striking similarities to *Gratiola.* Kral (1983) noted that *Amphianthus* Torrey, a rare monotypic North American genus whose taxonomic position has long been debated, is morphologically nearest to *Gratiola.* Eskuche (1974) described the monotypic genus *Braunblanquetia* Eskuche from Argentina and separated it from *Gratiola* based on a single morphological character. Clearly, any attempt to resolve the phylogenetic relationships of tribe Gratioleae and to accurately circumscribe *Gratiola* should include *Amphianthus*, *Braunblanquetia*, *Sophronanthe*, and *Tragiola*.

As noted above, the only worldwide taxonomic treatment of *Gratiola* was that of Bentham (1846) in DeCandolle's *Prodromus*. He recognized 20 species and instituted the first infrageneric classification recognizing two sections, sect. *Sophronanthe* and sect. *Gratiolaria* (=sect. *Gratiola*) (Table 2). The status of sect. *Sophronanthe* has been thoroughly described above. He divided sect. *Gratiola*, the larger of the two sections with

18 species, into two subsections, subsect. *Subdidynamae* and subsect. *Diandrae*. His subsection *Subdidynamae* included those species with a well-developed pair of filiform posterior staminodia with subcapitate apices. He divided this subsection into two groups based on pedicel length, the predominantly Northern Hemisphere inhabiting *Pedicellatae* with prominently pedicellate flowers and the largely Southern Hemisphere inhabiting *Sessiliflorae* with subsessile or short-pedicellate flowers. Bentham (1846) placed the remaining species in subsect. *Diandrae*, a mostly North American group (with the exception of the Australian *G. pedunculata*) characterized by short ecapitate or absent posterior staminodia.

Except for the removal of sect. *Sophronanthe* by Small (1903) as described previously, the infrageneric classification scheme for *Gratiola* s.s. devised by Bentham (1846) remained unchanged for nearly 90 years. Pennell (1935) took all of the North American species of Bentham's *Diandrae* and placed them in the newly created sect. *Nibora* (Raf.) Pennell. He differentiated sect. *Nibora* from sect. *Gratiola* based on the relative density of glands on the leaves (obscurely vs. conspicuously glandular-punctate), leaf base shape (tapering vs. clasping), relative capsule size (± equal to the sepals vs. exceeded in length by the sepals), seed coloration (yellowish vs. brownish), duration (annual vs. perennial), and geographic distribution (North America vs. all other areas occupied by the genus). Since Pennell last modified the infrageneric taxonomy of *Gratiola*, additional work on the genus has dealt only with differentiating species in restricted geographic regions. Some of these studies (e.g. Barker 1990) indicate that the morphological characters used by Pennell to erect sect. *Nibora* actually do not hold when taxa from outside of eastern North America, the focal region of Pennell's (1935) treatment, are considered. This raises questions about the actual relationships of the sections of *Gratiola.* In order to understand the relationships of the sections and species of *Gratiola*, a complete molecular phylogeny coupled with an analysis of morphological character evolution and historical biogeography is needed.

Goals of the Project—*Gratiola* is a poorly understood genus that has not previously been studied systematically. No phylogenetic work has been undertaken for *Gratiola* and the last worldwide taxonomic treatment is more than 160 years old. Therefore, *Gratiola* is in need of a detailed systematic investigation, the focus of this study. Below, the major components of this investigation are outlined:

- **Objective 1 – Preliminary Phylogeny of Tribe Gratioleae and Assessment of the Relationships between *Gratiola* s.l. and *Amphianthus***

  (1) provide a preliminary assessment of phylogenetic relationships among genera within Gratioleae using chloroplast *ndhF* sequences
  (2) determine the phylogenetic placement of *Gratiola* within the Gratioleae using chloroplast *ndhF* sequences
  (3) investigate the relationships of *Gratiola* and *Amphianthus* using non-coding chloroplast *trnS*-*trnG* intergenic spacer and *trnG* intron sequences.

- **Objective 2** – **Taxonomic Study of *Gratiola neglecta***

  (1) study herbarium specimens to investigate patterns of morphological variation in the wide-ranging and phenotypically variable species, *Gratiola neglecta*
  (2) conduct a morphological analysis to study morphological variation within *Gratiola neglecta*
  (3) conduct field work to examine populations of *G. neglecta*
  (4) use scanning electron microscopy to investigate micromorphological features in *G. neglecta*

- **Objective 3 – Phylogeny, morphological evolution, and biogeography of *Gratiola***

  (1) determine the phylogenetic relationships of the ca. 33 species of *Gratiola* using non-coding cpDNA sequences from the *trnS*-*trnG* intergenic spacer and *trnG* intron and the *trnQ-rps16* intergenic spacer
  (2) assess the relationships of *Braunblanquetia*, *Sophronanthe*, and *Tragiola* to *Gratiola*
  (3) test the monophyly of the sections of *Gratiola*
  (4) examine the evolution of morphological characters within *Gratiola*
  (5) infer historical biogeographical patterns within *Gratiola*

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APPENDIX; CHAPTER 1

Table 1.1. Genera of Plantaginaceae tribe Gratioleae, their geographic distribution, and approximate number of species. Bolded names represent genera sampled in previous molecular phylogenetic studies and determined to be in the Gratioleae based on DNA sequences.

Genus (synonyms and segregates)	**Distribution**	**# Species**
Achetaria Cham. Et Schlecht.	South America, Caribbean Is.	9
Adenosma R.Br.	se Asia, Australasia	15
Anamaria V.C.Souza	Brazil	1
Bacopa Aubl. (*Allocalyx*, *Ancistrostylis*, *Anisocalyx*, *Bryodes*, *Calytriplex*, *Cardiolophus*, *Heptas*, *Herpestis*, *Hydranthelium*, *Hydrotrida*, *Mella*, *Monocardia*)	Neotropics, Pantropical	~60
Benjaminia Mart. Ex Benj.	Brazil to Mexico	1
Boelckea Rossow	Bolivia	1
Cheilophyllum Pennell ex Britton	Caribbean Is.	8
Conobea Aubl.	Neotropics	2
Darcya B.L. Turner and Cowan	Central America	3
Deinostema T.Yamazaki	E Asia	2
Dizyogostemon (Benth.) Radik.	Brazil	2
Dopatrium Buch.-Ham. ex Benth.	E Asia to Africa	12-13
Geochorda Cham. et Schlecht.	Brazil	1
Gratiola L. (_Amphianthus_, *Braunblanquetia***,** *Fonkia*, *Sophronanthe*, *Tragiola***)**	Americas, Eurasia, Australasia	~33
Hydrotriche Zucc.	Madagascar	4
Ildefonsia Gardn.	Brazil	1
Leucospora Nutt.	North America	2
Limnophila R.Br.	E. Asia, Africa, Australasia	36
Maeviella Rossow	South America	1
Mecardonia Ruiz. and Pav.	N. and S. America	15
Morgania R.Br.	Australia	4
Otacanthus Lindl.	Brazil	6
Philcoxia P.Taylor and V.C.Souza	Brazil	3
Schistophragma Benth. ex Endl.	N. and S. America	2
Schizosepala G.M.Barroso	Brazil	1

Table 1.1. continued.

Genus (synonyms and segregates)	Distribution	# Species
***Scoparia* L.**	Neotropics	20
***Stemodia* L.** (*Chodophyton*, *Lindneria*, *Poarium*, *Stemodiacra*, *Valeria*, *Verena*)	Neotropics, Asia, Africa	~56
Tetraulacium Turcz.	Brazil	1

Table 1.2. Clades and taxa of *Gratiola* recognized in this study plus their geographic distributions. Underlined taxa were not included in this study.

Clades, Subclades, and Species	Geographic Distribution	No. Samples Included (*trnS-trnG-trnG* / *trnQ-rps16*)
Clade 1 ("*Sophronanthe*")		
Gratiola pilosa Michx. var. *pilosa*	se U.S.	(2 / 2)
Gratiola pilosa Michx. var. *epilis* Pennell	se U.S.	(1 / 0)
Gratiola hispida (Benth. ex Lindl.) Pollard	se U.S.	(2 / 2)
Clade 2 ("*Diandrae*")		
G. flava Leavenw. ex Pennell	se U.S.	(2 / 2)
G. neglecta Torr.	widespread North America	(2 / 2)
G. floridana Nutt.	se U.S.	(2 / 2)
G. quartermaniae D. Estes	e North America	(2 / 2)
G. graniticola D. Estes	se U.S.	(2 / 2)
G. ebracteata Benth.	w U.S.	(2 / 2)
G. heterosepala Mason and Bacigal.	w U.S.	(2 / 2)
G. oresbia B.L. Robins.	Mexico and Guatemala	(2 / 2)
G. amphiantha D. Estes and R.L. Small nom. nov. ined.	se U.S.	(2 / 2)

Table 1.2. continued.

Clades, Subclades, and Species	Geographic Distribution	No. Samples (*trnS-trnG-trnG* / *trnQ-rps16*)
Clade 3 ("*Nibora*")		
Gratiola virginiana L.	e U.S. and Mexico	(2 / 2)
G. japonica Miq.	e Asia	(2 / 2)
G. griffithii J.D. Hooker	e Asia	(0 / 0)
Clade 4 ("*Gratiola*")		
Gratiola peruviana Subclade		
G. peruviana L.	s South America	(2 / 2)
G. uliginosa Foncke	Argentina	(1 / 1)
G. bogotensis Cortes ex Pennell	Colombia, Ecuador, Venezuela	(2 / 2)
G. uruguayensis Herter and Melchior	s South America	(0 / 0)
Gratiola latifolia Subclade		
G. latifolia R.Br.	Australia and Tasmania	(3 / 2)
G. sexdentata R. Cunn.	New Zealand and Chatham Is.	(2 / 2)
G. aff. *nana*-Aust.	se Australia	(3 / 3)

Table 1.2. continued.

Clades, Subclades, and Species	Geographic Distribution	No. Samples (*trnS-trnG-trnG* / *trnQ-rps16*)
Gratiola pubescens Subclade		
G. pubescens R.Br.	Australia and Tasmania	(2 / 2)
G. aff. *pubescens*	Western Australia	(1 / 2)
Gratiola nana Subclade		
G. nana Benth.	Tasmania	(2 / 2)
G. aff. *nana*-Tasman.	Tasmania	(1 / 1)
G. concinna	New Zealand	(2 / 2)
G. aff. *concinna*	New Zealand	(1 / 1)
Gratiola officinalis Subclade		
G. officinalis L.	Eurasia	(2 / 2)
G. linifolia Vahl	Spain and Portugal	(1 / 1)
Gratiola aurea Subclade		
G. aurea Muhl.	e North America	(2 / 2)
G. viscidula Pennell	se U.S.	(3 / 1)
G. brevifolia Raf.	se U.S.	(2 / 2)
G. ramosa Walt.	se U.S.	(2 / 2)

Chapter 2

PRELIMINARY PHYLOGENETIC RELATIONSHIPS OF PLANTAGINACEAE TRIBE GRATIOLEAE WITH EMPHASIS ON THE RELATIONSHIPS OF THE MONOTYPIC GENUS *AMPHIANTHUS*

This paper is a slightly modified version of the following paper accepted for publication in the journal *Systematic Botany* (2007) by Dwayne Estes and Randall L. Small:

> Estes, D. and R.L. Small. Phylogenetic relationships of the monotypic genus *Amphianthus* (Plantaginaceae tribe Gratioleae) inferred from chloroplast DNA sequences. Systematic Botany: in press.

In the following chapter, my use of the words "we" and "our" refers to my co-author and me. My contributions to this paper include (1) the initial discovery of the problem and further development of the core ideas behind the project, (2) preparation of grant proposals to secure funding, (3) completion of all field and herbarium-related research, (4) most of the lab work, (5) and most of the writing.

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* intergenic spacer and *trnG* 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.*

INTRODUCTION

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, Rahmanzadeh et al. 2005). Scrophulariaceae s.l. is now generally considered to be comprised of 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, Rahmanzadeh 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 septicidally (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; Rahmanzadeh 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* Eskuche, *Geochorda* Cham. et Schlecht, *Ildefonsia* Gardn., *Maeviella* Rossow, *Schizosepala* G.M.Barroso, and *Tetraulacium* Turcz. In addition, *Sophronanthe* Benth. and *Tragiola* Small and 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; Rahmanzadeh 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 and Pav., *Otacanthus* Lindl., *Scoparia* L., and *Stemodia* L. Some additional genera, including *Angelonia* Humb. and 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 current paper, we will 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).

Currently, the placement of *Amphianthus* relative to the other six sampled Gratioleae genera remains unclear. 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 *Otacanthus* (Albach et al. 2005; Rahmanzadeh et al. 2005) or *Stemodia* (Oxelman et al. 2005) to be sister to *Gratiola.*

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

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) and specifically to determine the phylogenetic placement of *Amphianthus* within the Gratioleae using chloroplast *ndhF* sequences, (3) investigate the relationships of *Amphianthus* and *Gratiola* using non-coding chloroplast $trnS^{GCU}$-$trnG^{UUC}$-$trnG^{UUC}$ 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; Rahmanzadeh 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 Rahmanzadeh 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; Rahmanzadeh 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). Sequence data were obtained from three chloroplast regions: the gene *ndhF* (Fig. 2.1) and the *trnS-trnG* intergenic spacer and *trnG* intron (Fig. 2.1) (*trnS*^{GCU}-*trnG*^{UUC}-*trnG*^{UUC}). The *ndhF* region was used 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 µL) consisted of the following components: 1 µL template DNA (~ 10-100 ng), 1X buffer (TaKaRa, Madison, Wisconsin), 200 µmol/L each dNTP, 3.0 mmol/L $MgCl_2$ (1.5mmol/L for *trnS-trnG-trnG*), 0.1 µmol/L each primer, 0.2 µg/µL 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, non-overlapping 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 + Γ 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. Non-overlapping 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 accession number: S1776).

RESULTS

ndhF—The *ndhF* data matrix contained 47 taxa and 2091 characters and was aligned with little difficulty. 7.4% of the data matrix consisted of missing data. The Bayesian majority rule consensus tree is shown in Fig. 2.2. This tree was well-resolved and most nodes were strongly supported with posterior probability (PP) values ≥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. 2.2, 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. 2.2, Clade E, the *Gratiola* clade), three groups were identified. Group 1 consisted of *G. hispida* and *G. pilosa*, both members of *Gratiola* sect. *Sophronanthe* 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—The *trnS-trnG-trnG* dataset contained eight taxa and 1821 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. 2.3) was topologically consistent with Clade E from the *ndhF* analysis (Fig. 2.2). As in the *ndhF* analysis, three major groups were recovered, the only difference being 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 ≥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; Rahmanzadeh 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). Rahmanzedah 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, cytological, 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* (Olmstead et al. 2001, Albach et al. 2005, 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. 2.2) 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. 2.2) 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. 2.3) is consistent with Clade E of the *ndhF* tree (Fig. 2.2), 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 Gratioleae; 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 and 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 Gratioleae 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). Other Gratioleae genera (*Deinostema* T. Yamaz., *Limnophila*, *Dopatrium* Buch.-Ham. ex Benth., *Hydrotriche*, *Gratiola*), however, may also have cleistogamous flowers (Philcox 1970; Fischer 1997, 2004). *Amphianthus* is also not alone in the Gratioleae in its simultaneous production of cleistogamous and chasmogamous flowers; *Limnophila australis* B.S.Wannan and 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 Gratioleae, cleistogamy is not unique to *Amphianthus*.

Some taxonomists (Small 1933; Pennell 1935) have distinguished *Amphianthus* from other Gratioleae genera on the basis of its distinctive capsules which are laterally compressed and obcordate. Although the capsules of *Amphianthus* are relatively unique in appearance compared to other genera in the Gratioleae, 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 dimorphism 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. 2.2), *Amphianthus* is an annual. Pennell (1935) regarded this feature to be an important character 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. 2.3) 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 Gratioleae 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 the 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 Gratioleae 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 11 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. japonica* (2N=16; Probatova and Sokolovskaya 1981), *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. 2.2, 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 and R. L. Small, nom. nov. Basionym: *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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APPENDIX; CHAPTER 2

APPENDIX 2. 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.

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 and AJ619555. ***Callitriche hermaphroditica*** L., Olmstead and Reeves 1995, L36396. ***Chelone obliqua*** L., Young, Steiner, and dePamphilis 1999, AF123680. ***Collinsia grandiflora*** Lindley, AF188182. ***Digitalis grandiflora*** Mill, Olmstead and 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, and dePamphilis 1999, AF123683. ***Hydrotriche hottoniaeflora*** 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. and 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]. ***Ourisia poeppigii*** Benth., Oxelman et al. 2005, AJ619560, AJ619561, and AJ619562. ***Plantago lanceolata*** L., Olmstead and 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 and AJ619568. ***Scoparia dulcis*** L., USA, Florida, Putnam Co., *Beck s.n.* (TENN), [EF527450]. ***Scoparia plebeja*** Cham. and Schltdl., Bolivia, *Carretero 1099* (NY), [EF527452]. ***Scrophularia californica*** Cham. and Schldtl., Olmstead and Reeves 1995, L36411. ***Stemodia glabra*** Oerst., Kornhall and Bremer (unpubl. data), AJ617584 and 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 and Reeves 1995, L36419.

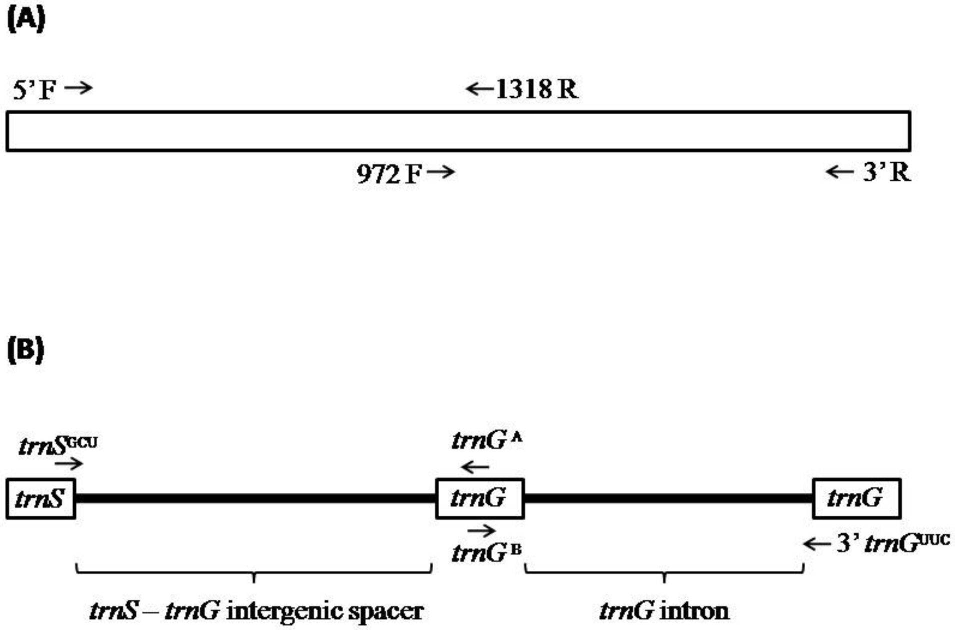
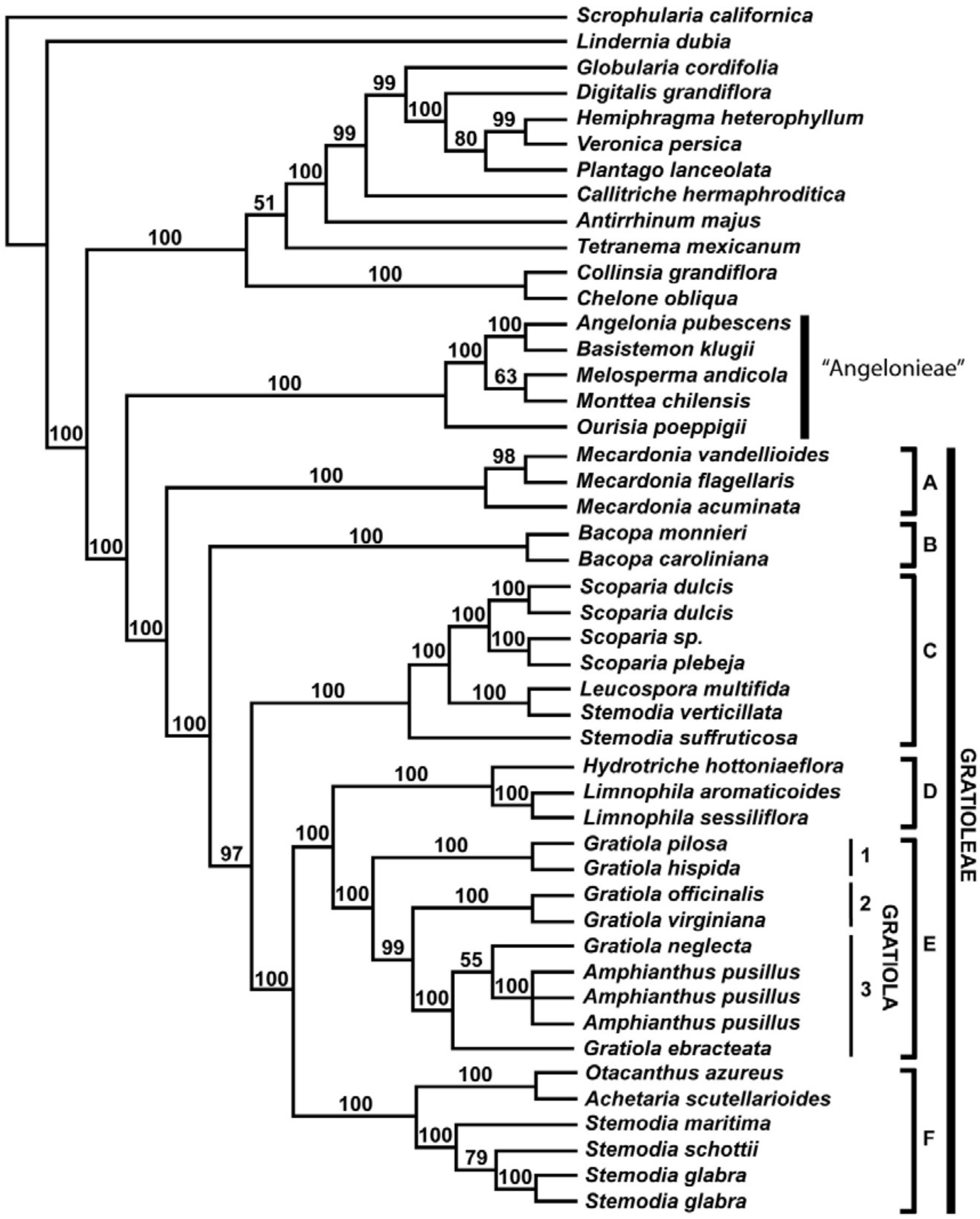


Fig. 2.1. Map of the chloroplast DNA regions surveyed in this investigation with arrows showing relative location of primer annealing sites. White boxes represent coding regions (exons); heavy black bars represent non-coding regions (intergenic spacers or introns). **A.** *ndhF* gene. **B.** *trnS*-*trnG* intergenic spacer and *trnG* intron.

Fig. 2.2. Bayesian majority rule consensus tree based on analysis of *ndhF* cpDNA sequences showing relationship of *Amphianthus* to *Gratiola* and the rest of tribe Gratioleae and the Plantaginaceae. Numbers above branches indicate posterior probabilities.


Scrophularia californica
Lindernia dubia
Globularia cordifolia
Digitalis grandiflora
Hemiphragma heterophyllum
Veronica persica
Plantago lanceolata
Callitriche hermaphroditica
Antirrhinum majus
Tetranema mexicanum
Collinsia grandiflora
Chelone obliqua
Angelonia pubescens
Basistemon klugii
Melosperma andicola
Monttea chilensis
Ourisia poeppigii
"Angelonieae"
Mecardonia vandellioides
Mecardonia flagellaris
Mecardonia acuminata
Bacopa monnieri
Bacopa caroliniana
Scoparia dulcis
Scoparia dulcis
Scoparia sp.
Scoparia plebeja
Leucospora multifida
Stemodia verticillata
Stemodia suffruticosa
Hydrotriche hottoniaeflora
Limnophila aromaticoides
Limnophila sessiliflora
Gratiola pilosa
Gratiola hispida
Gratiola officinalis
Gratiola virginiana
Gratiola neglecta
Amphianthus pusillus
Amphianthus pusillus
Amphianthus pusillus
Gratiola ebracteata
Otacanthus azureus
Achetaria scutellarioides
Stemodia maritima
Stemodia schottii
Stemodia glabra
Stemodia glabra
A
B
C
D
E
F
1
2
3
GRATIOLA
GRATIOLEAE
99
100
80
99
99
100
51
100
100
100
100
63
100
100
98
100
100
100
100
100
100
100
100
100
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100
97
100
100
100
100
99
100
55
100
100
100
100
100
79
100

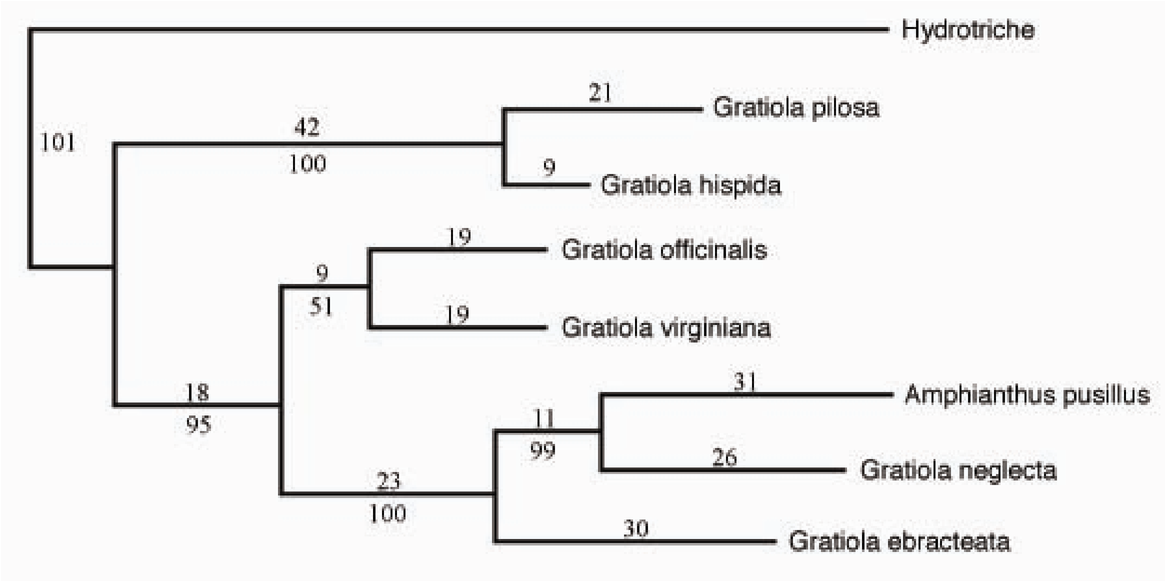

Fig. 2.3. Single most parsimonious tree showing phylogenetic relationships of *Amphianthus* and selected species of *Gratiola* using *trnS*-*trnG*-*trnG* cpDNA sequences. Numbers above branches are branch lengths; numbers below branches are bootstrap values.

Chapter 3

TWO NEW SPECIES OF *GRATIOLA* (PLANTAGINACEAE) FROM EASTERN NORTH AMERICA AND AN UPDATED CIRCUMSCRIPTION FOR *GRATIOLA NEGLECTA*

This paper is a slightly modified version of the following paper recently published in the *Journal of the Botanical Research Institute of Texas* by Dwayne Estes and Randall L. Small:

> Estes, D. and R.L. Small. 2007. Two new species of *Gratiola* (Plantaginaceae) from eastern North America and an updated circumscription for *Gratiola neglecta. Journal of the Botanical Research Institute of Texas* 1: 149–170.

The use of the words "we" or "our" refers to my coauthor and me. My contributions to this paper include (1) the initial discovery of both new species, (2) preparation of grant proposals to secure funding, (3) completion of all field and herbarium-related research, (4) morphometric analysis, (5) and most of the writing.

ABSTRACT

Gratiola section *Nibora*, a North American taxon as currently circumscribed includes six species: *G. ebracteata*, *G. flava*, *G. floridana*, *G. heterosepala*, *G. neglecta*, and *G. virginiana. Gratiola ebracteata* and *G. heterosepala* are restricted to western North America and the remaining four species are mostly eastern North American. The species with the largest range and greatest degree of morphological variability is *G. neglecta.* A recent investigation of *G. neglecta* involving fieldwork, examination of herbarium specimens, morphological analysis, and phytogeographic study, has resulted in the discovery of two undescribed species, *G. graniticola* sp. nov. and *G. quartermaniae* sp. nov., both of which are endemic to rock outcrop communities of eastern North America. In this paper, both new species are described, illustrated, and compared to their widespread congener, *G. neglecta.* An updated circumscription of *G. neglecta* is provided and a key distinguishing the new species from *G. neglecta* is included.

INTRODUCTION

Recent evidence from field and herbarium studies indicates that material previously referred to *G. neglecta* includes two undescribed species. The first new species, *G. quartermaniae* D.Estes sp. nov., has a highly fragmented distribution in eastern North America and is endemic to ephemerally wet sites associated with calcareous outcrops (cedar glades) and prairies. The second new species, *G. graniticola* D.Estes sp. nov., is endemic to north-central Georgia where it is restricted to vernal pools on granitic outcrops. In this paper, both new species are described, illustrated, and compared to their widespread congener, *G. neglecta.* Because *G. quartermaniae* and *G. graniticola* have been included within the concept of *G. neglecta* by previous authors, an updated circumscription of *G. neglecta* is provided.

MATERIALS AND METHODS

In order to clarify morphological variation within and between *G. neglecta*, *G. quartermaniae*, and *G. graniticola*, an investigation was conducted that incorporated fieldwork, examination of herbarium specimens, morphological analyses, and phytogeography. Fieldwork was conducted in portions of 26 states in the United States and the province of Ontario, Canada between 2001 and 2006. In addition, more than 4,000 herbarium specimens (including some digital images), representing all taxa from sect. *Nibora*, were examined from the following 49 herbaria: A, ALU, APSC, ASTC, AUA, BRIT, CAN, CITA, CLEMS, DAO, DUKE, EKY, FSU, GA, GH, H, ILLS, ISC, JEPS, JSU, K, KANU, LL, LSU, MIN, MISS, MO, MTSU, NCSC, NCU, NLU, NO, NY, NYS, OKL, PH, SBSC, SMU, TENN, TEX, TROY, TRT, UARK, UC, UNA, US, USCH, VDB, and VPI (herbarium acronyms follow Index Herbariorum, http://www.nybg.org/bsci/ih/search).

From the herbarium specimens examined during this project, a subset of 87 mature and complete specimens representing 55 *G. neglecta*, 15 *G. graniticola*, and 17 *G. quartermaniae*, was selected for use in a morphometric study. Specimens were chosen to represent the full geographic distribution, range of habitat, and morphological variation of each species. For each specimen, 10 quantitative vegetative and floral characters were measured (Table 1); these specimens are denoted by an asterisk in the lists of representative specimens examined. Seed measurements were taken from five of the above specimens (1 *G. graniticola*, 4 *G. neglecta*) plus an additional 14 specimens representing a total of 10 widespread populations of *G. neglecta*, four of *G. graniticola*, and five of *G. quartermaniae.* Twenty seeds from a single capsule were measured per population, and three quantitative characters were scored per seed (Table 1). Specimens used for seed measurements are indicated by a dagger (†) in the lists of specimens examined. For each scored character, summary statistics including mean, standard deviation, and range were calculated; these values are presented in Table 1. In the taxonomic key and species descriptions, measurements for characters are given as the mean ± one standard deviation with extreme values, based on additional observations, given in parentheses. In order to reveal discontinuities in the data and to determine which characters are most useful for delimiting taxa, pairwise comparisons of characters were conducted using scatter diagrams and box plots. Seeds and trichomes of all three species were also examined with the aid of scanning electron microscopy (SEM) to search for useful taxonomic characters. The geographic distribution of *G. neglecta, G. graniticola,* and *G. quartermaniae* was determined by examining the collection data included on herbarium specimens and plotting the county-level distribution of each species on outline maps. Each point on these maps is represented by at least one herbarium specimen examined.

RESULTS AND DISCUSSION

Morphology—*Gratiola neglecta*, *G. quartermaniae*, and *G. graniticola* form a morphologically cohesive group referred to here as the *Gratiola neglecta* complex. A fourth species, *G. floridana*, also belongs to this complex; however, it is quite distinct morphologically in spite of sharing a suite of features uniting it with the other three species. *Gratiola floridana* differs from the other members of the complex in its overall larger features including much larger flowers 13–25 mm long (vs. 5–14 mm), longer proximal fruiting pedicels averaging 23–43 mm long (vs. 12–25 mm), and longer seeds averaging 0.79–0.9 mm (vs. 0.4–0.6 mm). This species tends to inhabit forested sites whereas the others mostly grow in open communities. It is also the southernmost member of the complex ranging from northwestern Florida and southeastern Louisiana (historically) north into southeastern Tennessee. The distribution of *G. floridana* only slightly overlaps with the ranges of *G. neglecta* and *G. quartermaniae* in the northern portion of its range. Since *G. floridana* is one of the most distinctive species of the genus and has rarely been confused with *G. neglecta* or the two new species, it will not be discussed further.

Several characters distinguish *G. graniticola* from *G. neglecta* and *G. quartermaniae* (Table 2; Fig. 3.1, Fig. 3.2). *Gratiola graniticola* has shorter leaves (normal leaves that subtend pedicels are also referred to as bracts or bracteal leaves in this paper) that are widest at or below the middle (Fig. 3.2 A), shorter pedicels that are longer relative to their subtending bracts (Fig. 3.2, D–E), smaller corollas that have a purplish or pinkish posterior lobe and beard of whitish trichomes, bracteoles that are shorter than to only slightly exceeding the calyces, smaller more subglobose purple-tinged capsules (Fig. 3.2 F), smaller seeds (Fig. 3.2 G–H; Fig. 3.3 A), and bulbous-based trichomes (Fig. 3.3). *Gratiola neglecta* and *G. quartermaniae* have longer leaves (Fig. 3.2 A), longer pedicels that are mostly equal to or shorter than their subtending bracts (Fig. 3.2, D–E), larger corollas that usually lack purplish or pinkish coloration and that have a beard of yellow trichomes inside the corolla orifice, bracteoles that are mostly longer than the calyces, larger more ovoid and brownish capsules (Fig. 3.2 F), larger seeds (Fig. 3.2, G–H; Fig. 3.3 B–C), and slender-based trichomes (Fig. 3.3, E–F). A scatter diagram of leaf length vs. capsule length between *G. graniticola*, *G. neglecta*, and *G. quartermaniae* reveals two primary clusters that exhibit minimal overlap (Fig. 3.1 A). In this scatter plot, specimens of *G. graniticola* mostly group separately from the second unresolved cluster that consists of specimens of *G. neglecta* and *G. quartermaniae.* A scatter plot of proximal pedicel length/subtending bract length vs. leaf width also distinguishes *G. graniticola* from *G. neglecta* (Fig. 3.1 B).

Gratiola quartermaniae differs from *G. neglecta* in having a glabrous midstem, narrower (Fig. 3.2 B) and more falcate, fewer veined and fewer toothed leaves that have a larger length to width ratio (Fig. 3.2 C) and seeds that average longer, thicker, and darker (Fig. 3.2, G–H). In comparison to *G. quartermaniae*, *G. neglecta* has mostly pubescent (rarely glabrate in some New England estuarine populations) midstems, wider (Fig. 3.2 B), more veined and more toothed leaves that have a smaller leaf length to width ratio (Fig. 3.2 C). The seeds of *G. neglecta* are lighter in color and average slightly shorter and are not as thick as those of *G. quartermaniae* (Fig. 3.2, G–H). In Fig. 3.1 C, a scatter plot of leaf length/leaf width vs. number of teeth per margin for *G. neglecta* and *G. quartermaniae* reveals two clusters of specimens.

Distribution and Ecology—*Gratiola neglecta* has the largest distribution of the three species, being found throughout most of temperate North America (Fig. 3.4). It ranges from Nova Scotia and British Columbia, Canada, south in the United States to central Georgia, coastal Texas, northern Arizona, and northern California. The species is most common in the eastern United States particularly in the lower Mississippi, Missouri, and Ohio River valleys. West of the Mississippi River, the range of *G. neglecta* mostly follows the major river systems toward the Great Plains. From the upper Missouri River watershed, *G. neglecta* ranges south into the southern Rocky, Cascade, and Sierra Nevada mountains. Several populations in the western United States are associated with reservoirs; these may represent recent introductions by migrating waterfowl. Interestingly, *G. neglecta* has also been collected in France (*Simon s.n.* FSU; *Rastetter 11653* UC) and Finland (*Lampinen 5629* H; see Suominen 1984) where probably introduced.

Gratiola neglecta grows in a broader array of wetland communities and endures a greater range of environmental conditions than *G. graniticola* or *G. quartermaniae*. It grows from sea level to an elevation of 2400 m in the mountains of the western United States. Compared to the new species, *G. neglecta* occurs more frequently in the deeper soil of agricultural fields, openings in bottomland hardwood forests, wet meadows, mudflats, and pond margins. Rarely, *G. neglecta* occurs in salt marshes or on various types of shallow-soiled rock outcrops including igneous, sandstone, limestone, and granite formations.

Gratiola quartermaniae has a fragmented range (Fig. 3.5) and is most common in the limestone cedar glades of the Interior Low Plateau of middle Tennessee and northern Alabama. From this core range, it is disjunct to the alvars of southeastern Ontario, Canada, a distance of ca. 1200 km. Most of the Ontario populations are associated with the Napanee limestone plain but a few are found in the Dummer Moraine and Prince Edward Peninsula physiographic regions (Chapman and Putnam 1984). Numerous other species that are more common on calcareous outcrops in the southeastern United States also occur on Canadian alvars including several of the species commonly associated with *G. quartermaniae* in Tennessee and Alabama such as *Carex granularis* Muhl. ex Willd., *C. crawei* Dewey, *C. molesta* Mack. ex Bright, *Isanthus brachiatus* (L.) B.S.P., *Scutellaria parvula* Michx., and *Sporobolus vaginiflorus* (Torr. ex Gray) Wood. *Gratiola quartermaniae* is also disjunct to Will County, Illinois from its main range in central Tennessee, a distance of approximately 600 km. Two limestone glade near-endemics, *Dalea foliosa* (Gray) Barneby and *Astragalus tennesseensis* Gray ex Chapman, share this similar distribution pattern (Baskin and Baskin 2003). *Gratiola quartermaniae* is also disjunct to the Edward's Plateau of Texas, a distance of ca. 1200 km. Interestingly, *Juncus filipendulus* Buckley, a species that *G. quartermaniae* frequently occurs with in Alabama and Tennessee, is also disjunct to the Edward's Plateau where it occurs with *G. quartermaniae*. Therefore, while the disjunction patterns exhibited by *G. quartermaniae* are unusual, further examination indicates that in each of these areas *G. quartermaniae* occurs in similar habitat and always occurs with other calciphilous species, some of which have similar patterns of disjunction. This species should be searched for in other regions where calcareous outcrops and prairies occur such as the limestone glades of the southern Ridge and Valley of southeastern Tennessee and northwestern Georgia, the Blackbelt prairies of Mississippi and Alabama, the limestone glades of central and western Kentucky, the Ozark glades of southern Missouri and northern Arkansas, and alvar habitats in New York, Michigan, and Ohio.

Gratiola quartermaniae is found on limestone or dolomite outcrops and calcareous prairies. In these habitats, the species predominantly occurs in shallow clayey soils of ephemeral pools, seasonal streambeds, and periodically wet meadows on or immediately adjacent to outcrops. These sites are usually flat to slightly sloping and are located in areas that receive high to moderate levels of sunlight. They are wet in late winter and early spring but become severely desiccated by late spring and early summer. Rarely, *G. quartermaniae* occurs in situations otherwise more typical for *G. neglecta* such as low wet fields, open wet woods, and marsh edges, but these populations are always located within close proximity to glade habitat. Limestone glade endemics such as *Leavenworthia alabamica* Rollins, *L. crassa* Rollins, *L. torulosa* Gray, and

Lesquerella lyrata Rollins are sometimes found in disturbed non-outcrop habitats often in association with *G. quartermaniae.* In central Tennessee and northern Alabama, *G. quartermaniae* is almost always associated with limestone cedar glade endemics or calciphiles such as *Allium cernuum* Roth, *Carex crawei*, *C. granularis*, *Dalea foliosa*, *D. gattingeri* (Heller) Barneby, *Eleocharis bifida* S.G. Smith, *Hypericum sphaerocarpum* Michx., *Isoetes butleri* Engelm., *Juncus filipendulus*, *Leavenworthia* spp., *Ludwigia microcarpa* Michx., *Mecardonia acuminata* (Walt.) Small, *Sedum pulchellum* Michx., *Sporobolus vaginiflorus*, and *Talinum calcaricum* Ware. In areas where *G. quartemaniae* is disjunct as in Ontario, Illinois, and Texas, the species is associated with a number of additional calciphilous taxa, including a few of those listed above.

Gratiola graniticola is restricted to granite outcrops in 13 counties on the Piedmont Plateau of Georgia (Fig. 3.6). Of the approximately 17 Piedmont granite outcrop endemics (McVaugh 1943; Weakley 2007), *G. graniticola* is one of only five species, along with *Isoetes melanospora* Engelmann, *I. piedmontana* (N.E. Pfeiffer) C.F. Reed, *I. tegetiformans* Rury, and *Amphianthus pusillus* Torr., restricted to the ephemeral pools of the outcrops. Interestingly, *I. tegetiformans* and *G. graniticola* are the only Piedmont granite outcrop endemics completely restricted to Georgia.

All known populations of *G. graniticola* occur on granite outcrops in water-filled depressions lined with a thin layer of soil. These depressions are filled with water during the winter and spring months but dry out in the summer and fall. Species commonly associated with *G. graniticola* include *Croton willdenowii* G.L.Webster, *Cyperus granitophilus* McVaugh, *Diamorpha smallii* Britt. ex Small, *Eleocharis obtusa* (Willd.) Schult., *Isoetes piedmontana* (N.E. Pfeiffer) C.F. Reed, *Juncus georgianus* Coville, *Lindernia monticola* Muhl. ex Nutt., *Minuartia uniflora* (Walt.) Mattf., *Packera tomentosa* (Michx.) C.Jeffrey, *Pilularia americana* A.Braun, *Rhynchospora* sp., and *Schoenolirion croceum* (Michx.) A.Gray.

Gratiola quartermaniae is sympatric with *G. neglecta*; however, the two species generally occupy different habitat types. They occur syntopically at a few sites in middle Tennessee and northern Alabama where the typical glade habitat of *G. quartermaniae* occurs in close proximity to habitats preferred by *G. neglecta*. Each of these sites is located within ca. 500 m of a cedar glade or glade-like area. Plants at these sites appeared to belong either to *G. quartermaniae* or to *G. neglecta* with no obvious hybrids observed at most sites. One specimen (*Kral 52812* VDB, MO) collected from a seep over limestone in Cannon County, Tennessee appears to be typical *G. quartermaniae* in general morphology and habitat; however, the middle portion of the stems on this specimen are slightly pubescent and more typical of *G. neglecta* (Fig. 3.1 C). It is possible that this specimen represents a hybrid between *G. neglecta* and *G. quartermaniae.* Although *G. neglecta* was not found on any cedar glades in middle Tennessee or northern Alabama, the species has been collected from a variety of rock outcrop types elsewhere where it exhibits morphological features typical of non-outcrop populations. *Gratiola floridana* is sympatric with both *G. quartermaniae* and *G. neglecta* in northern Alabama's Moulton Valley (Lawrence and Morgan counties). Although these three species have been found within 1 km of each other, sites supporting all three species are unknown. *Gratiola floridana* and *G. quartermaniae* occur syntopically at one site in Lawrence County, Alabama (*Whetstone et al. 16471* JSU, mixed collection of *G. floridana* and *G. quartermaniae*). *Gratiola floridana* usually inhabits shaded muddy sites in forested bottoms or ravines but in northern Alabama it rarely occurs in habitats more typical of *G. quartermaniae*. No obvious hybrids between *G. floridana* and *G. quartermaniae* or between *G. floridana* and *G. neglecta* have been discovered.

The range of *G. graniticola* lies near the southern edge of the range of *G. neglecta* and the two species overlap only in northeastern Georgia (Elbert and Greene counties). Although the Greene County specimen of *G. neglecta* (*Allison 2630* GA) was collected from a granite outcrop, the two species have never been observed growing syntopically and no putative hybrids have been found. A disjunct population of *G. graniticola* reportedly occurs on a granite outcrop in Lancaster County, South Carolina (J. Allison, Georgia Natural Heritage Program, pers. comm.), but specimens needed to confirm this report have not been located.

KEY TO THE SPECIES OF THE *GRATIOLA NEGLECTA* COMPLEX

1. Flowers 13–25 mm long, adaxial surface of the corolla lobes pilose; proximal fruiting pedicels (20) 23–43 (55) mm long; seeds (0.6) 0.79–0.9 mm long, trichomes short stalked, the stalks approximately equaling or shorter than the glandular head ____ ***G. floridana***

1. Flowers 5–14 mm long, adaxial surface of the corolla lobes glabrous; proximal fruiting pedicels (5) 12–25 (37) mm long; seeds (0.3) 0.4–0.6 (0.7) mm long, trichome stalks ≥ 1.5 times as long as the glandular head.

    2. Mid-stem leaves (11) 20–41 (66) mm long; proximal fruiting pedicels (8) 13–25 (37) mm long, (0.3) 0.5–1 (1.6) times as long as the subtending bracteal leaves; bracteoles slightly longer to conspicuously longer than the sepals; posterior corolla lobe white (rarely inconspicuously tinged with pink or lavender); beard inside corolla orifice of yellow trichomes; mature capsules ovoid, brown; seeds (0.4) 0.5–0.6 (0.7) mm long and (0.18) 0.21–0.29 (0.37) mm thick, trichomes slender-based.

        3. Leaves narrowly elliptic or rhombic to oblanceolate, not conspicuously falcate, (2.7) 5–11 (18) mm wide at widest point; length to width ratio (2.5) 3.5–5 (6), each margin with (1) 3–5 (7) often conspicuous teeth, primary veins 3–5 (7); mid-stem moderately to densely glandular pubescent (rarely glabrate), seeds (0.18) 0.22–0.26 (0.29) mm thick ____ ***G. neglecta***

        3. Leaves linear, linear-lanceolate, to elliptic-lanceolate, often falcate, (1) 2.5–4 (4.5) mm wide at widest point, length to width ratio (5.5) 6–9.5 (11), entire or each margin with 1–2 (3) inconspicuous teeth, primary vein 1 (–3); mid-stem glabrous, seeds (0.19) 0.26–0.32 (0.37) mm thick ____________________________________ ***G. quartermaniae***

    2. Mid-stem leaves (6) 7–13 (18) mm long; proximal fruiting pedicels (5) 7–17 (22) mm long, (0.9) 1–2 (2.3) times as long as the subtending bracteal leaves; bracteoles shorter than to barely exceeding sepals; posterior corolla lobe conspicuously tinged with pink or purple; beard inside corolla orifice of white trichomes; mature capsules subglobose, purplish; seeds (0.3) 0.36–0.42 (0.5) mm long and (0.17) 0.20–0.24 (0.27) mm thick, trichomes bulbous based ____ ***G. graniticola***

TAXONOMIC TREATMENT

Gratiola neglecta Torrey (**Fig. 3.7**), Catal. Pl. New York. 10, 89. 1819. TYPE: [no locality data on specimen, but as noted by Stuckey (1979) this specimen was donated to the Schweinitz herbarium by John Torrey. Torrey (1819) gives the locality as "Inundated and moist places, New York."], [no collection date provided on sheet or in Torrey (1819)], [collector not specified on sheet but Pennell (1935) noted "it is almost certainly a plant of Torrey's collecting…"]. (LECTOTYPE: here designated, PH!; ISOLECTOTYPE: here designated, K-digital image!).

Conobea borealis Spreng., Neue Entdeck 3: 26. 1822.
Gratiola missouriana Beck, Amer. Jour. Sci. 10: 258. 1826.
Gratiola odorata Raf., Autik. Bot. 43. 1840.
Gratiola heterophylla Raf., Autik. Bot. 43. 1840.
Gratiola gracilis Benth., Prod. Syst. Nat. Regn. Veg. 10: 402. 1846.
Gratiola officinalis Michx. *ß caroliniensis* Pers., Syn. Plant. 1: 14. 1850.
Gratiola lutea Raf. var. *glaberrima* Fernald, Rhodora 34: 149. 1932. *Gratiola neglecta* Torr. var. *glaberrima* (Fernald) Fernald, Rhodora 51: 84. 1949.

Plants annual, solitary, erect herbs, (10) 16–27 (33) cm tall. **Roots** simple, fleshy, whitish with numerous rootlets. **Stems** erect, somewhat fleshy, simple or with few to many spreading-ascending branches, terete or slightly rounded-quadrangular in cross section, (0.8) 1.2–2.2 (2.9) mm in diameter at midstem; with (6) 7–10 (12) leafy nodes, mid-stem internodes (17) 28–45 (48) mm long, basal internodes not conspicuously shortened; stem green, usually densely short glandular-pubescent from below middle to apex, becoming glabrate near the base or rarely glabrate throughout, trichomes spreading, translucent, slender-based and gland-tipped. **Leaves** simple, oppositely-decussate, narrowly elliptic or rhombic to oblanceolate, or uncommonly falcate, spreading, 3–5 (7) veined, thin, mid-cauline blades (11) 24–44 (66) mm long and (3) 5–11 (18) mm wide, (2.5) 3.5–5 (6) times longer than wide, median leaves usually largest decreasing in size toward base and apex, apex acute, widest at or just distal to the midpoint, margins with (1) 2–5 (7) remotely spaced low and inconspicuous to sharp and evident teeth per margin, base acuminate and sessile or slightly clasping; blades green, glabrate to moderately glandular pubescent. **Flowers** solitary in axils of upper median and distal bracteal leaves, erect to spreading, zygomorphic, perfect; pedicels slender, ascending to divergent, (10) 12–30 (37) cm long, (0.27) 0.44–0.94 (1.33) times as long as the bracteal leaves, densely to sparsely pubescent with slender-based gland-tipped trichomes. **Bracteoles** 2, paired, closely subtending the calyx, lanceolate, narrowly elliptic, to oblanceolate, sometimes falcate, apex narrowly obtuse to acute, margins with 1–2 inconspicuous teeth, bases straight or tapering, longitudinally 3-nerved, in flower 2.5–7 mm long and 0.5–1 mm wide, enlarging as fruit matures and becoming foliose and up to 15 mm long and 2 mm wide, thin, green or minutely purple-tipped, sparsely to densely covered with slender-based gland-tipped trichomes on both surfaces. **Calyx** irregularly campanulate with 5 subequal, distinct, lanceolate, longitudinally 3-veined, slightly fleshy, green, (2.2) 3–4.4 (5) mm long and ca. 0.5 mm wide, apex narrowly obtuse, margins entire, sparsely to densely covered with gland-tipped trichomes. **Corolla** tubular, gamopetalous, zygomorphic, slightly marcescent, 6.5–12 mm long; corolla tube quadrangular, dorsal surface with a prominent hump near midpoint, the ventral surface canaliculate, to 9 mm long, to 2.1 mm in diameter proximal to dorsal hump, pale yellow, yellowish-cream, or yellowish-green, with many dichotomously forking brownish-violet lines extending from the tube base to the base of the corolla lobes, sparsely to moderately glandular pubescent pubescent externally with slender gland-tipped trichomes, inner surface near orifice at base of posterior corolla lobe with moderate to dense beard of clavately thickened yellow trichomes, proximal and median inner corolla tube pilose with eglandular trichomes; corolla lobes 5, generally slightly broader than high and obtuse to emarginated, spreading, white, 1.7–2.5 mm long and 3.2–3.8 mm wide; the posterior lobe generally largest, the two lateral lobes and lower lobe equal or slightly smaller, adaxial surfaces glabrous, abaxial surfaces glabrous or slightly glandular pubescent. **Stamens** 2, inserted near the middle of dorsal surface of the corolla tube, filaments to 1.2 mm long, anthers transversely oriented to the filaments, 0.6–0.8 mm long and 0.4–0.6 mm wide, connective greatly dilated around the two anther sacs, whitish; staminodes inserted ca. 1.5 mm above base of corolla or absent, when present ca. 0.3 mm long and not capitate. **Gynoecium** 5.3–6.8 mm long, subtended at the base by an orange nectary ring, ovary 1.4–3.3 mm long and to 2.1 mm in diameter, style 3.0–3.9 mm, stigma 2-lobed, dilated and flattened, ca. 0.7 mm long. **Capsules** ovoid, apex acute to obtuse, usually widest below the middle, (2.6) 3.6–5 (6) mm long, 3–5 mm in diameter, brown at maturity. **Seeds** several hundred per capsule, brownish-yellow, 10–13 ribbed, longitudinal ridges more conspicuous than the transverse ridges, asymmetrically ovoid to cylindric, often oblique at one end, reticulate with rectangular alveolae, alveolae covered by a thin iridescent membrane, (0.42) 0.48–0.60 (0.70) mm long and (0.18) 0.22–0.26 (0.29) mm in diameter, (1.7) 2–2.6 (3) times longer than wide. **Chromosome number**: 2N=18 (Gervais et al. 1999).

Phenology—Flowering and fruiting from March to October

Common Name— Clammy hedge-hyssop

Specimens Measured—CANADA. Ontario: Thunder Bay District, 8 km SW of Thunder Bay City, 17 Aug 1978, *Garton 18549* (ISC*). **Quebec**: Montmorency Co., Ange-Gardien, 23 Jul 1963, *Cinq-Mers et al. 69-169* (UC*). Portneuf Co., Portneuf, 7 Jul

1941, *Rouleau 1045* (PH*). **Saskatchewan**: 8 mi E of Saskatoon, 6 Jul 1950, *Ledginham 890* (SMU*).

U.S.A. Alabama: Greene Co., Smith Lake (swamp) ca. 14 mi due WNW of Eutaw, 1 May 1980, *Haynes 7775* (UNA*). Limestone Co., Beaverdam Creek 0.01 mi N of US Hwy 72 / Alt. 20 bridge, Wheeler Wildlife Refuge, 20 May 1980, *Meigs 555* (UNA*). **Arizona**: Apache Co., River Reservoir, Greer Lakes, 1.4 mi E of AZ Hwy 373, 2 airmiles NE of Greer, and 9 airmiles W of Eagar, 30 Aug 1988, *Ricketson & Raechal 4415* (MO*). **Arkansas**: Union Co., El Dorado, 3 May 1940, *Demaree 22048* (PH*†). **Connecticut**: Hartford Co., Suffield, 20 Jun 1923, *Weatherby s.n.* (NCSC*). **Delaware**: New Castle Co., 0.5 mi W of Glasgow, 15 Jun 1929, *Benner 3572* (PH*). **Georgia**: Bartow Co., Big Belfry Pond, 4.8 mi E of Adairsville, 5 May 1951, *Duncan 12316* (US*). Walker Co., Chickamauga, 16 May 1900, *Biltmore 3913a* (US*). **Illinois**: Johnson Co., Ferne Clyffe State Park; floodplain of Buck Branch, 21 May 1992, *Mibb 692* (NLU*). McHenry Co., McHenry, 15 Jun 1925, *Benke 4083* (US*). **Indiana**: Vanderburgh Co., 0.5 mi S of Staser, 26 May 1926, *Deam 42953* (PH*). **Kansas**: Cherokee Co., 0.5 mi W of Crestline, 6 Jun 1970, *Magrath 5352* (VDB*†). Greenwood Co., T28S, R13E, sec 9, edge of temporary pool of valley in scrub oak woodland, 13 May 1987, *McGregor 38094* (GA*). **Kentucky**: Warren Co., along Warren Co. Rt. 1288, ca. 1 mi from intersection with Warren Co. 961, 5 Jun 1968, *Nicely 1666* (NCSC*). **Louisiana**: Richland Parish, beside south side of I-20E about 1.7 mi W of the Rayville Exit (La. 137), 8 May 1990, *Thomas 115,966* (TENN†). **Massachusetts**: Berkshire Co., Mount Washington, 25 Aug 1923, *Meredith s.n.* (PH*). Worcester Co., Boylston, 24 Jun 1962, *Richardson s.n.* (MO*). **Minnesota**: Carlton Co., between Holyoke and Foxboro, 4 Jul 1942, *Lakela 4986* (SMU*). **Mississippi**: Carroll Co., field beside MS 7, at Avalon, 17 May 1973, *Thomas & Marx 34783* (SMU*†). Washington Co., ca. 3.5 mi NE Leland, 12 May 1988, *Bryson 7637* (VPI*). **Missouri:** Pulaski Co., Falls Hollow Sandstone Glade, Ft. Leonard Wood, 13 May 1994, *Hays 434* (MO*). **Montana:** Lake Co., 4 mi S and 2 mi W of Ronan, 8 Jul 1956, *Harvey 6517* (NCU*). **Nevada**: Elko Co., 0.8 road mi E of Deeth on the road to O'Neil Basin, backwaters of the Marys River, 4 Jul 1986, *Tiehm 10727* (BRIT*). **New Jersey**: Cumberland Co., Maurice River W of Bricksboro, 3 Jun 1934, *Long 43311* (PH*). **New Mexico**: Rio Arriba Co., vicinity of Chama, 9 Jul 1911, *Standley 6659* (US*). **New York**: Clinton Co., Rouses Point, 7 Aug 1910, *Williamson s.n.* (PH*). Monroe Co., near Rochester, 4 Jul 1913, *Baxter s.n.* (MO*). **North Carolina**: Cabarrus Co., Rocky River at NC Rd. 73, 25 May 1969, *Daggy 5478* (TENN†). Caswell Co., by Hyco Creek SE of Hightowers, 22 May 1958, *Bell 11947* (NCU*). Chatham Co., 3 mi W of Mann's Chapel on Co. Rd. 1536, 22 May 1974, *Massey & Levesque 3988* (NCU*). **North Dakota**: Cass Co., Harwood, 30 Jun 1937, *Stevens 246* (GA*); 7 mi W of Enderlin, 28 Aug 1968, *Barker 5213* (MO†). Richland Co., Wyndmere, 18 Jun 1965, *Stevens 2775* (US*). **Ohio**: Champaign Co., Thackery, 11

Jun 1914, *Leonard s.n.* (US*). Crawford Co., ca. 1.5 mi NW of Lykens, 30 Sep 1979, *Stuckey 9962* (PH*). Erie Co., W of Ceylon, Berlin Township, 15 Jul 1973, *Jones 73-7-15-802* (TENN†). **Oklahoma**: Le Flore Co., along Poteau River, near Howe, 25 May 1931, *Palmer 39340* (MO*). McCurtain Co., near Harris, about 2 mi. N of the Red River, 20 Apr 1946, *Nelson, Nelson, & Goodman 5579* (TEX*). **Oregon**: Crook Co., Farewell Bend, 17 Jul 1894, *Leiberg 456* (US*). **Pennsylvania**: Chester Co., French Creek near Hallman, 25 Jun 1927, *Stone s.n.* (PH*). **South Dakota**: Brookings Co., T112N R52W S32 SW4 SW4, restored prairie pothole wetland, 15 Jul 1991, *Galatowitsch s.n.* (ISC*). Custer Co., Custer, 25 Jul 1892, *Rydberg 924* (US*). **Tennessee**: Gibson Co., floodplain of North Fork of Forked Deer River near jct. with Hwy 104, 6 Jul 1979, *Boom, Whitten, and Wofford 529* (TENN†). Giles Co., NW side of Ardmore, north of Hwy 7 along north side of Austin Witt Rd. E of intersection of Austin Witt Rd. and Union Hill Church Rd., 5 May 2001, *Estes 02059* (TENN*). Hardin Co., side of Pittsburgh Landing Rd., S of Walker Branch, 18 May 1989, *Guthrie & Tennesen 2235* (NCU*). Weakley Co., E side of TN 89 along floodplain of Cane Creek, ca. 1.5 mi N of Palmerville, 25 May 1981, *Webb 3919a* (VDB*). **Texas**: Franklin Co., 3 mi E of Mount Vernon, off US 67, 3 May 1945, *Lundell 13701* (LL*). Jasper Co., 9.3 mi NE of Burkeville, 14 Apr 1960, *Shinners 27909* (SMU*). **Virginia**: Giles Co., Flat Top Mtn. near the upper end of Pearis Thompson Branch, NE of Holly Brook, 7 Aug 1990, *Wieboldt 7368* (NCU*). Warren Co., Waterlick, 19 Jun 1924, *Pennell 12113* (US*). **Washington**: Klickitat Co., Lyle, small shoal in Columbia River on the east side of the mouth of the Klickitat River, 26 Aug 1993, *Halse 4697* (K*). Spokane Co., margin of Newman Lake, 2 Jul 1927, *St. John 8811* (MO*). Whitman Co., wet pond beds, Pullman, 1 Aug 1896, *Elmer 163* (US†). **West Virginia**: Tucker Co., 0.25 mi S of Burley's Camp, Cabin Mtn. Range, 8 Jul 1941, *Allard 9055* (US*†). Wetzel Co., near Littleton, 1 Jul 1961, *Haught 7127* (BRIT*). **Wisconsin**: Lincoln Co., Tomahawk Twp., 18 Jul 1950, *Seymour 11687* (MO*). Taylor Co., near Rib River, 22 Jun 1957, *Schlising 648* (UC*).

Gratiola quartermaniae D. Estes., sp. nov. (**Fig. 3.8)**. TYPE: CANADA. ONTARIO. Hastings Co., Tyendinaga Township, "Toddary" alvar, Daley Road, ca. 7.5 km N of Lonsdale, 44.3404 N, 77.14539 W, moist open areas on alvar, with *Eleocharis compressa*, *Rumex crispus*, *Eleocharis obtusa*, 22 Jun 2006, *Oldham, Norris, & Van Sleeuwen 32809* (HOLOTYPE: TENN; ISOTYPES: BRIT, CAN, DAO, NHIC, NY, MO).

Gratiola quartermaniae a G. neglecta Torr. differt herba magis sparsim pubescente; caulibus plerumque simplicibus vel infrequenter ramosis, ad medium glabris; foliis angustioribus falcatis, lineari-lanceolatis vel elliptico-lanceolatis, marginibus integris *vel inconspicue dentata, laminis plerumque uni- vel trinervis; seminis parum longioribus crassioribusque, magis fusce brunneis.*

Gratiola quartermaniae differs from G. neglecta in herbage more sparsely pubescent; stems mostly simple to infrequently branched, glabrous at middle; leaves narrower and falcate, linear-lanceolate to elliptic-lanceolate; margins entire or inconspicuously toothed; blades mostly uni- or trinerved; seeds slightly longer and thicker, darker brown.

Plants annual, solitary, erect herbs, (6) 11–22 (30) cm tall. **Roots** simple, fleshy, whitish with numerous rootlets. **Stems** erect, fleshy, simple or with few ascending branches, terete or slightly rounded-quadrangular in cross section, (0.6) 1–1.9 (2.3) mm in diameter at midstem; with 7–10 (11) leafy nodes, mid-stem internodes (12) 19–35 (38) mm long, basal internodes shortened, 1–7 mm long; green or suffused with reddish or reddish-pink pigments, especially near the base or upper nodes; glabrous or nearly so from the base to above the middle, becoming sparsely glandular pubescent among the upper flower-bearing nodes with spreading, translucent, slender-based gland-tipped trichomes. **Leaves** simple, oppositely-decussate, similar in shape but gradually reduced in size from base to apex, lowermost often congested due to the shortened internodes and sometimes early deciduous, linear, linear-lanceolate to elliptic-lanceolate, often falcate, spreading or ascending, mostly with one evident main vein, sometimes trinerved with two short secondary veins, rarely the two secondary veins well-developed, slightly fleshy-thickened, mid-cauline blades (16) 18–32 (43) mm long and (1) 2.5–4 (4.5) mm wide, (5.5) 6–9.5 (11) times longer than wide, apex tapering to an acute or narrowly obtuse, widest near the middle, base sessile or slightly clasping; margins entire or each margin with 1–2 (3) remote, low, bluntly pointed teeth beyond the middle; blades green, the basal blades sometimes suffused with red; glabrous or nearly so. **Flowers** solitary in the axils of middle and upper bracteal leaves, erect to spreading, zygomorphic, perfect; pedicels slender, ascending to divergent, (8) 13–22 mm long, 0.5–1.1 (1.6) times as long as the subtending bracteal leaves, sparsely pubescent with slender-based gland-tipped trichomes. **Bracteoles** 2, paired, closely subtending the calyx, equaling or to 2.3 times longer than the sepals, linear-oblanceolate to linear-lanceolate and often falcate, one-nerved or inconspicuously trinerved with two small lateral nerves, in fresh material bracteoles often appearing nerveless, fleshy-thickened, in flower 2.8–8.2 mm long, lengthening in fruit to 11.8 mm long, 0.7–1.0 mm wide, apex obtuse, margins entire, surface green, sparsely to moderately covered with slender-based gland-tipped trichomes. **Calyx** irregularly campanulate with 5 subequal, distinct, lanceolate sepals, each inconspicuously longitudinally three-nerved, in fresh material appearing single-nerved or apparently nerveless, fleshy-thickened, green, 2.7–5.1 mm long and 0.7–1.0 mm wide, apex obtuse, margins entire, sparsely covered with slender-based gland-tipped trichomes. **Corolla** tubular-funnelform, gamopetalous, zygomorphic, slightly marcescent, 6–13.7 mm long; corolla tube quadrangular, dorsal surface with a prominent hump near midpoint, the ventral surface canaliculate, to 9.3 mm long, 1.3–2.7 mm in diameter proximal to dorsal hump, greenish-yellow, creamy yellow, or bright yellow, with many brownish-purple lines extending the length of the tube, sparsely pubescent externally with slender-based gland-tipped trichomes, inner surface near orifice at base of posterior corolla lobe with moderate to dense beard of clavately thickened yellow trichomes, proximal and median inner corolla surfaces pilose with eglandular trichomes up to 0.8 mm long; corolla lobes 5, generally slightly broader than high and emarginate, white, 2.0–3.6 mm long and 2.4–5.4 mm wide; the posterior lobe generally largest, the two lateral lobes and lower lobe equal or slightly smaller, adaxial surfaces glabrous, abaxial surfaces glabrous or slightly glandular pubescent. **Stamens 2**, inserted near the middle of the dorsal surface of the corolla tube, filaments 0.8–1.5 mm long, anthers transversely oriented to the filaments, 0.8–1.3 mm long and 0.5–0.9 mm wide, connective whitish and greatly dilated around the two anther sacs; staminodes inserted 1.4–1.8 mm above base of corolla or absent, when present to 0.3 mm long, not capitate. **Gynoecium** 5.9–7.6 mm long, subtended at the base by an orange nectary disc, ovary 1.6–4.3 mm long and 1–2.8 mm in diameter, style 3.1–4.6 mm, stigma 2-lobed, dilated and flattened, 0.6–0.9 mm long. **Capsules** ovoid, apex acute, usually widest below the middle, (3.4) 3.6–4.7 (5.1) mm long, 2.9–4.5 mm in diameter, brown at maturity. **Seeds** several hundred per capsule, grayish-brown to reddish-brown, 10–13 ribbed, longitudinal ridges more conspicuous than the transverse ridges, asymmetrically ovoid to oblong-cylindric, often oblique at one end, reticulate with rectangular alveolae, alveolae covered by a thin iridescent membrane, (0.43) 0.55–0.63 (0.71) mm long and (0.19) 0.26–0.32 (0.37) mm in diameter, (1.5) 1.8–2.3 (2.6) times longer than wide. **Chromosome** number unknown.

Phenology—Flowering and fruiting from April to early June in Alabama, Tennessee, Texas and from June to August in Illinois and Ontario, Canada

Etymology—This species is named in honor of Dr. Elsie Quarterman, retired Vanderbilt University plant ecologist, who has dedicated her career to the study of the ecology of the limestone cedar glades and the species that inhabit them.

Common Name—Quarterman’s hedge-hyssop; limestone hedge-hyssop

Conservation Status—*Gratiola quartermaniae* is most common in the limestone cedar glades of middle Tennessee where it is known from ca. 30 populations in nine counties. Although it appears to be secure in Tennessee, the mid-state area where this species occurs is one of the most rapidly developing regions in the southeastern U.S. and the once abundant glade habitat preferred by this species is increasingly being destroyed. Consequently, while *G. quartermaniae* is not sufficiently rare in Tennessee now to warrant state or federal conservation status, its populations should be monitored in the next few decades. In Alabama, Illinois, Texas, and Ontario this species appears to be quite rare and is restricted to small geographic areas. In these regions it should be afforded protection at the state or provincial level.

Representative Specimens—CANADA. Ontario: Hastings Co., Belleville, May 1861, *Macoun* [number illegible] (K); vicinity of Belleville, Jun 1867, *Macoun 17454* (CAN*); flats near the Iron Bridge at Belleville, Jun 1868, *Macoun 41730* (CAN); Belleville, 10 Jun 1871, *Macoun 123* (TRT); Belleville, 24 Jun 1871, *Macoun 1261* (DAO); Pt. Anne, Belleville, Ontario, 13 Jun 1972, *Morton 5091* (CAN, QK, TRT, WAT); Hungerford Township, Larkins Alvar, ca. 9.5 km SE of Tweed, ca. 1.5 km SW of Larkins, S of Marlbank Rd., 30 Jun 2006, *Oldham, Norris, & Van Sleeuwen 32877* (DAO, MICH, MO, NHIC, TENN, US); Richmond Township, Roblin Dump alvar, ca. 1.5 km SE of Roblin, ca. 9 km SSE of Marlbank, 30 Jun 2006, *Oldham, Norris, Sutherland & Van Sleeuwen 32869* (CAN, MICH, MO, NHIC, NY, TENN, TRTE, US, UWO). Lennox and Addington Co., Camden East Township, ca. 10 km NW of Newburgh, ca. 15 km N of Napanee, road to Roblin Hell Holes, off Centreville Road, 30 Jun 2006, *Oldham 32868* (BRIT, CAN, DAO, HAM, MICH, MO, MT, NHIC, TENN, US, VDB). Peterborough Co., alvar about 2 miles north of Nogies Creek in Harvey Tp., 11 Jul 1974, *Catling & McKay s.n.* (CAN, TRT); 1.79 air mi NE of Nogies Creek, 1.1 air mi NNW of jct. of Co. Rd. 36 and Quarry Rd., 0.37 road miles NW from jct. of Quarry Rd. and Ledge Rd., 18 Jun 2005, *Estes 07955* (CAN, DAO, NY, MICH, TENN, VDB). Prince Edward Co., Big Sand Bay, Long Point, 7 Jun 1963, *Brassard & Hainault 2702* (CAN*, TRT); about 2 miles SE of Milford, 5 Aug 1951, *Soper & Heimburger 5412* (TRT); South Marysburgh Township, Hilltop Rd., ca. 5 km SE of Milford, near South Bay, 19 Jun 2006, *Oldham 32786* (DAO, MICH, MO, NHIC, NY, TENN, TRT, US).

U.S.A. Alabama: Franklin Co., ca. 5-6 mi E of Russellville along N side of New Hwy 24, just W of jct. of New Hwy 24 and County Rd. 83, 15 May 2003, *Estes 04625* (TENN). Lawrence Co., by Ala. 36 ca. 2 mi. e. jct. Ala. 157, 6 May 1978, *Kral 61662* (JSU, VDB*); approximately 4 miles NW of Mt Hope, ca. 1.5-2 miles E of Franklin County line, W of Town Creek, at Prairie Grove Glades preserve, 15 May 2003, *Estes 04611* (TENN†); ca. 0.2 to 0.4 mi ESE of Landersville, south of junction of Hwy 24 and County Rd. 55, growing in wet ditch over limestone on west side of County Rd. 55, 34º28’09” N, 87º23’46”W, 29 Apr 2004, *Estes 05928* with *Webb* (CAN, MO, TENN, UNA). Morgan Co., 5.6 mi. W of Falkville, 23 Apr 1968, *Kral 30494 B* (GA, VDB*); seep in sandy clay field 1 mi E jct AL 157 by AL 36, W of Danville, 14 Apr 1978, *Kral*

61500 (JSU, MO, VDB); N side of Morgan Co Rd 55, 0.9 mi E of Massey (McKendree Church), 2.1 mi W of Lebanon Church and 6 mi W of int. US 31 at Falkville, 28 Apr 1989, *Orzell & Bridges 9380* (TEX*). **Illinois**: Will Co., Romeo, 18 Jun 1898, *Umbach s.n.* (US). **Tennessee**: Bedford Co., N side Deason by US 231, 28 Apr 1974, *Kral 52571* (MO, VDB); 0.2 mi N of US 41A at Rover along Bunker Hill Rd, 3 Jun 1993, *Kral 82558 with Rust* (VDB); approximately 5 miles NE of Unionville, ca. 0.75 mile S of Newtown, near intersection of Longview Rd. and Putnam Well Rd., on east side of Longview Rd., 22 May 2003, *Estes 04583 with Wofford et al.* (CAN, GH, TENN†). Cannon Co., by US 71S, 0.5 mi E of Readyville, 20 May 1974, *Kral 52812* (VDB*, MO); Coffee Co., Manchester prairie, 4 mi E of Manchester on US 41, 7 June 1966, *Baskin & Caudle 258* (VDB). Davidson Co., Hamilton Creek Recreation Area, SE side of Nashville, W of Percy Priest Lake, E side of Ned Shelton Rd., 15 June 2003, *Estes 04894* (EKY, GA, JSU, TENN*, UNA). Giles Co., S of Pulaski, Cedar Grove community, growing on W side of Hwy 166, south of Everly Branch and just N of Cedar Grove Church, 18 Apr 2003, *Estes 04454* (TENN*). Marshall Co., 2.1 mi ESE Pottsville on TN 99, 2 June 1969, *Kral 34776* (MO, VDB*); N side TN 99, just inside W county border, 14 May 1988, *Kral & Kral 74722* (VDB); approximately 4 miles NE of Chapel Hill near Beasley community, ca. 100-200 yards east of intersection of Hwy 99 and Beasley Rd., S side of Beasley Rd., 22 May 2003, *Estes 04582 with Wofford et al.* (GH, MO, NCU, NY, TENN†, TEX, UC). Maury Co., ca. 2 mi NW of Pottsville, 1.5 mi NE of jct of Hwy 412 and Rally Hill Rd., E side of Rally Hill Rd., 22 May 2003, *Estes 04672 with Wofford et al.* (TENN†). Rutherford Co., 10 mi. E Beech Grove along US 41, 9 Jun 1970, *Kral 26889* (FSU*, SMU, VDB, TENN); SE of Eagleville, 1 mile off S.R. 99, 28 May 1996, *Rust 66* (VDB*); WSW of Fosterville, ca. 2 mi W of US Hwy 231, 0.33 mi N of Squire Hall Rd., E side of Harrison Rd., 22 May 2003, *Estes 04586 with Wofford et al.* (NCU, TENN†, VDB); E of Murfreesboro, approximately 1 mi SE of Halls Hill Pike, S side of Factory Rd., Flat Rock Cedar Glade and Barrens State Natural Area, 22 May 2002, *Estes 03337* (TENN*); approximately 4 miles E of Murfeesboro on Hall Hill Pike, turn S onto Smith Hall Rd. (a dead-end road), E side of road, 22 May 2002, *Estes 03336* (TENN*); N Murfeesboro, ca. 1 mile W of intersection of E Northfield Blvd. and Hwy 96, 22 May 2003, *Estes 04574 with Wofford et al* (TENN*); approximately 4-5 miles E of Murfreesboro, W side of Factory Rd., Flatrock Cedar Glades/Barrens State Natural Area, 1 May 2003, *Walck s.n.* (TENN*); base of Garrett Knob, 29 May 2003, *Bailey & Lincicome s.n.* (TENN). Wilson Co., Lebanon, 2 Jun 1923, *Pennell 11377* (PH); Cedars of Lebanon State Forest and Natural Area, N of Moccasin Rd. / Proctor Trail, 8 May 2003, *Bailey s.n.* (TENN). **Texas**: Bell Co., 6 mi SE of Belton, *Wolff 2317* (SMU). Llano Co., Llano River east of Packsaddle Mountain, 4 May 1947, *Whitehouse 18477* (SMU, UC, US). Williamson Co., Round Rock, 24 March 1890, *Bodin s.n.* (PH, MIN-digital image); ca. 3.9 mi SSW of Liberty Hill, along CR 284, 1.3 mi W of jct CR 282, S side rd, 29 Apr 2005, *Turner & Turner 122* (BRIT, MO, TENN, TEX); southern part of co., just NW of Round Rock, FM 1431 at jct Sam Bass Rd., SE corner, 150 m S of FM 1431, 29 Apr 2005, *Turner & Turner 119* (BRIT, GH, MO, TENN, TEX).

Gratiola graniticola D. Estes, sp. nov. (**Fig. 3.9**). TYPE: U.S.A. GEORGIA. DeKalb Co.: Rock Chapel, GA hwy 124 at Rock Chapel County Park, gneiss flatrock, W side of highway, vernal pools, 2 May 1984, *Allison 2101** (HOLOTYPE: GA).

Gratiola graniticola a G. neglecta Torr. differt herba trichomatibus brevioribus basi bulbosis vestita; caulibus gracilioribus, simplicibus vel infrequenter ramosis; foliis brevioribus angustioribusque, lanceolato-ovatis vel anguste oblongis, marginibus subintegris vel inconspicue dentatis, basibus magis valde amplectentibus; pedicellis folia bractealia subtendentia aequantibus vel eos duplo longioribus; bracteolis calycibus brevioribus vel eis vix superantibus; floribus minoribus lobis posterioribus purpurascentibus, barba in corollae orificio e trichomatibus albidis translucentibusve constante; capsulis minoribus, magis subglobosis purpura suffusis; seminibus minoribus magis obscure cinereis.

Gratiola graniticola differs from G. neglecta Torr. in herbage covered with shorter bulbous-based trichomes; stems more slender, simple or infrequently branched; leaves shorter and narrower, lanceolate-ovate to narrowly oblong, margins subentire or inconspicuously toothed, bases more strongly clasping; pedicels equaling the subtending bracteal leaves or two times longer than them; bracteoles shorter than to barely exceeding the calyces; flowers smaller with purplish posterior lobes, beard inside the corolla orifice of whitish or translucent trichomes; capsules smaller, more subglobose, and purple-tinged; seeds smaller and darker gray.

Plants annual, solitary, erect herbs, (7) 9–21 (29) cm tall. **Root** simple, fleshy, whitish with numerous rootlets. **Stems** erect, somewhat fleshy, simple or with few ascending branches, terete or slightly rounded-quadrangular in cross section, (0.7) 0.9–1.2 (1.5) mm in diameter at midstem; with (6) 7–10 (12) leafy nodes, mid-internodes (15) 17–30 (36) mm long, basal internodes shortened (1.5–8 mm); green or suffused with reddish or reddish-pink pigments, especially near the base and upper nodes; glabrous or glabrate near base becoming increasingly pubescent upward, with spreading, translucent, conical or bulbous-based, glandular trichomes. **Leaves** simple, oppositely decussate, similar in shape but gradually reduced in size from base to apex, lowermost often congested due to the shortened internodes and sometimes early deciduous, lanceolate-ovate to narrowly oblong usually widest at or below the middle, horizontally spreading with tips curved upward, with one evident main vein or trinerved with two short secondary veins, slightly fleshy-thickened, blades (6) 7–13 (18) mm long and 1–3 (5) mm wide, (2.8) 3.5–5.7 (7.4) times longer than wide, apex narrowly obtuse, margins entire or with 1–2 (3) pairs of remote, low, bluntly pointed teeth beyond the middle, base usually amplexicaulate; blades green or leaf tips, teeth, and basal leaves often suffused with reddish pigments; proximal leaves glabrate, median and distal leaves moderately pubescent with bulbous based trichomes. **Flowers** solitary in axils of upper bracteal leaves, erect to spreading, zygomorphic, perfect; pedicels slender, ascending, (5) 8–17 (22) mm long, (0.9) 1–2 (2.3) times as long as the subtending bracteal leaves, sparsely to moderately pubescent with bulbous based trichomes. **Bracteoles** 2, paired, closely subtending the calyx, usually shorter than or equaling the sepals, lanceolate and often falcate, longitudinally 3-nerved (sometimes single nerved) though not often evident when fresh, fleshy-thickened, 2–4.5 mm long and 0.5–1.0 mm wide, apex obtuse, margins entire, surface green, apex purple-tipped, abaxial surface convex, moderately covered on both surfaces with bulbous-based trichomes. **Calyx** irregularly campanulate with 5 subequal, distinct, lanceolate sepals, longitudinally 3-nerved (sometimes single nerved) though not often evident when fresh, fleshy-thickened, green on the surface with a minute purple tip, 2–4.2 mm long and 0.5–1.3 mm wide, apex obtuse, margins entire, moderately covered, especially abaxially, with conical or bulbous-based trichomes. **Corolla** tubular-funnelform, gamopetalous, slightly marcescent, and zygomorphic, 6.8–9.0 mm long; corolla tube quadrangular, dorsal surface with a hump near midpoint, 5.5–6.8 mm long and 1.3–1.9 mm in diameter, outer surface pale yellowish-green or cream-colored, often purplish or pinkish dorsally, faintly to conspicuously purple-lined exteriorly, scarcely pubescent with conical or bulbous-based trichomes, inner surface near orifice at base of posterior corolla lobe with sparse beard of clavately thickened whitish to translucent trichomes, proximal and middle inner corolla surfaces pilose with eglandular trichomes; corolla lobes 5, each usually broader than high and often emarginated at apex, spreading, the lower three white or cream-colored, the upper two strongly suffused with purple or pink, the lobes 1.0–1.7 mm high and 1.5–2.3 mm wide, adaxial surfaces glabrous, abaxial surfaces glabrous. **Stamens** 2, inserted near the middle of the dorsal surface of the corolla tube, filaments to 1.2 mm long, anthers transversely oriented to the filament, 0.5–0.7 mm long and 0.5–0.6 mm wide, connective greatly dilated around the two anther sacs, whitish; staminodes inserted ca. 1–1.3 mm from base of corolla tube or absent, when present minute and ca. 0.2 mm long, not capitate. **Gynoecium** 4.4–4.8 mm long, subtended at the base by an orange nectary ring, ovary 1.6–2.0 mm long and 1.2–1.7 mm in diameter, style 1.9–2.2 mm long, stigma 2-lobed, dilated and flattened, 0.5–0.6 mm long. **Capsules** subglobose to slightly ovoid, (2.4) 2.8–3.6 mm long, 2.1–3.7 mm in diameter, purple tinged when mature. **Seeds** several hundred per capsule, brown to grayish-brown, 10–13 ribbed, longitudinal ridges more conspicuous than the transverse ridges, asymmetrically ovoid to short cylindric, often oblique at one end, surface reticulate with rectangular alveolae, alveolae covered by a thin iridescent membrane, (0.3) 0.36–0.42 (0.47) mm long and (0.17) 0.2–0.24 (0.27) mm wide, (1.3) 1.6–2.1 (2.5) times longer than wide. **Chromosome** number unknown.

Phenology—Flowering and fruiting from April to May

Etymology—The ephithet *graniticola* was chosen to reflect the granite flatrocks that this species inhabits.

Common Name—Granite hedge-hyssop

History of Taxon—*Gratiola graniticola* was apparently first collected in 1928 (*Wherry & Benedict s.n.* PH) from "pools on granite ledges" in Gwinnett County, Georgia. A decade later, Pyron and McVaugh (*2866* GA, PH), collected a specimen of *G. graniticola* from granitic areas in Oglethorpe County, Georgia. McVaugh sent a specimen of this Oglethorpe County collection to F.W. Pennell who wrote "your collection, with that of Wherry and Benedict…differ from *G. neglecta* Torr. by bracts shorter relative to pedicels, capsules smaller (3 mm long), upper corolla-lobes purple or purplish, and seeds smaller and grayer" (McVaugh 1943). He added that these specimens seemed to match his description and photograph of *G. gracilis* Benth., a species described by Bentham (1846) from Texas.

Bentham (1846) described *G. gracilis* Benth. from material collected by Drummond near Harrisburgh, Texas (near present-day Houston) in ca. 1834. Unfortunately, Drummond failed to note the habitat from which he collected the plants. A second specimen annotated by Pennell as *G. gracilis* was collected by Lindheimer (*43* MO) from nearby Galveston in ca. March (May?, illegible) 1842. Like Drummond, Lindheimer did not provide specific locality or habitat information. Despite being known only from herbarium specimens, *G. gracilis* was maintained as a species by Small (1903) and Pennell (1921). Later, Pennell (1935) reduced *G. gracilis* to synonymy with *G. neglecta* noting the characters Bentham used to distinguish *G. gracilis* from *G. neglecta* "are all variable features that occur without geographic correlation."

During this study, a photograph of the holotype of *G. gracilis* (*Drummond coll. 3, n. 284*, K) and an isotype (GH) were examined. As Pennell noted, these specimens do share some features with those plants from the Georgia granite outcrops, most notably in the length of the leaves and the ratio of the length of the pedicel and subtending bract. While of rare occurrence, *G. neglecta* can have relatively short leaves and bracteal leaves shorter than the pedicels (e.g. *Guthrie 1002* VDB, Lake Co., TN). The three *G. gracilis* specimens also differ from *G. graniticola* in that they lack purple coloration on the corollas and capsules, features diagnostic for *G. graniticola*. In terms of habit, the stems of the *G. gracilis* specimens are more branched like those of *G. neglecta* compared to those of *G. graniticola*, which are mostly simple. Lastly, *G. graniticola* is endemic to granite outcrops and has not been found in non-granitic areas. Since there are no granite outcrops in southeastern Texas, it is reasonable to assume that the plants collected by Drummond and Lindheimer likely came from a different habitat type. Based on the evidence presented above, we follow Pennell and recognize *G. gracilis* as a synonym of *G. neglecta.*

Conservation Status—*Gratiola graniticola* should be considered a rare species in Georgia due to the small number of populations and limited distribution.

Representative Specimens Examined—U.S.A. Georgia: Barrow Co., Winder, GA Hwy 81, roughly 0.25 mi S of junction with US Hwy 29, E side of highway, 30 Apr 1984, *Allison 2095* (GA*); same site, 19 May 2003, *Estes 04590 with Allison* (TENN*†). Butts Co., about 2.7 mi NNE of Jackson, GA Hwy 36, ca. 0.5 mi S of Cedar Rock Church, E side of highway, 13 May 1984, *Allison 2175* (GA*); same site, 10 Apr 2004, *Estes 05742* (TENN). Columbia Co., about 4.25 mi ESE of Appling, ca. 0.45 mi NNW of confluence of Little Kiokee Creek and Benton Branch, adjacent to Heggies Rock Preserve, 10 May 1987, *Allison 2842* (GA*). DeKalb Co., across from Rock Chapel Park, 4 mi N of railroad track in Lithonia, along State Hwy 124, 16 Apr 1978, *Patrick 592 with Wofford et al.* (TENN*); Lithonia, ca. 0.3 mi N of intersection of Interstate 20 and Hwy 124, NW side of the intersection of Hwy 124 and Conyers Street on small concealed granite outcrop, 10 Apr 2004, *Estes 05733* (TENN); same site as previous, 01 May 2004, *Estes 05954* (TENN*, MO, NY). Greene Co., 8.2 mi SSE of Greensboro, 5.8 mi W of White Plains, 2 May 1987, *Allison 2834* (GA*); ca. 9 mi SSE of Greensboro, ca. 1.5 mi SW of Mosquito Crossing, S side of Leach Flatrock Rd., 33.46738 N, 83.13214 W, 19 May 2003, *Estes 04585 with Allison* (NCU, TENN†). Gwinnett Co., 6 mi SW of Grayson, 3 May 1928, *Wherry & Benedict s.n.* (PH); 4.25 mi E of Snellville, 2.25 mi SSE of Grayson, Langley Rd., 0.34 mi by air NW of junction with US Hwy 78, E side of road, 13 Jun 1984, *Allison 2306* (GA). Hancock Co., 3.5 mi SE of Sparta, 11 May 1952, *Duncan 13533* (GA, digital image); ca. 1 mi or less NE of Sparta, 0.3 mi N of Hwy 16, 0.3 mi W of Twomile Creek, 33.29098 N, 82.95428 W, *Estes 04659 with Allison* (TENN*). Hart Co., 5.3 mi NNE of Vanna, 1.5 mi NNE of Goldmine, about 0.2 mi E of county road 141 at a point ca. 0.45 mi NW of junction with county road 140, 15 Apr 1986, *Allison 2625* (GA*); same site, 19 May 2003, *Estes 04588 with Allison* (NCU, TENN†). Newton Co., ca. 3 mi NE of Covington, ca. 1.25 mi NE of the intersection of Hwy 142 and Alcovy Rd., S side of Alcovy Rd., 19 May 2003, *Estes 04584 and Allison* (TENN†); same site, 10 Apr 2004; *Estes 05738* (TENN). Oglethorpe Co., Echols' Mill, May 1938, *Pyron & McVaugh 2866* (GA, PH); ca. 0.5 mi E of Echols' Mill, ca. 9.3 mi N

45 deg. of Lexington, 7 May 1978, *Treiber & Nesom 1518* (NCU*). Pike Co., 1.6 mi S of Hollonville on Concord Road, E side of road, 19 May 1984, *Allison 2254* (GA*); same site, 01 May 2004, *Estes 05953* (MO, NY, TENN). Upson Co., NE corner of county, about 0.4 mi S of Lamar County line and just E of Barnesville-Yatesville Rd., 18 May 1984, *Allison 2235* (GA*). Walton Co., 4.9 mi WNW of Walnut Grove, Ace Moon Road (county road 197), just S of junction with Sharon Church Road (county road 106), E side of road, 11 May 1984, *Allison 2141* (GA*); by GA 138, 1 mi. NE of Walnut Grove, 17 May 1989, *Kral 72517* (FSU, GH, VDB*).

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APPENDIX; CHAPTER 3

TABLE 3.1. Morphological characters measured for *Gratiola graniticola*, *G. neglecta*, and *G. quartermaniae* and their means ± standard deviations and ranges (parentheses). *N*=sample size.

Veg. Characters	***G. graniticola*** (*N*=15)	***G. neglecta*** (*N*=55)	***G. quartermaniae*** (*N*=17)
Stem height (cm)	14.8 ± 5.9 (7.4–29.4)	19.9 ± 5.5 (10.2–33.2)	16.5 ± 5.1 (5.8–29)
Stem diameter (mm)	1.2 ± 0.2 (0.7–1.4)	1.6 ± 0.5 (0.8–2.9)	1.4 ± 0.4 (0.6–2.3)
Leaf length (mm)	10 ± 2.8 (6.3–17.7)	30.8 ± 10.3 (11–66)	25.1 ± 7.0 (16–43)
Leaf width (mm)	2.1 ± 1.0 (1.1–5.2)	7.7 ± 2.6 (2.7–18)	3.3 ± 0.8 (1.8–4.5)
Leaf length/leaf width (ratio)	4.6 ± 1.0 (2.8–7.45)	4.1 ± 0.80 (2.6–6.1)	7.7 ± 1.8 (5.5–11.2)
No. teeth per leaf margin	1.0 ± 0.7 (0–3)	3.5 ± 1.2 (1–7)	1.2 ± 1.1 (0–3)
Proximal pedicel length (mm)	12.4 ± 4.9 (5.3–22)	20.6 ± 7.7 (10.5–37)	17 ± 4.2 (8–22)
Bract length (mm)	8.7 ± 2.2 (5.3–11.2)	28.8 ± 9.5 (11.5–66)	21.8 ± 6.6 (12.5–33)
Pedicel length/bract length (ratio)	1.5 ± 0.4 (0.9–2.3)	0.8 ± 0.3 (0.3–1.3)	0.8 ± 0.3 (0.5–1.6)

Table 3.1. continued.

Veg. Characters (cont.)	***G. graniticola*** (*N*=15)	***G. neglecta*** (*N*=55)	***G. quartermaniae*** (*N*=17)
Capsule length (mm)	3.2 ± 0.3 (2.9–3.6)	4.3 ± 0.6 (2.6–5.7)	4.1 ± 0.6 (3.4–5.1)

Seed Characters	***G. graniticola*** (*N=80*)	***G. neglecta*** (*N=200*)	***G. quartermaniae*** (*N=100*)
Seed length (mm)	0.40 ± 0.03 (0.31–0.47)	0.54 ± 0.06 (0.42–0.7)	0.59 ± 0.04 (0.43–0.71)
Seed diameter (mm)	0.22 ± 0.02 (0.17–0.27)	0.24 ± 0.02 (0.18–0.29)	0.29 ± 0.03 (0.19–0.37)
Seed length/seed width (ratio)	1.86 ± 0.24 (1.32–2.53)	2.3 ± 0.27 (1.67–3.03)	2.05 ± 0.27 (1.47–2.6)

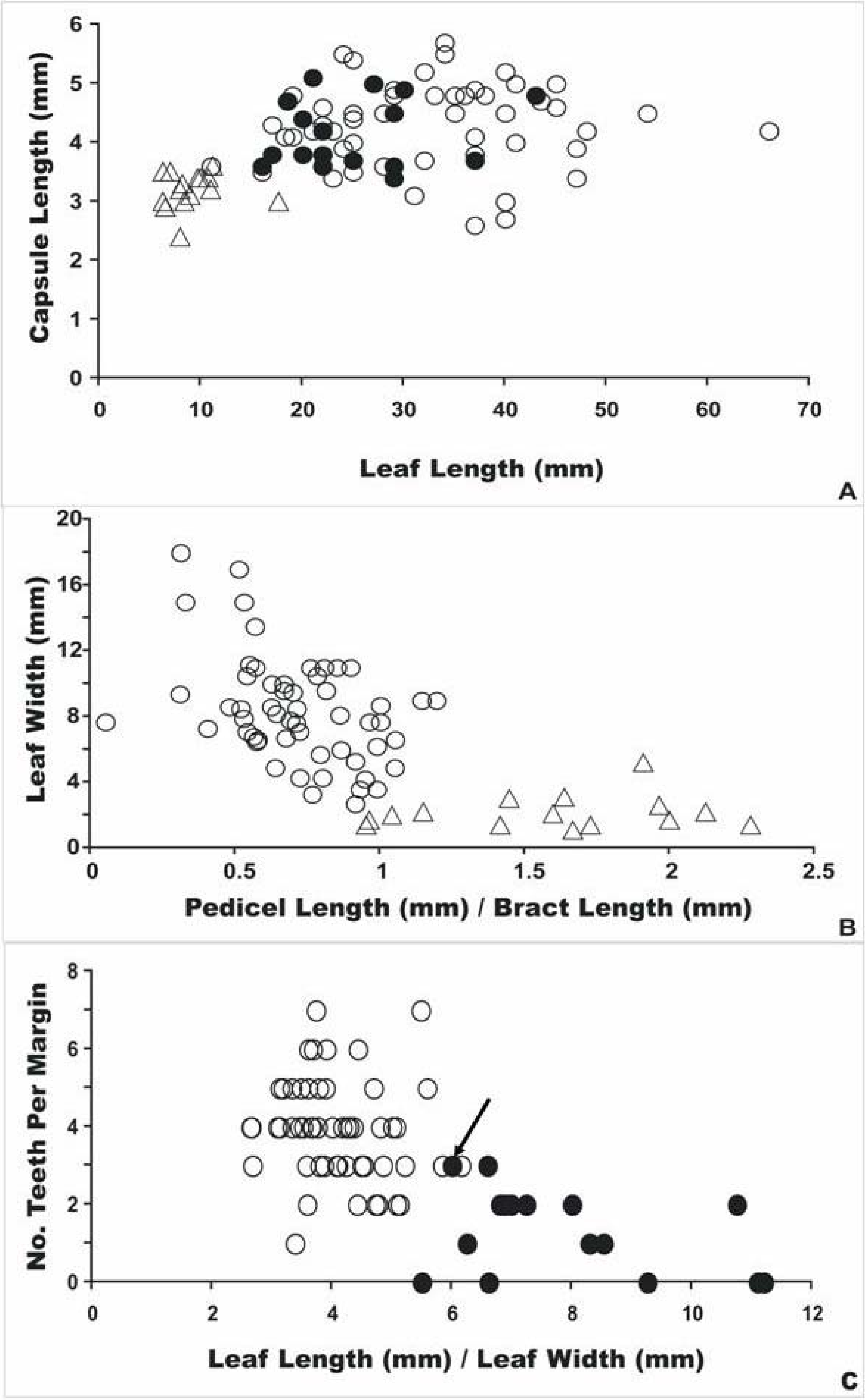
Table 3.2. Qualitative morphological characters useful for distinguishing *G. graniticola*, *G. neglecta*, and *G. quartermaniae*.

Character	***G. graniticola***	***G. neglecta***	***G. quartermaniae***
Stems	simple–rarely branched	branched–rarely simple	simple–rarely branched
Leaf shape	lanceolate-ovate to narrowly oblong	narrowly elliptic, rhombic, or oblanceolate	linear, linear-lanceolate, or elliptic-lanceolate
Basal leaf disposition	± congested	not congested	± congested
Widest point of leaf	middle or below middle	middle or beyond middle	middle
Proximal bract to pedicel ratio	bract < pedicel	bract ≥ pedicel	bract ≥ pedicel
Mid-stem vestiture	glandular-pubescent	glandular-pubescent	glabrous
Trichome shape	bulbous-based	slender-based	slender-based
Ratio bracteole length/calyx length	bracteoles ≤ to slightly exceeding calyx	bracteoles ≥ calyx	bracteoles ≥ calyx
Posterior corolla lobe color	purplish or pinkish	white (rarely pinkish)	white (rarely pinkish)

Table 3.2. continued.

Character	***G. graniticola***	***G. neglecta***	***G. quartermaniae***
Beard color	white	yellow	yellow
Capsule shape	subglobose	ovoid	ovoid
Capsule color	purplish	brown	brown
Seed color	grayish-brown	yellowish-brown	grayish-brown
Habitat	granite outcrops	various wetland types, rarely on outcrops	limestone/dolomite outcrops, calcareous prairies

Fig. 3.1. Scatter plots of leaf length vs. capsule length (**A**), proximal pedicel length/subtending bract length vs. leaf width (**B**), leaf length/leaf width vs. number of teeth per margin (**C**) for *G. graniticola* (open triangles), *G. neglecta* (open circles), and/or *G. quartermaniae* (closed circles). Note that open circles in panel **C** represent specimens with pubescent mid-stems, and solid circles represent plants with glabrous mid-stems with one exception; the solid circle marked with an arrow has features typical of *G. quartermaniae* except for having a pubescent mid-stem. This specimen (*Kral 52812*, VDB, Cannon Co., TN) was collected from a seep over limestone at the edge of the range of *G. quartermaniae* and may represent a hybrid.


Capsule Length (mm)
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
Leaf Length (mm)
A
Leaf Width (mm)
20
16
12
8
4
0
0
0.5
1
1.5
2
2.5
Pedicel Length (mm) / Bract Length (mm)
B
No. Teeth Per Margin
8
6
4
2
0
0
2
4
6
8
10
12
Leaf Length (mm) / Leaf Width (mm)
C

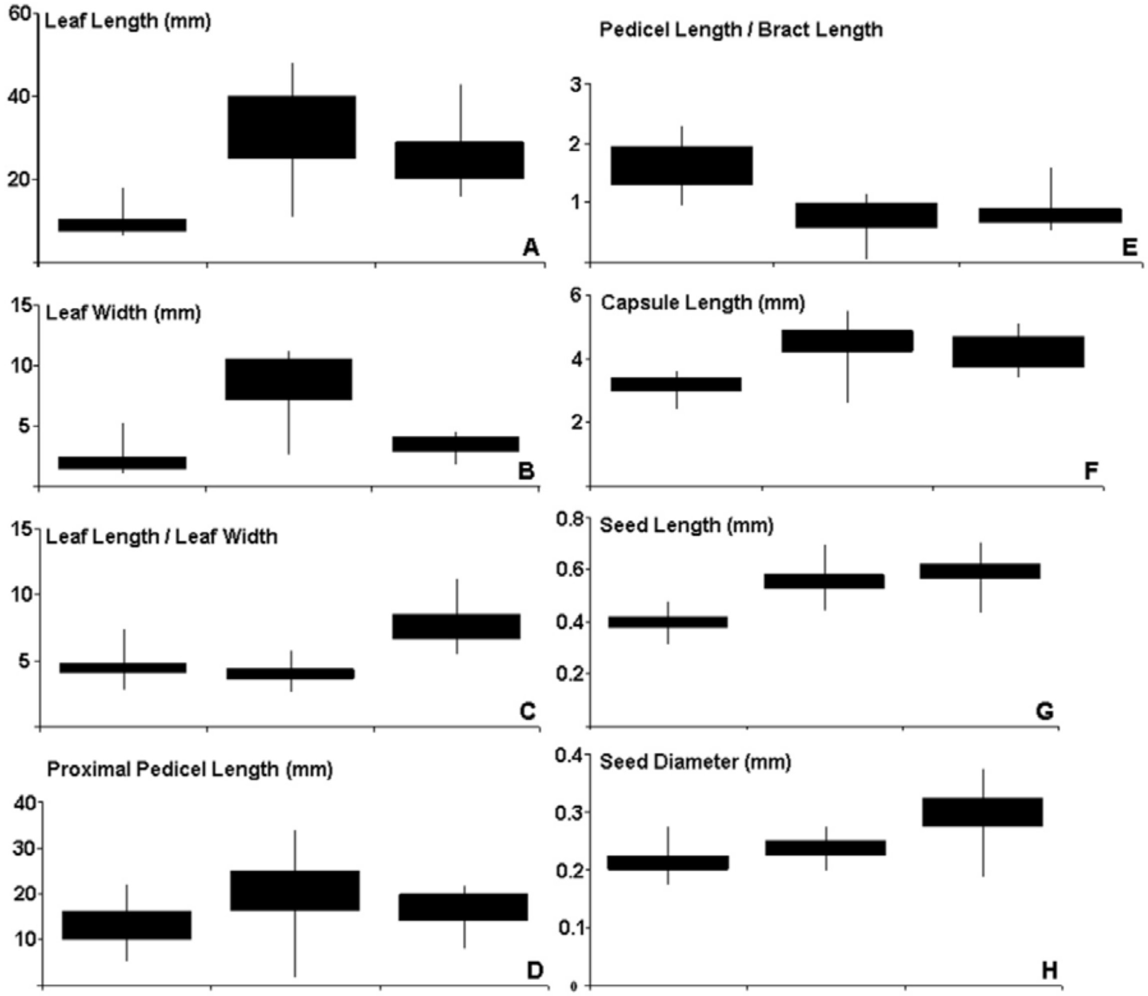


Fig. 3.2. Graphical comparison of eight selected characters for *Gratiola graniticola* (left), *G. neglecta* (center), and *G. quartermaniae* (right): leaf length (**A**), leaf width (**B**), ratio of leaf length to leaf width (**C**), proximal pedicel length (**D**), ratio of proximal pedicel length to subtending bract length (**E**), capsule length (**F**), seed length (**G**), and seed diameter (**H**).

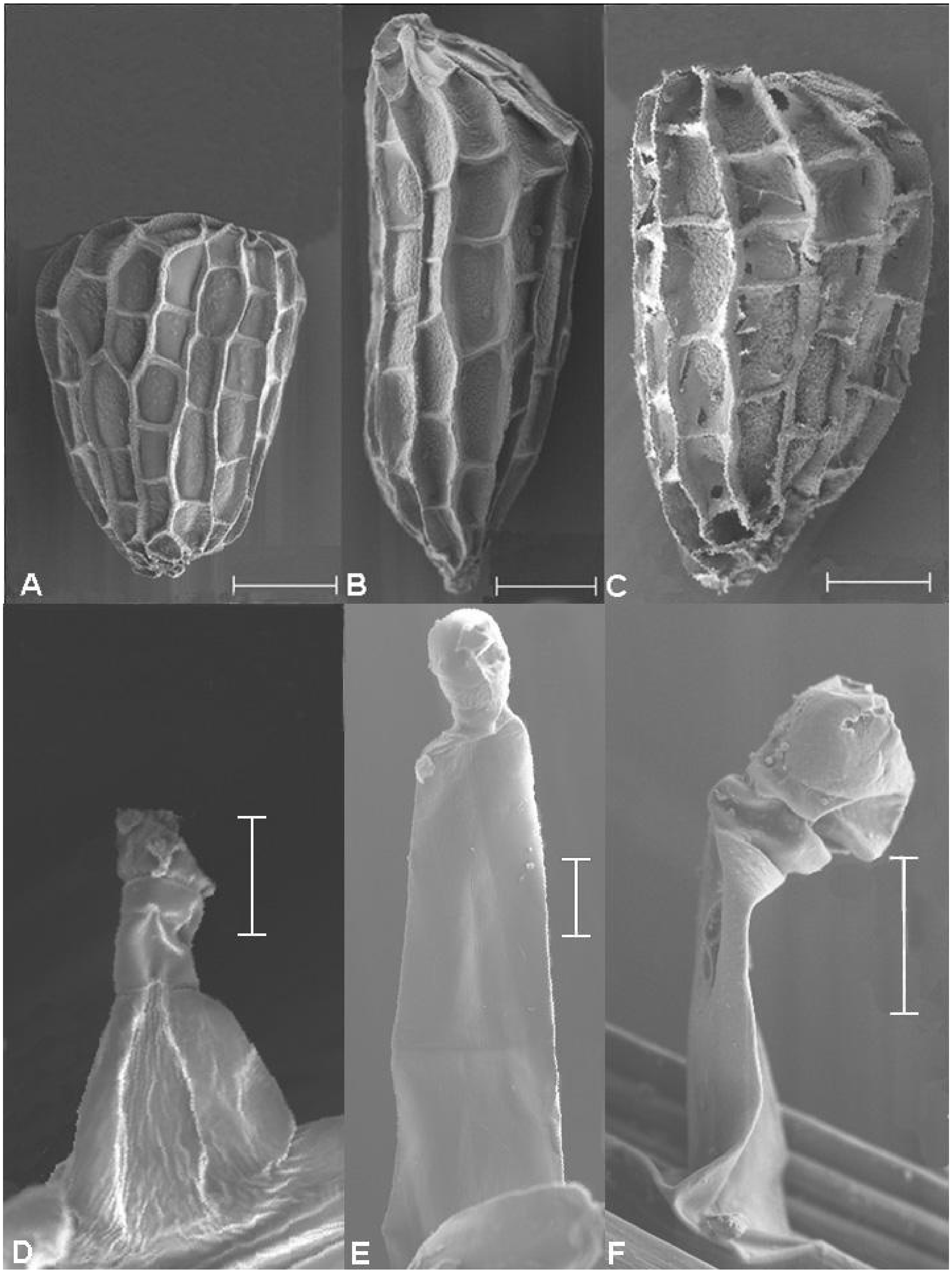


Fig. 3.3. Seeds of *Gratiola graniticola* (**A)**, *G. neglecta* (**B)**, and *G. quartermaniae* (**C)**; scale bars = 100 μm. Trichomes of *G. graniticola* (**D**), *G. neglecta* (**E**), and *G. quartermaniae* (**F**); scale bars = 20 μm.

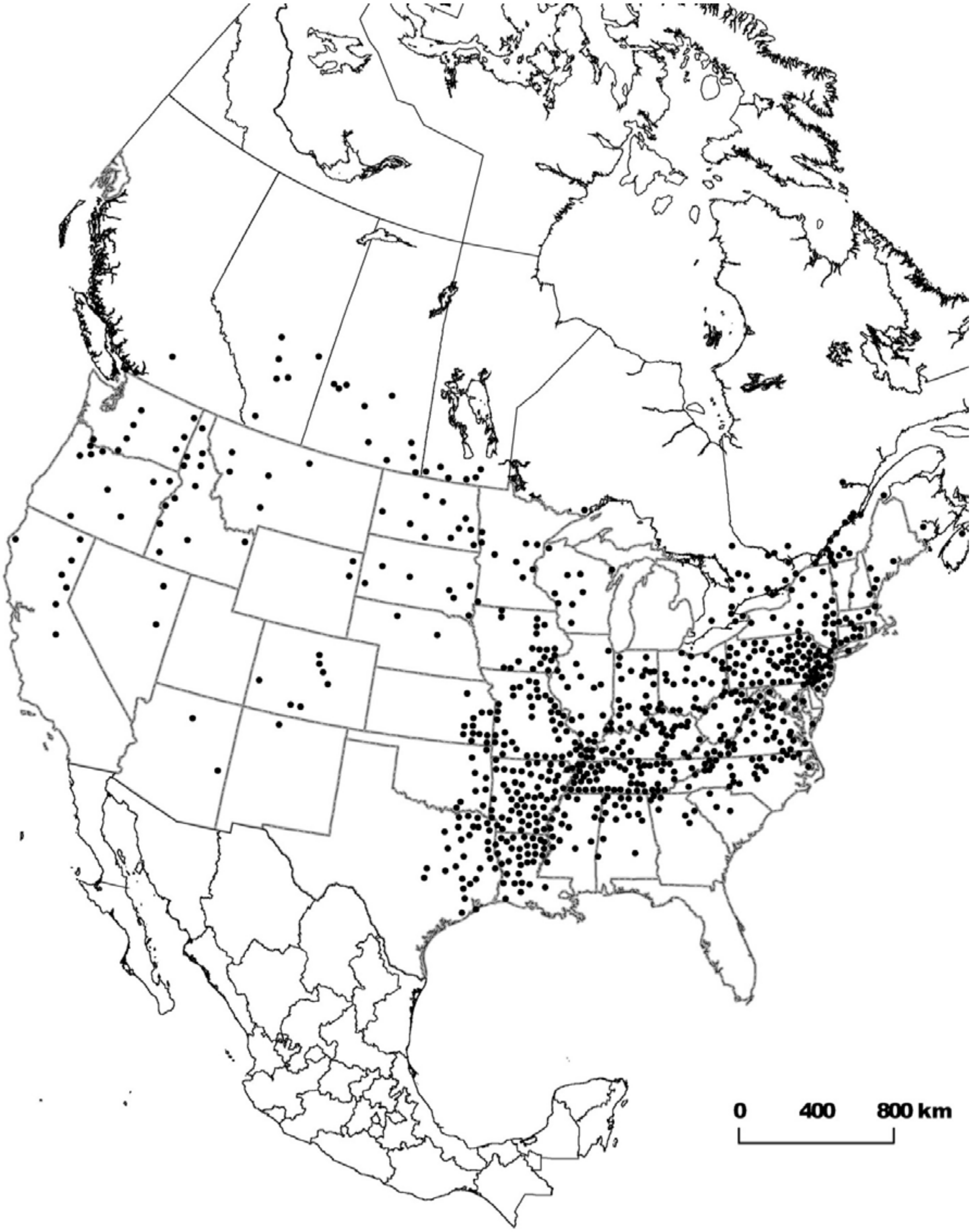


Fig. 3.4. Geographic distribution of *Gratiola neglecta* in North America.

Fig. 3.5. Geographic distribution of *Gratiola quartermaniae.*

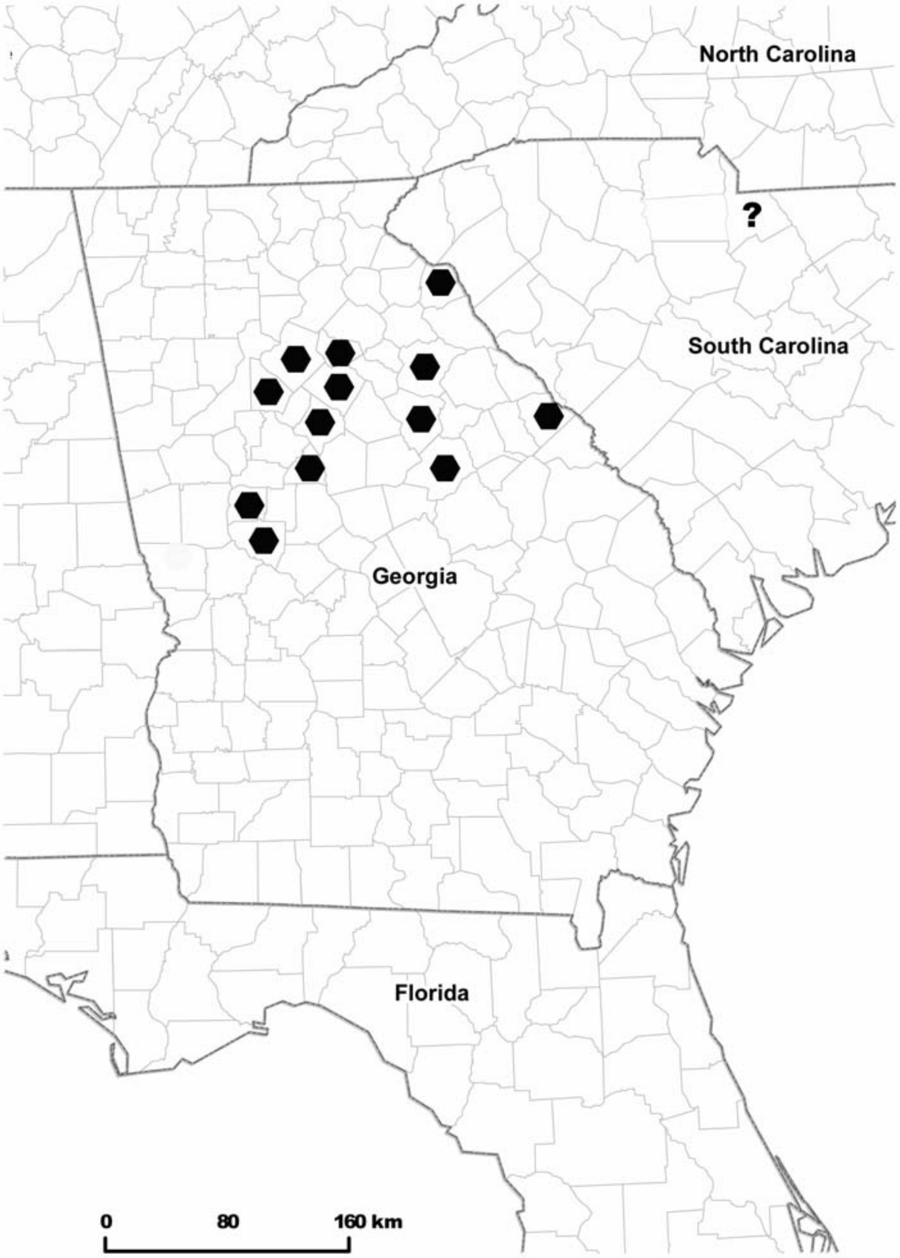


Fig. 3.6. Geographic distribution of *Gratiola graniticola.* The question mark represents an unconfirmed report from Lancaster Co., South Carolina.

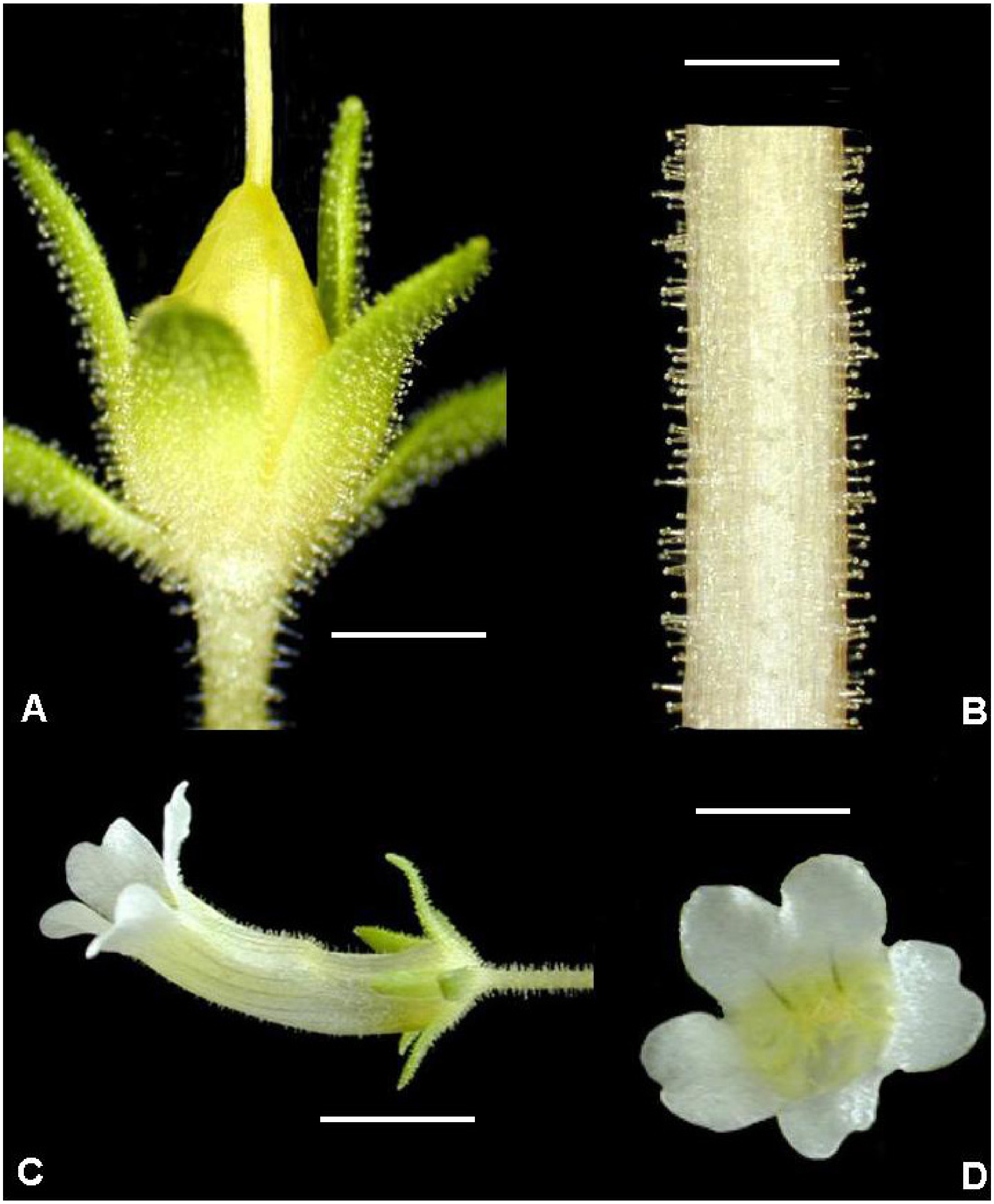


Fig. 3.7. *Gratiola neglecta.* **A.** Flowering calyx (corolla removed) and bracteoles (scale bar = 2 mm). **B**. Close-up of mid-stem (scale bar = 2 mm). **C**. Flower, lateral view (scale bar = 4 mm). **D**. Flower, front view (scale bar = 2.5 mm).

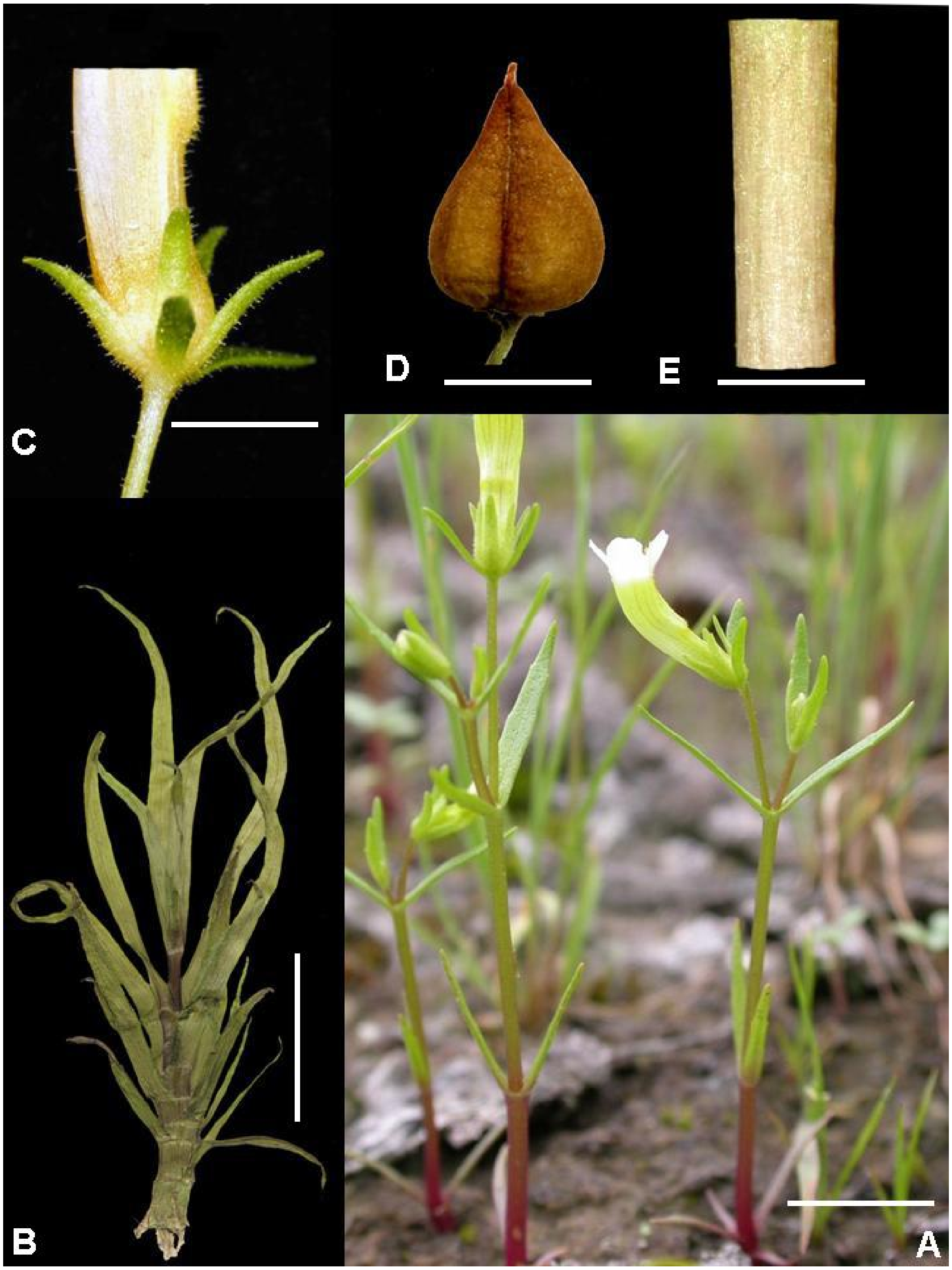


Fig. 3.8. *Gratiola quartermaniae.* **A**. Habit of *G. quartermaniae* at type locality (photo by M. Oldham, 2 Jun 2006). **B.** Pre-flowering specimen showing congested basal leaves (pressed specimen from *D. Estes 04359* TENN; scale bar = 2 cm). **C**. Flowering calyx and bracteoles (scale bar = 4 mm). **D**. Capsule (from *Oldham et al. 32877* TENN; scale bar = 3.5 mm). **E**. Close-up of mid-stem (scale bar = 2 mm).

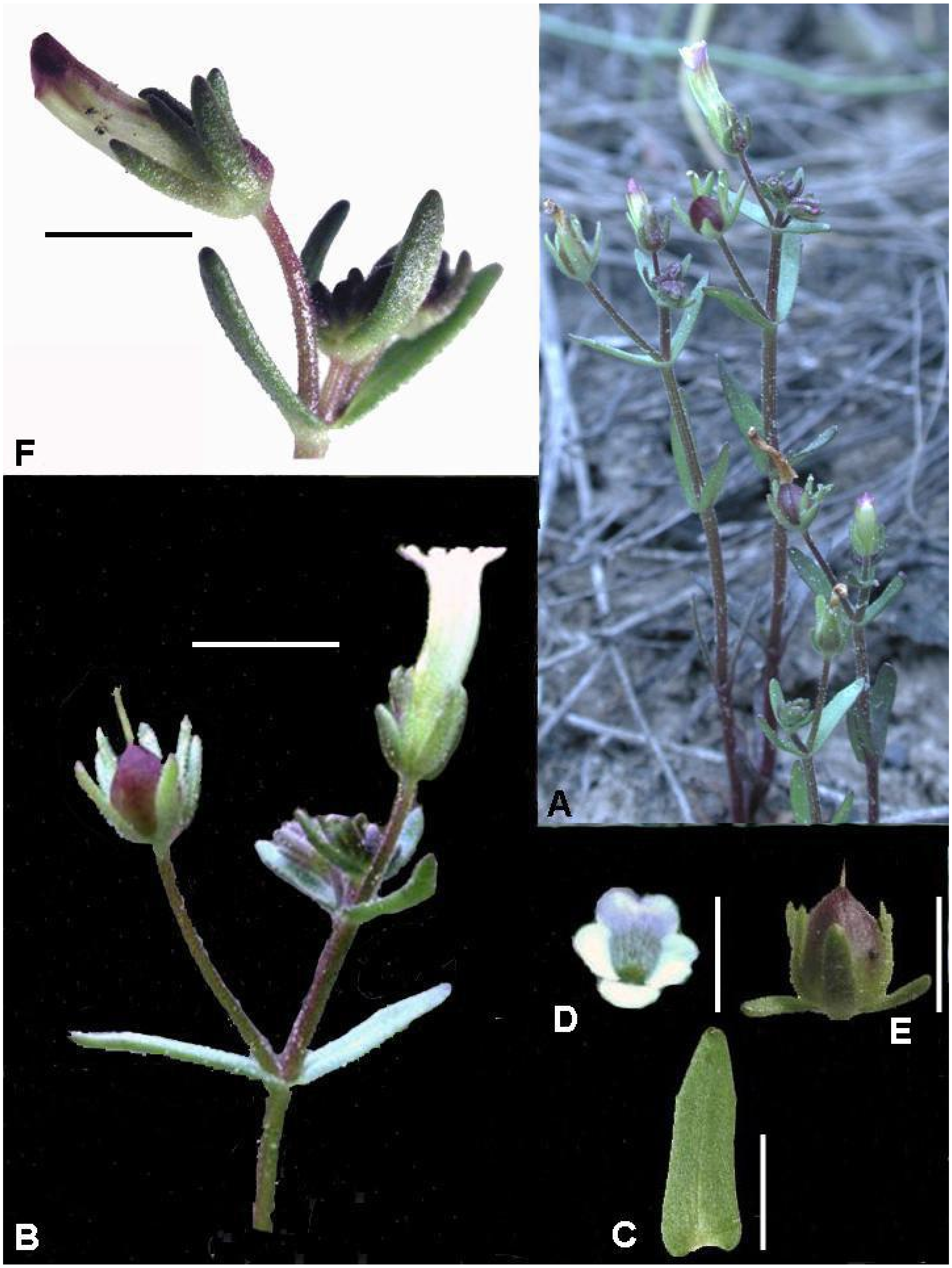


Fig. 3.9. *Gratiola graniticola.* **A**. Habit, in Butts Co., Georgia, 10 Apr 2004. **B.** Upper stem with flower and immature capsule. **C.** Leaf (scale bar = 5 mm). **D**. Flower, front-view (scale bar = 2 mm). **E**. Immature capsule with subtending calyx and bracteoles (scale bar = 3.5 mm). **F.** Unopened flower showing purplish corolla lobes.

Chapter 4

MOLECULAR PHYLOGENETIC RELATIONSHIPS, MORPHOLOGICAL EVOLUTION, AND BIOGEOGRAPHY OF *GRATIOLA* (PLANTAGINACEAE) WITH NEW INSIGHTS ON THE STATUS OF *FONKIA*, *SOPHRONANTHE*, AND *TRAGIOLA*

ABSTRACT

Gratiola (Plantaginaceae), the fourth largest genus of the predominantly Neotropical tribe Gratioleae, comprises 36 wetland-adapted species (four undescribed) distributed throughout temperate, tropical-montane, and, rarely, subtropical regions of the Americas, Eurasia, and Australasia. Little systematic work has been conducted on the genus and the last worldwide taxonomic treatment was written more than 160 years ago. During this time, one new section has been established, three monotypic genera have been segregated from *Gratiola*, several new species have been described, and a few more species remain undescribed. Given this, the genus is in need of considerable systematic work. In this study, a phylogenetic hypothesis is presented for the genus based on chloroplast DNA sequence data from the *trnS*-*trnG* intergenic spacer and *trnG* intron region as well as the *trnQ-rps16* intergenic spacer. All but two of the 36 species of *Gratiola* were sampled resulting in a robust phylogenetic assessment. The phylogeny was then used to examine the evolution of morphological characters within the genus as well as historical biographical patterns. The results from the phylogenetic analyses indicate that *Gratiola* sensu lato consists of four major clades. Another finding from this study was that sect. *Sophronanthe* is monophyletic, however, sect. *Nibora* and sect. *Gratiola* are not. Many of the species formerly recognized as part of sect. *Nibora* form their own strongly supported clade and should be placed in their own section. The results from the morphological analysis indicate that *Gratiola* s.l. is not supported by any morphological synapomorphies. Both *Gratiola* s.s. and "*Sophronanthe*" are each supported by morphological synapomorphies. Results from the biogeographical analysis indicate that *Gratiola* as well as the four major clades of the genus likely originated in North America. Finally, the monotypic genus *Fonkia* was found to be phylogenetically embedded within *Gratiola* supporting the recognition of this taxon as *Gratiola uliginosa*, and this study provides additional support for the recognition of *Sophronanthe* (including *Tragiola*) as a distinct genus.

INTRODUCTION

Gratiola is the fourth largest (behind *Bacopa*, *Stemodia*, and *Limnophila*) and most widely-distributed genus of the predominantly Neotropical tribe Gratioleae, which contains about 40 genera (Fischer 2004; Estes and Small, Chapter 2, in press). The family placement of *Gratiola* has been in a state of flux recently. Prior to Olmstead et al.'s (2001) dismantling of the Scrophulariaceae, *Gratiola* had long been assigned to that family (Bentham 1846; Bentham and Hooker 1876; Wettstein 1891; Pennell 1935), but recent authors have assigned *Gratiola* to the Gratiolaceae (Rahmanzadeh et al. 2005) or, more frequently, to the enlarged Plantaginaceae (Albach et al. 2005; Fritsch et al. 2007), the latter family placement is adopted here. Estes and Small (Chapter 2, in press), demonstrated that *Gratiola* sensu lato (s.l.) is sister to a clade comprised of the mostly aquatic Afro-Asian genus, *Limnophila* R.Br., and the small, aquatic, Madagascaran genus *Hydrotriche* Zucc. Fritsch et al. (2007) showed that *Dopatrium* Buch.-Ham. ex Benth, a wetland-adapted genus of Africa and southeastern Asia (Fischer 1997), is sister to *Hydrotriche* and is thus also closely related to *Gratiola*. The phylogenetic placement of *Gratiola* within the Gratioleae found by Estes and Small (Chapter 2, in press) and Fritsch et al. (2007) contradicts the classification of Fischer (2004), in which *Gratiola* is grouped into a different subtribe, the Gratiolinae, apart from the above genera, which Fischer placed in the Dopatriinae. The 36 species (including 4 undescribed species and 1 non-autonymic variety) of *Gratiola* s.l. are found in temperate, tropical-montane, and, rarely, subtropical regions of the Americas, Eurasia, and Australasia (Pennell 1935; Estes, unpubl. data). Like most genera of the Gratioleae, the species of *Gratiola* s.l. predominantly occur in wetland or aquatic habitats. Morphologically, the genus is characterized in the tribe in the combination of its two fertile stamens, anthers with predominantly discoid-dilated connectives, and bibracteolate calyces.

Circumscription of *Gratiola*—The circumscription of *Gratiola* has been a source of taxonomic debate for over a century. In its broadest circumscription, *Gratiola* has been defined to include *Sophronanthe* Benth. (=*G. hispida*) and *Tragiola* Small and Pennell (=*G. pilosa*) (Bentham 1846; Radford et al. 1968; Correll and Johston 1970; Gleason and Cronquist 1991; Godfrey and Wooten 1981; Clewell 1985; Wofford 1989; Wunderlin and Hansen 2003), but other taxonomists have regarded either one or both of these taxa as distinct monotypic genera (Small 1933; Pennell 1935; Fischer 2004, in part). Estes and Small (Chapter 2, in press) demonstrated that the two species formerly assigned to *Sophronanthe* and *Tragiola* are sister to each other and are, in turn, sister to *Gratiola* s.s. However, they were not able to conclusively test the relationships of *Sophronanthe* and *Tragiola* to *Gratiola* s.s. because they only sampled four species from *Gratiola* s.s.

Aside from debating the generic status of *Sophronanthe* and *Tragiola* relative to *Gratiola*, the circumscription of the genus has otherwise been quite stable until recently when Estes and Small (Chapter 2, in press) showed that *Amphianthus* Torr., previously considered a monotypic genus endemic to southeastern United States (Torrey 1837, Pennell 1935), is phylogenetically embedded within a paraphyletic *Gratiola*. As a result, Estes and Small (Chapter 2, in press) expanded the circumscription of *Gratiola* to include *Amphianthus.*

The phylogenetic relationships of some other genera within tribe Gratioleae are still poorly understood, especially that of four predominantly South American monotypic genera considered by Fischer (2004) to be allied to *Gratiola* and included by him in subtribe Gratiolinae, *Benjaminia* Mart. ex Benj., *Boelckea* Rossow, *Braunblanquetia* Eskuche, and *Maeviella* Rossow. Rossow (1983) included *Braunblanquetia* in synonymy under *Fonkia* Phil. and maintained that this monotypic genus is distinct from *Gratiola.* However, the characters used to separate *Fonkia* from *Gratiola* also occur in some species of *Gratiola*; therefore, the validity of this genus is necessarily called into question and demands further investigation both from a morphological comparison and from a molecular phylogenetic perspective.

Infrageneric Classification—Within *Gratiola* s.s. there are many unresolved questions needing attention. The last comprehensive taxonomic treatment of the genus was prepared 160 years ago by Bentham (1846). He recognized 20 species and instituted the first infrageneric classification recognizing two sections, sect. *Sophronanthe* and sect. *Gratiolaria* (=sect. *Gratiola*), the latter he divided into two subgroups, the *Diandrae* and the *Subdidynamae*. Later, Pennell (1935) transferred all of the species of Bentham's *Diandrae*, except the Australasian *G. pedunculata* R.Br., into the newly created sect. *Nibora* (Raf.) Pennell. The infrageneric structure of *Gratiola* established by Pennell (1935) has not since changed. Estes and Small (Chapter 2, in press), sampling only four species from *Gratiola* s.s., demonstrated that sect. *Nibora* is not monophyletic. Additional phylogenetic work is needed to test the monophyly of sect. *Gratiola* and the position of the remaining species of Pennell's (1935) sect. *Nibora* as well as the phylogenetic relationships of the 36 species of the genus.

Morphological Character Evolution—Pennell (1935) was perhaps the first taxonomist to address the morphology of the Gratioleae and *Gratiola* in an evolutionary context. Based on the tribe's "simplicity of structure," he considered the Gratioleae to be the most primitive group within the traditional Scrophulariaceae and he supposed that "there was an early separation…between the Gratioleae and the remaining tribes (Antirrhineae and Cheloneae), a supposition that has since been confirmed by the phylogenetic work of Albach et al. (2005). According to Pennell (1935), the primitive characteristics of the Gratioleae include their primarily septicidal capsule dehiscence, reticulate ovoid seeds, distinct plate-like stigmas, didynamous stamens, distinct sepals, presence of pedicel bractlets, simple racemose inflorescences (with bracts like normal foliage leaves), and opposite phyllotaxy. Within certain genera of the Gratioleae, several of these features have undergone significant evolutionary modification and were considered by Pennell (1935) to represent the derived condition. For example, in *Gratiola*, the capsules frequently dehisce loculicidally as well as septicidally and the anterior pair of stamens has been reduced to two sterile staminodia, both features considered by Pennell to be evidence of degeneration from the ancestral gratiolean condition. Pennell (1935) noted that within *Gratiola* "it is difficult to see definite stages of evolutionary progress between the species." The sections and species of *Gratiola* have been differentiated by habit and duration, leaf shape and morphology, presence or absence of bractlets and relative size compared to calyx, pedicel length, corolla color, presence or absence of staminodia, relative capsule size and shape, and seed morphology. Many of these characters vary across the genus and are perhaps too variable to be useful for classification purposes, but others are important for diagnosing particular groups. A morphological analysis coupled with a molecular phylogenetic study is needed to understand how these morphological characteristics are distributed among species and how they have changed during the course of the evolution of the genus.

Biogeography of *Gratiola* sensu lato—The Gratioleae, including several of its larger genera (i.e. *Bacopa*, *Mecardonia*, *Stemodia*), is best represented in the Neotropics, the region that Pennell (1935) surmised was the likely area of origin for the tribe. Work by Albach et al. (2005) supports a New World origin for the Gratioleae. The sister group to the Gratioleae, the "Angelonia Clade" of Estes and Small (Chapter 2, in press) is also a Neotropical group, with all of its genera, except *Ourisia*, being restricted to that region. Meudt and Simpson (2006) showed that *Ourisia*, a genus of ca. 30 spp. of South America and Australasia, originated in South America. Unlike many of the genera of the Gratioleae, *Gratiola* is one of only a few that is also widely distributed in portions of the Old World. Given that *Dopatrium*, *Hydrotriche*, and *Limnophila*, the closest known relatives to *Gratiola*, are each restricted to the Old World, an Old World origin for *Gratiola* seems plausible. A little less than half of the species of *Gratiola* are found in Australia and eastern Asia and the rest are found in the New World, mostly in southeastern North America. Not only are there more species of *Gratiola* s.l. in the New World, but North America also contains representatives from all three taxonomic sections whereas in the Old World and in South America only section *Gratiola* is represented. The greater taxonomic and corresponding genetic diversity of *Gratiola* s.l. in the New World, particularly in North America, could be interpreted as evidence that the genus originated there. All of this indeed raises some intriguing biogeographic questions especially concerning the area of origin of the genus and its major clades. Reconstruction of the historical biogeography of *Gratiola* can only be achieved with a complete phylogeny of the genus in place.

The objectives of this study are to (1) assess the relationships of *Sophronanthe*, *Tragiola*, and *Fonkia* to *Gratiola* s.s., (2) determine the phylogenetic relationships of the species of *Gratiola*, (5) examine the evolution of morphological characters within *Gratiola*, and (6) infer historical biogeographical patterns within *Gratiola.*

MATERIALS AND METHODS

Taxon Sampling—Specimens used for this study were collected from wild populations or from herbarium vouchers (Appendix 4.1). Sequences from 30 described species, four undescribed species, and one non-autonymic variety were included in this study. For all but five species and one variety, at least two accessions of each were included. Our sampling represents all 16 North American species, 4 of 5 Eurasian species, 2 of 3 South American species, all 7 described Australasian species, and 4 undescribed Australasian species (Estes, unpubl. data). Furthermore, sampling coverage includes all species of sect. *Gratiola* except the South American *G. uruguayensis*, all species of sect. *Nibora* except the eastern Asian *G. griffithii*, and both species of sect. *Sophronanthe*. In addition, the monotypic Argentine endemic *Fonkia uliginosa* was sampled because it was formerly placed in *Gratiola* and because the characters Rossow (1983) used to separate *Fonkia* from *Gratiola* are not unique to *Fonkia* and occur in some species of *Gratiola*. Two different outgroups were used in this study. In the first, *Hydrotriche* was used as the outgroup based on the results of Estes and Small (Chapter 2, in press) and Fritsch et al. (2007) which showed *Hydrotriche* to be in a clade sister to *Gratiola*. In the second, *Gratiola hispida* (=*Sophronanthe pilosa*) and *G. pilosa* (=*Tragiola pilosa*) were used as outgroups based on the results of Estes and Small (Chapter 2, in press) which demonstrated that these two taxa are sister to *Gratiola* s.s.

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). Sequence data was obtained from two chloroplast regions, the *trnS-trnG* intergenic spacer (*trnS*^{GCU}-*trnG*^{UUC}) + the *trnG* intron and the *trnQ-rps16* intergenic spacer (Fig. 4.1). The *trnS-trnG-trnG* region was used because Shaw et al. (2005) demonstrated that this one of the most phylogenetically informative of 21 noncoding cpDNA regions surveyed in *Gratiola*. The use of *trnQ-rps16* is predicated on recent findings by Shaw et al. (2007) that showed this region to be one of the most highly variable of a new set of noncoding cpDNA regions evaluated. PCR and sequencing primers for *trnS-trnG-trnG* are described in Shaw et al. (2005); those for *trnQ-rps16* are described in Shaw et al. (2007). PCR reaction volumes (25 µL) consisted of the following components: 1 µL template DNA (~ 10-100 ng), 1X buffer (TaKaRa, Madison, Wisconsin), 200 µmol/L each dNTP, 3.0 mmol/L $MgCl_2$ (1.5mmol/L for *trnS-trnG-trnG*), 0.1 µmol/L each primer, 0.2 µg/µL bovine serum albumin, and 1.25 units of *rTaq* or *ExTaq* (TaKaRa). PCR cycling conditions for *trnS-trnG-trnG*: template denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 15 sec, primer annealing at 54.5°C for 15 sec, extension at 72°C for 1.5 min; followed by a final extension step of 72°C for 5 min. PCR cycling conditions for *trnQ-rps16*: template denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 15 sec, primer annealing at 55.5°C for 15 sec, primer extension at 72°C for 1.5 min, followed by a final extension step of 72°C for 5 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 will be deposited in GenBank (Appendix 1).

Sequence Editing and Alignment—The sequences were assembled into contigs and edited in Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, Michigan), aligned using ClustalX (Thompson et al. 2001), and adjusted by eye in MacClade 4.0 (Maddison and Maddison 2001). For the *trnS-trnG-trnG* data matrix, 68 characters from the 5' end and 44 characters from the 3' end were deleted because the regions flanking the primer annealing sites were often incomplete and difficult to align. For the *trnQ-rps16* data matrix, 37 such characters from the 5' end and 29 characters from the 3' end were removed. Non-overlapping parsimony informative indels were coded as binary characters and added to the ends of each data matrix following the simple indel coding procedure described by Simmons and Ochoterena (2000).

Phylogenetic Analyses—Three phylogenetic analyses were conducted, one using just the *trnS-trnG-trnG* data set, the second using only the *trnQ-rps16* data set, and the third using a combined data set of both matrices. The purpose of the first two analyses was to assess the relative utility of each of these regions for providing phylogenetic resolution within *Gratiola.* Next, the trees derived from each independent analysis were compared to check for potential topological incongruence. Topological incongruence was assessed by visual comparison of the trees from each individual analysis. Only those nodes with at least 70% BS were considered as discussed by Kellogg et al. (1996). Specifically, the trees were examined and checked for the presence of hard incongruences (i.e. when two different topologies are each strongly supported) versus soft incongruences (i.e. two different topologies are produced because of poor support). Inspection of the strict consensus trees for both *trnS-trnG-trnG* and *trnQ-rps16* revealed that the two trees were congruent with respect to resolved nodes ≥70% BS.

After determining that combining the two datasets would be appropriate, the combined dataset was subjected to further testing to determine the effect of outgroup choice on ingroup resolution. First, the combined dataset was analyzed using *Hydrotriche* as the outgroup and *Gratiola* s.l. as the ingroup. Next, *Hydrotriche* was excluded from the analysis and *Gratiola pilosa* and *G. hispida*, the two species of Small's (1933) *Tragiola* and *Sophronanthe*, respectively, which were shown by Estes and Small (Chapter 2, in press) to be sister to *Gratiola* s.s., were both used as outgroup taxa.

The analyses of both the individual and combined data sets were performed under the optimality criterion of maximum parsimony using PAUP* v. 4.0 b10 (Swofford 2002) with the following options: maxtrees set to 5,000 (combined analysis) or 1,000 (the two individual analyses), 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. The consistency index (CI) and retention index (RI) were calculated to assess the amount of homoplasy present in the data. Relative clade support was estimated using 10,000 "fast stepwise-addition" bootstrap (Felsenstein 1985) replicates in PAUP*. The data and phylogenetic trees generated during this project will be deposited in TreeBASE.

Character State Evolution—A survey of the taxonomic literature (Bentham 1846; Small 1903; Small 1933; Godfrey and Wooten 1981; Pennell 1935; Barker 1986, 1990) and examination of herbarium specimens of all species of *Gratiola* resulted in the identification of 20 discrete morphological characters (Table 2) useful for distinguishing species. Each character was scored for each taxon included in our phylogenetic analysis. Morphological data were mapped onto one of the single MP trees produced by the molecular phylogenetic analysis of the combined *trnS-trnG-trnG* / *trnQ-rps16* data set using MacClade 4.0 (Maddison and Maddison 2001). The purpose of these analyses was to enable us to (1) identify morphological features useful for taxonomic classification and (2) understand the evolution of these morphological characters (i.e., which character states are synapomorphic vs. plesiomorphic, and which characters are homoplastic, e.g., due to convergent evolution).

Biogeographical Analysis—The phylogeny generated during this study was used to explore historical biogeographical patterns within *Gratiola*. The following questions were specifically addressed: (1) Did *Gratiola* originate in the New World or Old World? (2) Where did each of the major clades originate? To answer these questions, we employed character-state mapping implemented in MacClade v. 4.0 (Maddison and

Maddison 2001) with geographic areas coded as discrete characters (e.g., see Albach et al. 2005). For this analysis, seven geographic regions (Table 1) were optimized onto one of the equally most-parsimonious trees from the combined analysis. The following eight geographical regions that encompass the current distribution of *Gratiola* species were employed in the analysis: North America (0), South America (1), Eurasia (2), Australia (3), New Zealand (4), and Tasmania (5). In addition, three outgroup genera (*Dopatrium*, *Hydrotriche*, *Limnophila*) identified by Estes and Small (Chapter 2, in press) and Fritsch et al. (2007) as forming a clade sister to *Gratiola* were included in the biogeographical analysis. Most of the species of *Dopatrium* are endemic to Africa and all of the species of *Hydrotriche* are endemic to Madagascar; therefore in the biogeographical analysis these genera were assigned an African (6) distribution. *Limnophila* was coded as having a Eurasian distribution since most of the species occur in southeastern Asia.

RESULTS

Results of outgroup comparison—In using *Hydrotriche* as the outgroup for the phylogenetic investigation, four major clades were recovered within *Gratiola* s.l. with one clade formed by *G. hispida* and *G. pilosa* being sister to a much larger clade comprising the rest of the species of the genus (tree not shown). These results are consistent with those obtained by Estes and Small (Chapter 2, in press) and Fritsch et al. (2007). Next, *Hydrotriche* was excluded from the analysis and *G. hispida* and *G. pilosa* were used as outgroups with the remaining species of *Gratiola* from the other three major clades forming the ingroup. By eliminating *Hydrotriche* from the analysis and using *G. hispida* and *G. pilosa* instead, resolution and support within *Gratiola* improved.

Analysis of the trnQ-rpS16 intergenic spacer—The *trnQ-rps16* sequences generated during this study represent previously unpublished sequences (Appendix 1). Summary statistics for the analysis of the individual *trnQ-rps16* data matrix are presented in Table 1. Alignment of this data set was hampered slightly by the relatively large number of indels present in the matrix. Of these, 25 were parsimony-informative. In two regions (318-387; 1048-1180) of the alignment, the sequences could not be aligned with confidence and homology could not be assessed; therefore, the 203 bp from these regions were excluded from the analysis. Less than 1% of the data matrix consisted of missing data. The strict consensus tree from the analysis of the *trnQ-rps16* region is shown in Fig. 4.2A.

Individual analysis of the *trnQ-rps16* region recovered four groups (Clade 1, 2, 3, 4) (Fig. 4.2A). Clade 1 is fully resolved and consists of two species, *G. hispida* and *G. pilosa*. These two species form a clade sister to the rest of *Gratiola* (Clades 2, 3, 4). The taxa in Clade 2 are sister to Clade 3+4 and are fully resolved with the exception of *G. neglecta*, *G. floridana*, and *G. quartermaniae* (hereafter referred to as the *Gratiola* *neglecta* Complex). Clade 3, a small clade sister to Clade 4, is fully resolved and includes two species, *G. japonica* and *G. virginiana.* Most of the taxa in Clade 4 are grouped into five moderately to well-supported subclades. Unfortunately, the relationships between these subclades mostly remain unresolved, and three species, *G. fluviatilis*, *G. linifolia*, and *G. officinalis*, are left unassigned to a particular clade.

Analysis of the trnS-trnG intergenic spacer and trnG intron—The *trnS-trnG-trnG* sequences produced during this study represent previously unpublished sequences; these will be deposited in Genbank (Appendix 1). The *trnS-trnG* intergenic spacer and the *trnG* intron were analyzed as a single unit. Summary statistics for the analysis of the *trnS-trnG-trnG* matrix are presented in Table 1. This region was slightly difficult to align due to the large numbers of indels present. Of the numerous indels within the *trnS-trnG-trnG* region, 53 were parsimony-informative. Only in one region (603-629) was it too difficult to assess homology; therefore, this region was excluded from the analysis. Approximately 1% of the data matrix consisted of missing data. The strict consensus tree from the maximum parsimony analysis of the individual *trnS-trnG-trnG* dataset is shown in Fig. 4.2B.

Analysis of the *trnS-trnG-trnG* region recovered the same four major clades (Clades 1, 2, 3, 4; Fig. 4.2B) found in the analysis of the *trnQ-rps16* region (Fig. 4.2A). The major differences between the individual analyses is that the relationships of the major subclades of Clade 4 are better resolved in the *trnS-trnG-trnG* analysis and the relationships of individual species within each subclade are mostly better resolved. In the *trnQ-rps16* analysis, the relationships of *G. fluviatilis*, *G. linifolia*, and *G. officinalis* were unresolved and these taxa were not assigned to a particular subclade. In the *trnS-trnG-trnG* analysis, *G. fluviatilis* and *G. linifolia* were grouped together but with low support; their position as well as the position of *G. officinalis* in the phylogeny was still equivocal.

Congruence between trnQ-rpS16 and trnS-trnG-trnG—Relationships within and among several of the clades in the *trnQ-rps16* tree were not resolved making it difficult to assess congruency between the *trnQ-rps16* tree and the *trnS-trnG-trnG* tree. In any case, the lack of congruency appeared to be due to lack of phylogenetic signal (soft incongruence), not conflicting and strongly-supported phylogenetic resolutions (hard incongruence). A comparison of the strict consensus trees from the individual analyses of both *trnQ-rps16* (Fig. 4.2A) and *trnS-trnG-trnG* (Fig. 4.2B) reveals that no major differences exist between the two phylogenetic trees. In general, relationships in the *trnS-trnG-trnG* tree were more resolved and more highly supported than in the *trnQ-rps16* tree, but for the most part, support values were still somewhat low.

Analysis of the Combined Dataset—The combined data set contained 68 accessions of 35 taxa (34 spp. and 1 variety) and 3058 characters. Additional statistics for the combined analysis are presented in Table 1. The same indels that were coded in the individual analyses of each region were also coded in the combined analysis. Likewise, the same ambiguously aligned regions excluded in those individual analyses were excluded in the combined analysis. The strict consensus tree from the combined analysis is shown in Fig. 4.3A and one of the most-parsimonious trees from the combined analysis is shown in Fig. 4.3B.

In general, the topologies of the trees from the combined analysis (Fig. 4.3) are consistent with those of the trees from the individual analyses (Fig. 4.2). For example, in both the combined and individual analyses, the same major clades are recovered. Support for Clades 1, 2, and 3 is about equal in the combined and individual analyses. Support for Clade 4 improved from less than 50% BS in the *trnQ-rps16* tree and 66% BS in the *trnS-trnG-trnG* tree to 73% BS in the combined analysis, a value that is still quite low. Resolution and support for the major subclades within Clade 4 increased only slightly compared to the individual analyses.

Morphological Character Evolution—Based on the analysis of 20 morphological characters, no single morphological synapomorphy could be identified for *Gratiola* s.l. as currently defined (Figs. 4.4, 4.5). In *Gratiola* s.l. all species have contiguous anther thecae but this character is not a synapomorphy for *Gratiola* s.l. because contiguous anther thecae are also found in *Hydrotriche* (Fischer 2004). Both *Dopatrium* and *Limnophila* have disjunct or divergent anther thecae (Fischer 2004). *Gratiola* s.l. for the most part also differs from the three outgroup genera (*Dopatrium*, *Hydrotriche*, and *Limnophila*) in having sepals that are free to the base (vs. partially connate), however, partially connate sepals occur in four species of *Gratiola* (*G. amphiantha*, *G. heterosepala*, *G.* aff. *pubescens*, and *G. uliginosa*) where fused sepals have apparently evolved independently (Fig. 4.6).

Examination of morphological characters also indicates that the two species of the Clade 1(“*Sophronanthe*”) are morphologically quite divergent from the rest of *Gratiola* (Fig. 4.5). Perhaps of most interest is the fact that the members of Clade 1 lack the discoid-dilated anther connectives that are found in *Gratiola* s.s. but that are absent from all other members of the Gratioleae including *Dopatrium*, *Hydrotriche*, and *Limnophila* (Fig. 4.7). The species of “*Sophronanthe*” also differ from the other three main clades of *Gratiola* s.s. in their anther thecae orientation (Fig. 4.8). In “*Sophronanthe*,” the thecae are oriented parallel to the filament of the stamen but in the rest of *Gratiola* the thecae are oriented perpendicular to the filament. The members of this clade can also be distinguished from the rest of *Gratiola* by their papillose leaf surfaces (vs. non-papillose), revolute leaf margins (vs. flat), septate-ciliate leaf margins (vs. eciliate), indurate capsule walls (vs. non-indurate), and bifid (vs. entire) capsule valves (Fig. 4.9). Within *Gratiola* s.l., the members of "*Sophronanthe*" are the only species with a caudex-type root system (Fig. 4.10). No synapomorphic characters were identified for either Clade 2 ("*Diandrae*") or Clade 4 ("*Gratiola*"). One synapomorphic character was identified for Clade 3 ("*Nibora*") (Fig. 4.5). Both of the species of this clade (*G. virginiana* and *G. japonica*) share nearly identical linear-cylindric seeds, a seed type that is restricted within *Gratiola* to these two species plus the morphologically very similar *G. griffithii*, which was not included in the molecular study. Other characters used by previous taxonomists to differentiate the sections or particular species of *Gratiola* were also analyzed but were found to be variable within the genus but still taxonomically informative; these include life-history type (i.e. annual vs. perennial, Fig. 4.11), pedicel type (distinct and elongate vs. subsessile, Fig. 4.12), presence or absence of bracteoles (Fig. 4.13), and staminode type (Fig. 4.14).

Biogeographical Analysis—The results of the biogeographical analysis indicate that the major clades and subclades within *Gratiola* are generally correlated with particular geographic regions (Fig. 4.15). For example, both species of "*Sophronanthe*" are restricted to southeastern North America. Likewise, all species of "*Diandrae*" are restricted to North America. Within the "*Diandrae*," the species of the northwestern United States, *G. ebracteata* and *G. heterosepala*, are sister to each other whereas the closely related complex of *G. neglecta*, *G. floridana*, *G. quartermaniae*, *G. graniticola*, and *G. flava* are eastern North American. Within the "*Gratiola*" Clade, the major subclades exhibit strong biogeographical ties. All of the South American species group together in a strongly supported clade indicating that they are indeed a monophyletic group. This study also indicates that the Australasian species likely comprise a monophyletic group since in most of the trees recovered they are grouped together in a single, yet poorly-supported, clade. Of the Australasian species, the Tasmanian *G. nana* and *G.* aff. *nana*-Tasman. group with *G. concinna* and *G.* aff. *concinna* of New Zealand. *Gratiola pubescens* and *G.* aff. *pubescens*, a predominantly Western Australian group, form a clade. One of the few clades in this study that is not limited to one major geographic region is the "*Nibora*" Clade. *Gratiola virginiana*, of "*Nibora*," is restricted to eastern North America and central Mexico whereas its sister species, *G. japonica*, is found in east-central Asia.

DISCUSSION

Based on the analysis of DNA sequence data, *Gratiola* is composed of four primary clades (Fig. 4.4) provisionally referred to here as Clade 1 ("Sophronanthe"), Clade 2 ("Diandrae"), Clade 3 ("Nibora"), and Clade 4 ("Gratiola"). The "*Sophronanthe*" Clade, includes two species and one non-autonymic variety native to southeastern North

America. Clade 1 matches Bentham's (1846) sect. *Sophronanthe* and is equivalent to Small's (1903) genus *Sophronanthe.* This clade includes *G. hispida*, the type species of *Sophronanthe.* The "*Diandrae*" Clade comprises nine species native to North America, a few of which were classified in Bentham's (1846) *Diandrae* group and later as part of Pennell's (1935) sect. *Nibora*. Clade 3, the "*Nibora*" Clade of this study, is different in circumscription from Pennell's (1935) sect. *Nibora* and includes two sampled species, one native to North America and a second native to eastern Asia; a third unsampled species, *G. griffithii*, also likely belongs to this clade. Clade 3 is referred to as the "*Nibora*" Clade because it contains the type of Pennell's sect. *Nibora*, *G. virginiana*. The "Gratiola" Clade includes 21 species (including four that are undescribed) grouped into seven subclades that are distributed in portions of eastern North America, South America, Australasia, and Eurasia. Clade 4 is referred to as the "*Gratiola*" Clade because it contains the type of the genus, *G. officinalis*.

In Fig. 4.4, the various subgeneric categories recognized by Bentham (1846) and Pennell (1935) are matched against the phylogeny generated during this study. Bentham's subsection *Diandrae* is shown to be polyphyletic because the North American species (*G. flava*, *G. floridana*, *G. neglecta*) are placed within Clade 2 and *G. pedunculata* is placed within Clade 4. Likewise, Bentham's subsect. *Subdidynamae* is rendered paraphyletic due to the placement of *G. pedunculata.* The divisions of Bentham's subsect. *Subdidynamae*, the *Pedunculatae* and the *Sessiliflorae*, also appear not to be monophyletic. When Pennell (1935) established the sect. *Nibora*, he designated the type to be *G. virginiana* . In Fig. 4.4, Pennell's sect. *Nibora* is clearly polyphyletic because *G. virginiana* is a member of Clade 3, which is more closely related to the members of Clade 4 than it is to the remaining members of Pennell's sect. *Nibora* of Clade 2.

In light of the conflict between phylogeny and the existing infrageneric classification of *Gratiola*, it is evident that a revision of the existing classification is needed so that monophyletic sections are recognized. First, Pennell's section *Nibora* needs to be recircumscribed to include *G. virginiana* (the type of sect. *Nibora*), *G. japonica*, and *G. griffithii. Gratiola griffithii* was not included in this study but its association with *G. virginiana* and *G. japonica* is obvious as they all three share a unique seed type within *Gratiola* as well as a number of other nearly identical features such as thick succulent stems and similar globose capsules. A new section is needed to accommodate the other taxa previously referred to sect. *Nibora* including the three other species (*G. flava*, *G. floridana*, *G. neglecta*) assigned to sect. *Nibora* by Pennell (1935), two species (*G. ebracteata*, Bentham 1846; *G. oresbia*, Robinson 1909) known at the time but not treated in Pennell's work because they occur outside the area covered by his treatment, three new species described after Pennell's treatment (*G. heterosepala*, Mason and Bacigalupi 1954; *G. graniticola* and *G. quartermaniae*, Estes and Small 2007), and one species in the process of being transferred to this group (*G. amphiantha*; Estes and

Small, Chapter 2, in press). Formal restructuring of the infrageneric taxonomy of *Gratiola* to correspond with the results of this phylogenetic analysis will be undertaken in a forthcoming worldwide revision of *Gratiola* (Estes, unpubl. data). For now, tentative informal clade names are used here until the nomenclatural issues regarding these sections can be resolved.

Clade 1: *"Sophronanthe"*—The "*Sophronanthe*" clade is strongly supported (100% BS, 82 character state changes) as a monophyletic group sister to the rest of *Gratiola* (Figs. 4.2, 4.3, 4.4), corroborating the findings of Estes and Small (Chapter 2, in press). The "*Sophronanthe*" clade comprises two species and one non-autonymic variety—*G. hispida*, *G. pilosa* var. *pilosa*, and *G. pilosa* var. *epilis*. The taxa in this clade share a number of morphological synapomorphies (Figs. 4.5, 4.8, 4.9, 4.10) including a caudex-type root system, presence of overwintering-offshoots, rigid stems, hirsute non-glandular vestiture, rigid leaves with thickened revolute margins, papillose leaf surfaces, calyces with unequally-sized sepals, stamens with vertically-oriented parallel anther thecae, non-dilated anther connectives, and capsule valves that are indurate and bifid. This group also shares a distinctive seed morphology not found elsewhere in *Gratiola* (Estes, unpubl. data). Both *G. hispida* and *G. pilosa* have seeds with low radial walls and tangential walls with several low sinuous ridges whereas all other *Gratiola* investigated (Estes, unpubl. data) have seeds with high radial walls and tangential walls that are either smooth or honeycombed-reticulate. The morphological differences between the "*Sophronanthe*" Clade and the rest of *Gratiola* are more pronounced and more numerous than that found among the other three clades of *Gratiola* s.s. The only chromosome count that has been made for this group is for *G. pilosa* var. *pilosa*, which has a number of 2N=22 (Lewis et al. 1962). This number is unique within *Gratiola*, but until a count is available for *G. hispida* and *G. pilosa* var. *epilis* it is unclear whether chromosome number is taxonomically significant for this clade. All three taxa are restricted to southeastern North America, specifically to the southeastern United States. Ecologically, the varieties of *G. pilosa* inhabit mesic or wet sites similar to other species of *Gratiola*, but *G. hispida* frequently grows in deep, sandy, often drier soils, and is the most xerophytic member of the genus.

The two species of "Sophronanthe" have had a complex taxonomic history. Some taxonomists have considered them both to be *Gratiola* (Bentham 1846; Fig. 4.4), others have placed them in the genus *Sophronanthe* (e.g. Small 1903), and some have placed each species in its own monotypic genus, *Sophronanthe* and *Tragiola* (Small 1933, Pennell 1935; Fig. 4.4). The species that many contemporary botanists (Godfrey and Wooten 1981, Wunderlin and Hansen 2003, Clewell 1985) recognize as *G. hispida* was originally described by Bentham (1836) as the monotypic *Sophronanthe hispida* Benth. Ten years later, Bentham (1846) reduced *Sophronanthe* to a section within *Gratiola* and placed both *G. hispida* (at the time known as *G. subulata* Baldwin) and *G. pilosa* in this new section. More than 50 years later, Small (1903) reinstated *Sophronanthe* as a genus and moved both *G. hispida* and *G. pilosa* out of *Gratiola* and into *Sophronanthe*. Three decades later, Small (1933) reconsidered the status of *Sophronanthe* and removed *Sophronanthe pilosa* (Michx.) Small (=*Gratiola pilosa*) placing it in its own monotypic genus, *Tragiola* Small and Pennell, as *Tragiola pilosa* (Michx.) Small and Pennell. Pennell (1935) also maintained *Sophronanthe* and *Tragiola* as distinct monotypic genera noting that these genera "stand sharply distinct from *Gratiola*" and "with the removal of *Sophronanthe* and *Tragiola*, *Gratiola* remains a quite natural genus." Most subsequent taxonomists have not followed the concepts of Small (1903, 1933) or Pennell (1935) but instead have recognized *Gratiola* s.l. as including *Sophronanthe* and *Tragiola* (Fernald 1950, Radford et al. 1968, Correll and Johnston 1970, Godfrey and Wooten 1981, Gleason and Cronquist 1991).

With a complete phylogeny of *Gratiola*, it is now possible to evaluate objectively the taxonomic placement, and corresponding nomenclature, of *G. pilosa* and *G. hispida.* Using the phylogeny in Fig. 4.4 as a guide, three taxonomic scenarios are possible under the principles of monophyly. First, because *G. pilosa* and *G. hispida* form a monophyletic clade that is, in turn, sister to a monophyletic *Gratiola* s.s., combining these two sister groups into the single genus, *Gratiola* s.l. would result in a larger group that is still monophyletic. Therefore, Scenario 1 would entail retaining *G. pilosa* and *G. hispida* within *Gratiola*, in keeping with the classification of Bentham (1846). Although this scheme offers the advantage of nomenclatural conservation (i.e. recognizing fewer names), it masquerades the relatively large amount of molecular and morphological divergence between these groups, described above. The second possible scenario involves recognizing *Gratiola* s.s. as well as a monotypic *Sophronanthe* and a monotypic *Tragiola*, following the classification of Small (1933) and Pennell (1935). The problem with this scenario is that it seems to overemphasize the relatively minor differences between *Sophronanthe* and *Tragiola* (leaf and corolla shape) and totally negates the numerous synapomorphies shared by these two taxa. The third possible scenario involves recognizing *Sophronanthe* (including both *S. hispida* and *S. pilosa*) and *Gratiola* as distinct sister genera following the classification of Small (1903). Under this scheme, *Tragiola* would be placed in synonymy with *Sophronanthe*. This last scenario is the one that is supported here. A formal reinstatement and taxonomic revision of *Sophronanthe* is currently being prepared by Estes (in prep.).

Clade 2: *"Diandrae"*—The "*Diandrae*" Clade is a strongly supported clade (100% BS, 35 character state changes) that is sister to "*Nibora*" + "*Gratiola*" (Fig. 4.4). This group is restricted to North America and includes nine annual species: *G. ebracteata*, *G. heterosepala*, *G. amphiantha*, *G. oresbia*, *G. flava*, *G. graniticola*, *G.*

floridana, *G. neglecta*, and *G. quartermaniae*. Pennell (1935) included four of the species (*G. ebracteata*, *G. flava*, *G. floridana*, *G. neglecta*) of "*Diandrae*" in his section *Nibora* (Raf.) Pennell. The other six species were not included in sect. *Nibora* by Pennell (1935) because they either had not yet been described or because their distribution was outside the region covered in his treatment. Mason and Bacigalupi (1954) described *G. heterosepala* and although they didn't directly assign this species to sect. *Nibora*, they associated it with the related *G. ebracteata*, thus implying its sectional affiliation. *Gratiola graniticola* and *G. quartermaniae*, both recently described by Estes and Small (2007) were assigned to sect. *Nibora.* Estes and Small (Chapter 2, in press) also assigned *G. amphiantha* nom. nov. ined. to sect. *Nibora.* Robinson (1909), in describing *G. oresbia*, assigned it to sect. *Gratiola*, but based on the morphology of this species and its phylogenetic placement (Figs. 4.4), it clearly is allied to the other species of "*Diandrae*." Pennell also assigned *G. virginiana* to sect. *Nibora* and, in fact, designated this species as the type for the section. Based on Fig. 4.4, *G. virginiana* is solidly supported as a part of Clade 3 and is not allied with the species of "*Diandrae*" as thought by Pennell (1935). Therefore, the name sect. *Nibora* must be applied only to Clade 3 and the taxa in Clade 2 should be placed in their own section. Designation of a new section to accommodate the species will be accomplished in a forthcoming worldwide revision of the genus (Estes, in prep.).

As a group, the taxa in the "*Diandrae*" are characterized by an annual habit and generally small stature, fleshy fibrous roots (Figs. 4.10, 4.11), inconspicuously glandular-punctate leaves, and ovoid seeds. All of the species of the "*Diandrae*" are also characterized by tangential walls of the seeds with a smooth surface overlying a honeycomb-reticulate layer (Estes, unpubl. data); however, further work is needed to determine whether this characteristic is restricted to the "*Diandrae*." The chromosome number of 2N=18 for two species in this clade, *G. amphiantha* (Lunsford 1939; Konda 1972) and *G. neglecta* (Gervais et al. 1999), has not been reported elsewhere in the genus, but additional counts from the other seven species of "*Diandrae*" are needed to determine whether chromosome number is taxonomically significant for this group.

Within Clade 2 there are three subgroups. The first subgroup contains *G. ebracteata* and *G. heterosepala.* This small clade is strongly supported (99% BS, 14 character state changes) and is sister to the rest of the clade. Both *Gratiola ebracteata* and *G. heterosepala* have ebracteate flowers (Fig. 4.13), corollas that only slightly exceed the sepals, and similar cylindric-ovoid seeds (Estes, unpubl. data). Furthermore, they are both restricted to western North America where they inhabit wetland sites. *Gratiola heterosepala*, is a species of conservation concern and is one of the more recently described species of the genus (Mason and Bacigalupi 1954). It is one of only four species with partially connate sepals (Fig. 4.6).

The second subgroup in Clade 2 is comprised by *G. amphiantha* and *G. oresbia.* Molecular support for this sister relationship is strong (100% BS, 16 character state changes), but when the morphology of these species is taken into consideration, the close connection is less obvious. *Gratiola amphiantha* and *G. oresbia* are similar in that they both have stems that are frequently short-statured and clustered, giving the appearance of a dense tuft, and they have ebracteate flowers that are often borne embedded in the dwarfed stems (in *G. oresbia* the stems may also frequently be elongate as in other *Gratiola*). However, these similarities are certainly easily overshadowed by the striking differences between them. For example, *G. amphiantha* is perhaps the most morphologically outstanding member of the genus with its conspicuously dimorphic leaves, tiny flowers, and distinctive compressed-obcordate capsules. In contrast, *G. oresbia* is morphologically quite similar to the newly described *G. quartermaniae* (Estes and Small 2007) of the *Gratiola neglecta* species complex (discussed below), especially in its linear-lanceolate leaves that are frequently clustered toward the base of the stems, nearly glabrous herbage, and ovoid capsules. *Gratiola oresbia* is one of only three species in the genus (along with *G. aurea* and *G. flava*) with completely golden-yellow corollas, and its corollas are several times larger than those of *G. amphiantha*. *Gratiola oresbia* is one of only two species in Mexico (along with *G. virginiana*) and the only species in Central America; in both regions it inhabits mountainous areas. *Gratiola amphiantha* is among the rarest species in the genus. It is classified as a federally threatened species by the U.S. Fish and Wildlife Service (1993) and is known from only a few dozen populations in Alabama, Georgia, and South Carolina, in the southeastern United States.

The third subgroup in Clade 2 contains five species: *G. flava, G. graniticola*, *G. neglecta*, *G. quartermaniae*, and *G. floridana.* Compared to the four species in the other two subgroups of the "*Diandrae*" which lack bractlets beneath the calyx (Fig. 4.13), these five are characterized by bibracteolate calyces and they all share a primarily eastern North American distribution. The narrowly distributed *G. flava*, of eastern Texas and extreme northwestern Louisiana in the southeastern United States (MacRoberts et al. 2007), is one of only three species in the genus with completely golden-yellow corollas (along with *G. aurea* and *G. oresbia*). *Gratiola flava* is unique within the genus in its distinctive seeds which are the shortest in the genus and have thick radial walls with knob-like protuberances (Estes, unpubl. data). It also is unique in its conical capsules which become well-exposed due to the reflexing of the sepals at maturity. Sister to *G. flava* is the *Gratiola neglecta* complex, which as noted by Estes and Small (2007) includes four morphologically similar species: *G. floridana*, *G. graniticola*, *G. neglecta*, and *G. quartermaniae.* Within this complex, *G. graniticola* is strongly supported (100% BS, 14 character state changes) as sister to an unresolved but strongly supported clade of *G. floridana*, *G. neglecta*, and *G. quartermaniae*.

Clade 3: "section *Nibora*"—Clade 3 is a species-poor group that is sister to the "Gratiola" clade. It is represented by two species in our study, *G. virginiana* and *G. japonica.* A third species, *G. griffithii*, although not included in our study, clearly belongs to this clade based on its distinctive morphology. The members of "*Nibora*" share a set of distinctive morphological features including thick, succulent and nearly glabrous stems, fleshy fibrous roots, short and subsessile and often clavately thickened pedicels, thin-walled globose capsules, and linear-cylindric seeds. Both *G. virginiana* and *G. japonica* have the same chromosome number of 2N=16 (Lewis et al. 1962, Probatova and Sokolovskaya 1981); no chromosome count has been made for *G. griffithii.* This complex represents yet another example of the widely cited eastern North American / East Asian biogeographic pattern (Wen and Shi 1999, Wen et al. 2002, Nie et al. 2006, Zhou et al. 2006). *Gratiola japonica* is native to China, Japan, Korea, and eastern Russia. *Gratiola griffithii* is one of the least known species of the genus and perhaps one of the rarest as well, being known only from a few existing herbarium specimens from southwestern China and northeastern India (Estes, unpubl. data). *Gratiola virginiana* is widely distributed in southeastern North America and is also disjunct to central Mexico (Puebla and Veracruz) where it occurs in association with other species disjunct from the southeastern United States and thus represents another example of the temperate flora disjunction between these two regions (Miranda and Sharp 1950, Morris et al. 2007).

Clade 4 "section *Gratiola*"—Clade 4 is sister to "*Nibora*" and includes six subclades: *Gratiola aurea subclade*, *Gratiola officinalis subclade*, *Gratiola peruviana subclade*, *Gratiola nana subclade*, *Gratiola pedunculata subclade*, *Gratiola pubescens subclade*, and the *Gratiola latifolia* subclade. Each of the subclades is supported by a combination of molecular synapomorphies, morphology, and biogeography. Most of these subclades are recovered in the strict consensus tree, but the relationships between the subclades are not completely resolved (Fig. 4.3). The four Australasian subclades—*Gratiola nana*, *G. pedunculata*, *G. pubescens,* and *G. latifolia* subclades—form a monophyletic group in the strict consensus tree (Fig. 4.3), but in the bootstrap tree (not shown) this clade falls apart. Of the above subclades, the only one that is not supported at all in the strict consensus tree is the *G. officinalis* subclade. The two species of the *G. officinalis* subclade (*G. officinalis* and *G. linifolia*) are tentatively grouped together here based on their general morphological similarity. However, phylogenetically, the position of these two species is equivocal because in the strict consensus tree (Fig. 4.3A) they are not grouped together but in some of the many equally parsimonious (e.g. Fig. 4.3B) these two species are sister to each other. Below, the systematics of each subclade is discussed.

<u>*Gratiola aurea* Subclade.</u> This subclade is strongly supported (100% BS, 30 character state changes) and comprises four species (Fig. 4.3B): *G. aurea*, *G. brevifolia*, *G. viscidula,* and *G. ramosa*. *Gratiola ramosa* is sister to the other three species, and *G. viscidula* is sister to *G. aurea* + *G. brevifolia*. The sister group to the subclade is not entirely clear. In the strict consensus tree from the combined analysis, a sister group was not identified because the relationships between the seven major subclades were not resolved. In the 50% majority-rule bootstrap tree (not shown), *G. fluviatilis*, a narrowly endemic species of Japan, is sister to the "*Gratiola aurea* Subclade" but support for this relationship is low (56% BS). Morphologically, the members of the "*Gratiola aurea* Subclade" are similar in habit and general morphology. They are all perennials with relatively slender, cord-like rhizomes. Each species has relatively small capsules that are mostly < 3 mm long and much shorter than the subtending calyx. The anterior pair of stamens in these species is reduced to staminodes as in all *Gratiola*, but unlike the other clades of sect. *Gratiola* which have filiform and capitate staminodia, the members of this group have staminodia that are shorter and ecapitate. Polyploidy appears to have played an important role in the evolution of this group as *G. brevifolia* and *G. aurea* have a chromosome number of 2N=28 (Lewis et al. 1967, Kapoor et al. 1987), *G. viscidula* a chromosome number of 2N=16 (Konda 1972), and *G. ramosa* a chromosome number of 2N=14 (Lewis et al. 1962). At present, this group is considered to be restricted to eastern North America; however, there is a specimen annotated as *G. aurea* from Japan that is housed in the herbarium at the University of California at Berkeley (UC). Ohwi (1965), in the *Flora of Japan*, does not refer to this specimen or to any species that likely could be confused with it. It is unclear whether this specimen represents a very rare and undescribed Asian member of the "*G. aurea* Subclade" or whether this specimen represents true *G. aurea* that has been introduced into Japan either naturally (i.e. by migrating birds) or anthropogenically. More work is needed to clarify the status of this anomalous Asian *Gratiola.*

<u>*Gratiola officinalis* Subclade.</u> This tentatively recognized group contains two species, *G. officinalis* and *G. linifolia* (Fig. 4.3B). Both species share a general affinity with the members of the *G. aurea* complex but differ most noticeably in their larger capsules and staminodes. These two species also bear a resemblance to *G. pedunculata* of Australia. Interestingly, both *G. officinalis* and *G. pedunculata* have the same chromosome number of 2N=32 (Fernandes et al. 1977, Murray and De Lange 1999). These two species are the only representatives of the "*Gratiola*" Clade in Eurasia. *Gratiola officinalis* has the largest range of any species in the genus and occurs from western Europe east to China and Siberia. *Gratiola linifolia* is endemic to Spain and Portugal on the Iberian Peninsula of western Europe. According to Pennell (1935), *Gratiola* is not known to occur in Africa, but during the course of this investigation

specimens labeled as *G. linifolia* var. *mauritanica* were examined that apparently are from the Atlas Mountains of Morocco. No samples of *G. linifolia* var. *mauritanica* were included in this study and it is not clear whether this variety is even a valid taxonomic entity. More work is needed to determine the status of these African populations. Although *G. officinalis* and *G. linifolia* appear to be closely related based on general morphology, support for their close phylogenetic relationship is poor. In fact, this clade is the least supported of all the subclades in sect. *Gratiola*. In the strict consensus tree (Fig. 4.3A), *G. officinalis* and *G. linifolia* are not grouped together in a clade but rather their position, relative to the other subclades, is unresolved. Individual examination of the 234 equally parsimonious trees from the combined analysis reveals that in all of these trees *G. officinalis* and *G. linifolia* are grouped together, yet their placement within the "*Gratiola*" Clade varies. In some trees, these two species are placed sister to the South American + Australasian Clade whereas in others, they form a clade sister to the rest of the "*Gratiola*" Clade. Further investigation is needed to determine the relationships of these two species to each other and to the rest of the "*Gratiola*" Clade.

Gratiola peruviana Subclade. This clade is strongly supported (95% BS, 17 character state changes) and comprises at least three poorly known species, *G. peruviana*, *G. bogotensis*, and *G. uliginosa* (Fig. 4.3B). In the strict consensus tree (Fig. 4.3A) and the 50% majority-rule bootstrap tree (not shown), the sister group to the *"Gratiola peruviana* Subclade" is equivocal. Examination of the equally parsimonious trees from the combined analysis indicates that the "*Gratiola peruviana* Subclade" is sister to the Australasian Clade. Within this subclade, the two accessions of *G. bogotensis*, from Ecuador and Colombia, form a strongly-supported clade (100% BS, 19 character state changes) that is sister to *G. uliginosa* + *G. peruviana*. Interestingly, *G. peruviana* is not monophyletic. The accession of *G. peruviana* from Argentina is sister to the rest of the subclade [*G. bogotensis* (*G. uliginosa* + *G. peruviana*)], but the second accession of *G. peruviana*, from Chile, is sister to *G. uliginosa*. The results of this study indicate that the species currently known as *G. peruviana* in South America may, in fact, consist of at least two species. Additional phylogenetic work is needed to clarify the relationships of the "*Gratiola peruviana Subclade*" and more taxonomic work is needed to accurately circumscribe *G. peruviana.*

Early on, Bentham (1846) acknowledged the heterogeneity in *G. peruviana*, noting specifically that specimens from Chile were nearly glabrous whereas those from Brazil were viscid-puberulent. Based on a preliminary study of herbarium specimens from this region, it appears there is indeed much variation in *G. peruviana* and that some of these variations may eventually need to be described as separate taxa. One such variation from the northern Andes of Colombia and Ecuador was described by Pennell (1920) as *G. bogotensis.* Pennell considered *G. bogotensis* to be "a near ally or possible geographical variety" of *G. peruviana*. He distinguished *G. bogotensis* from Chilean populations of *G. peruviana* by their more fleshy stems, narrower leaves, and shorter pedicels. Holmgren and Molau (1984) considered *G. peruviana* to be a more southern species of lower elevations that occurs from southern Brazil and Argentina to Chile that differs from *G. bogotensis* in its larger leaves and white corolla. Although these characteristics seem quite subtle, especially in light of the plasticity that many species of *Gratiola* seem to exhibit, phylogenetic data presented here supports Pennell's concept that *G. bogotensis* and *G. peruviana* are different.

Gratiola uliginosa is the most morphologically distinct species of the "*Gratiola peruviana* Subclade" with its dwarf repent habit, glabrate stems, small leaves, small flowers, and small capsules. It is also only one of four species in the genus with partially connate sepals (Fig. 4.6; along with *G. amphiantha*, *G. heterosepala*, and *G.* aff. *pubescens*). As pointed out by Reiche (1911), the habit of this species is very similar to *G. nana* of Tasmania; it also bears a superficial resemblance to *G. concinna* of New Zealand and *G.* aff. *nana*-Aust. of southeastern mainland Australia. Based on habit and general morphology alone, it seems plausible that *G. uliginosa* could be closely related to these other three species. Furthermore, given the well-documented biogeographical connection (Meudt and Simpson 2006) between the regions where these species occur (southern South America, Tasmania, New Zealand, southeastern Australia), it would seem plausible that these species could have become isolated by the break-up of Gondwanaland. However, the phylogenetic data presented here conflicts with this potential scenario demonstrating that *G. uliginosa* is not closely related to the other three Australasian taxa, but is instead most closely related to the other South American taxa. Therefore, any similarity between *G. uliginosa* and these Australasian taxa appears to be due to convergent evolution. This makes sense given that most of the taxa involved inhabit similar high-elevation habitats where short-stature plants are apparently better adapted.

The unusual morphology of *G. uliginosa* has apparently caused quite a bit of confusion for taxonomists resulting in this species being assigned to three different genera since the late 1800s. Philippi (1865) first described this species and assigned it to the monotypic genus *Fonkia* Phil. He differentiated *Fonkia* from *Gratiola* based on the supposed absence of anterior staminodia in the former, but apparently didn't realize that in some species of *Gratiola* this condition may be variable even among individuals of a population (Pennell 1935). Reiche (1911) transferred this taxon to *Gratiola* (*G. peruviana* var. *uliginosa*) and considered it to be merely a variety of the variable *G. peruviana*. Eskuche (1974), evidently not realizing the history of this taxon and its former assignment to both *Fonkia* and *Gratiola*, described this species as the monotypic *Braunblanquetia littoralis* Eskuche. He distinguished *Braunblanquetia* from *Gratiola* by the partially connate sepals in the former. Eskuche didn't realize that some *Gratiola* species also have a partially fused calyx (*G. heterosepala*, Mason and Bacigalupi 1953; *G. amphiantha*, Estes and Small Chapter 3, in press; *G.* aff. *G. pubescens*; Estes, unpubl. data), and more importantly, that the taxon he had described as *Braunblanquetia* had already been described by Phillippi (1865). Rossow (1983) pointed out that *Braunblanquetia* and *Fonkia* represent taxonomic synonyms, and since *Fonkia* has nomenclatural priority, he reinstated *Fonkia* and treated *Braunblanquetia* as a synonym. Rossow (1983) differentiated the reinstated *Fonkia* from *Gratiola* by the lack of clavate hairs in the corolla throat, position of the staminodia, and features of the anther connective. Based on cpDNA sequence data this species belongs to *Gratiola.*

Gratiola nana Subclade. The *"Gratiola nana* Subclade" includes two described species, *G. nana* and *G. concinna*, and two apparently undescribed species (designated here as *Gratiola* aff. *nana*-Tas. and *G.* aff. *concinna*) (Fig. 4.3B). Support for this clade is strong (91% BS, 5 character state changes). According to the strict consensus tree (Fig. 4.3A), this clade is sister to the rest of the Australasian taxa [*G. latifolia* subclade (*G. pedunculata* subclade + *G. pubescens* subclade)], but in the 50% majority-rule bootstrap tree (not shown) the sister to this clade is unresolved. *Gratiola nana* and *G.* aff. *nana*-Tas. are both endemic to Tasmania whereas *G. concinna* and *G.* aff. *concinna* are both endemic to New Zealand. Morphologically, the members of the *"Gratiola nana* Subclade" are characterized by their small stature, prostrate stems that root at the nodes, and small leaves. Hair et al. (1967) reported a chromosome number of 2N=30 for *G. concinna* (under the name of *G. nana*); this remains the only number reported for this clade.

Until now, most taxonomists have assigned all *Gratiola* populations in southeastern Australia, Tasmania, and New Zealand with short prostrate stems to *G. nana* (Burbidge and Gray 1970; Barker 1990; Bentham 1846, 1869; Cheeseman 1906; Allan 1969). However, this phylogenetic investigation indicates that populations in New Zealand are distinct from Tasmanian populations where the type of *G. nana* is from. *Gratiola nana*-like plants from New Zealand have been treated as *G. concinna.* Although Cheeseman (1906) considered *G. concinna* to be a synonym of *G. nana*, he pointed out that New Zealand populations are quite variable, with specimens from the South Island differing in a few notable features compared to North Island plants. Preliminary morphological data (Estes and de Lange, unpubl. data) supports Cheeseman's (1906) observations of this variability and it appears that South Island plants actually represent a distinct taxon. Due to the somewhat limited utility of cpDNA sequences in resolving relationships between closely related taxa, it is not surprising that our phylogenetic data do not match the morphological data. Perhaps further investigation with a low-copy nuclear DNA marker would help resolve the relationships of this group. However, it is clear that *G. concinna* in the broad sense is distinct from *G. nana*, both on a molecular and morphological level (Estes and de Lange, in prep.). A preliminary examination of herbarium specimens from New Zealand identified as *G. nana* indicates that a third possibly undescribed taxon exists in extreme southern New Zealand. Estes and de Lange (unpubl. data) are currently working to assess the status of these populations. Populations of what have been called *G. nana* from the mainland of southeastern Australia (Australian Capital Territory, New South Wales, Victoria) also are phylogenetically distinct from true *G. nana* of Tasmania. These Australian mainland *G. nana*-like (*G.* aff. *nana*-Aust.) populations actually are more closely related to *G. latifolia* and *G. sexdentata* of the "*Gratiola latifolia* Subclade." Differences between these mainland plants and Tasmanian *G. nana* have previously been noted by Burbidge and Gray (1970). A sample from a "*G. nana*" population from southern Tasmania that, according to W.R. Barker (University of South Australia, pers. comm.), potentially represents an undescribed species was also included in this study. This accession, labeled *G.* aff. *nana*-Tasman. in Fig. 4.3A is strongly supported (98% BS) as a member of the clade that contains *G. nana* s.s., however, within this clade *G. nana* and *G.* aff. *nana*-Tasman. are not resolved. Perhaps one of the most important findings of this study is that *G. nana*, which had been considered a single variable species, actually appears to represent five or possibly six species, nearly doubling the number of *Gratiola* taxa known from Australasia.

Gratiola pubescens Subclade. This subclade includes one described species, *G. pubescens* and one apparently undescribed species (here designated as *Gratiola* aff. *pubescens*) (Fig. 4.3B). Both are characterized by having rather narrow and densely glandular-pubescent leaves, subsessile pinkish or purplish flowers, and a predominantly Western Australian distribution. *Gratiola* aff. *pubescens* is quite distinct from *G. pubescens*; its stems are solitary and simple (vs. often branching from base), its leaves are clustered distally on the stem giving it a glomerate appearance (vs. leaves well distributed on the stem), and its sepals are partially fused and completely enclose the capsules forming an involucre-like structure (vs. sepals free and capsules exposed). *Gratiola* aff. *pubescens* may also be an annual, and if so, would represent the only annual species in Australasia. Of the two taxa, *G. pubescens* is the most widely distributed, ranging from southern Western Australia to coastal areas of southeastern Australia, northern Tasmania, and possibly northern New Zealand. *Gratiola* aff. *pubescens* appears to be quite rare and has been collected from only a few places in southern Western Australia. This species is currently being investigated and will be described by Estes (unpubl. data). In the strict consensus tree (Fig. 4.3A), the two accessions of *G. pubescens* are grouped together and received moderate support (80% BS); however, the two accessions of *G.* aff. *pubescens* are unresolved with respect to each other and to the two *G. pubescens* accessions. The sister group to the "*Gratiola pubescens* Subclade" is apparently the "*Gratiola pedunculata* Subclade" although support for this relationship is not strong.

Gratiola pubescens has for many years been considered synonymous with *Gratiola peruviana* (Bailey 1901). For example, Bentham (1869) treated it as a "narrow-leaved" pubescent form of *G. peruviana.* Only in the past two decades has *G. pubescens* been provisionally revived and treated as a distinct species (Barker 1986, 1992). This study suggests that *G. pubescens* is indeed a distinct species. This is corroborated by herbarium specimen examination and preliminary morphological data collected by Estes for a worldwide revision of the genus (Estes, unpubl. data).

Gratiola pedunculata Subclade. This subclade comprises two species, *G. pedunculata* and *G. pumilo* (Fig. 4.3B). Of all of the Australasian taxa, these are the only two species with conspicuous pedicels (mostly > 2 mm), although the pedicels in *G. pumilo* can sometimes be shorter. Murray and de Lange (1999) reported a chromosome number of 2N=32 for *G. pedunculata*, a number shared only with the Eurasian *G. officinalis*. No count has yet been made for *G. pumilo*. *Gratiola pedunculata* has the largest distribution of any of the Australasian species. It is most abundant in eastern Australia but its range extends west to South Australia and it is apparently disjunct in southern Western Australia. Murray and de Lange (1999) also recently reported that this species has apparently dispersed naturally to the extreme northern tip of the North Island of New Zealand, perhaps via waterfowl migrating from Australia. *Gratiola pumilo* is much more restricted in distribution and appears to be most abundant in parts of New South Wales, Victoria, and South Australia, especially in the Murray River watershed. Support for the "*Gratiola pedunculata* Subclade" is strong (100% BS, 17 character state changes), and both species in this clade are phylogenetically distinct. The sister group to this clade is most likely the "*Gratiola pubescens* Subclade" according to the strict consensus tree (Fig. 4.2); however, the 50% majority-rule bootstrap tree (not shown) is less resolved and this sister relationship is not supported.

Bentham (1846) assigned *G. pedunculata* to the *Diandrae* group, which otherwise consisted of only North American species (Fig. 4.4), based on the supposed absence of anterior staminodes in this species, a feature that characterizes the North American members of the *Diandrae*. However, Barker (1986) later noted that this species has long staminodia, a feature that separates it readily from the North American "*Diandrae*." A preliminary morphological investigation by Estes (unpubl. data) corroborates Barker's findings and confirms that the staminodia of *G. pedunculata* are similar to other Australian species and members of the "*Gratiola*" Clade. The assignment of *G. pedunculata* to the "*Gratiola*" Clade is supported by phylogenetic data (Figs. 4.3, 4.4) in addition to staminodial morphology. Among Australian *Gratiola*, Barker (1992) considered *G. pedunculata* to be the only clear-cut species noting that all others are questionably separable at the species level, including *G. pumilo.* For many years, *G. pumilo* was considered to be a variety of *G. peruviana* (*G. peruviana* var. *pumila*) (Bentham 1869); however, within the last couple of decades this species has been tentatively revived and treated as a distinct species (Barker 1986, 1992). Barker (1986), however, did note that this species may possibly be conspecific with *G. sexdentata* of New Zealand. This study supports the recognition of *G. pumilo* as a distinct taxon and clearly demonstrates that it is not related to *G. peruviana* as previously thought (Bentham 1869) nor is it related to *G. sexdentata*.

Gratiola latifolia Subclade. The "*Gratiola latifolia* Subclade" comprises two described species, *G. latifolia* and *G. sexdentata*, and one undescribed species, *G.* aff *nana*-Aust. Among Australasian *Gratiola*, these taxa are characterized by their predominantly glabrous stems and subsessile flowers (Fig. 4.3B). The three species of this subclade are largely allopatric. *Gratiola latifolia* is widespread in southeastern Australia and northern Tasmania, *G. sexdentata* is restricted to New Zealand and the Chatham Islands (Peter de Lange, pers. comm.), and *G.* aff. *nana*-Aust. is found only in the Australian Alps of southeastern mainland Australia (Victoria, New South Wales, Australian Capital Territory). In the strict consensus tree (Fig. 4.3A) *G. latifolia* and *G. sexdentata* form a strongly supported clade (92% BS, 8 character state changes), but support for each species is low or lacking. The three accessions of *G.* aff. *nana*-Aust. also form a strongly supported clade (100% BS, 9 character state changes). In the strict consensus tree (Fig. 4.3A), *G.* aff. *nana*-Aust. is sister to *G. latifolia* + *G. sexdentata* but bootstrap support for this sister relationship is low (58% BS, 5 character state changes).

The taxonomy of *G. latifolia*, the species included here in the "*Gratiola latifolia* Subclade," is poorly known. Bentham (1869) referred to this taxon as a "broad-leaved" glabrous form of *G. peruviana.* Other authors, including Burbidge and Gray (1970), considered *G. latifolia* to be a distinct species endemic to Australia and Tasmania. Other authors (Bailey 1901; Cheeseman 1925; Beadle et al. 1972; Barker 1986, 1992) followed Bentham (1869) in considering *G. latifolia* to be a synonym of *G. peruviana.* Currently, the status of Australian populations variously identified as *G. peruviana* or *G. latifolia* remains unsettled. According to the phylogeny in Fig. 4.3A, the Australian plants are phylogenetically distinct from *G. peruviana* indicating that the Australian plants should be referred to *G. latifolia.*

Gratiola sexdentata, like most of the other Australasian taxa, has also had a varied taxonomic past. Hooker (1867) considered it to be "most closely allied to the South American *G. peruviana*" and to be distributed throughout New Zealand as well as in southeastern Australia and Tasmania. Cheeseman (1906, 1925) included *G. sexdentata* as a synonym of *G. peruviana.* Allan (1961) recognized *G. sexdentata* as a distinct species and added that there are apparently two forms of this species in New Zealand, one being small and erect with quadrangular stems, entire, obtuse, and ovate leaves, and small flowers, and the other being larger and more prostrate or sub-erect with serrate, acute leaves and larger flowers. Further investigation of these forms may be warranted, but they were not considered during this study. Based on study of herbarium specimens (Estes, unpubl. data), *G. sexdentata* is restricted to New Zealand and the Chatham Islands (de Lange, pers. comm.) and does not occur in Australia or Tasmania. Based on the phylogeny in Fig. 4.3A, *G. sexdentata* is allied to the Australian/Tasmanian *G. latifolia* (formerly part of *G. peruviana*) as suspected by previous workers. Although molecular support for it being a distinct species is equivocal in this study, it seems sufficiently distinct morphologically and geographically to warrant recognition at the species level.

The taxon treated here as *G.* aff. *nana*-Aust. has historically always been identified as *G. nana* although Burbidge and Gray (1970) did point out that the *"G. nana*" of the Australian Capital Territory (=*G.* aff. *nana*-Aust.) differed from Tasmanian *G. nana* in their narrower leaves and shorter corolla tube lacking hairs in the throat. Like *G. nana*, this new species has a prostrate habit and relatively small leaves. Other synapomorphies are unknown presently, but additional morphological work is currently being conducted by Estes and de Lange (unpubl. data). From the phylogeny (Fig. 4.3A), it is clear that these plants are distinct from *G. nana* and the other members of the "*G. nana* Subclade."

Morphological Differences between "*Sophronanthe*" and *Gratiola* s.s.—Based on the morphological analysis conducted in this study, *Gratiola* s.l. (including the "*Sophronanthe*" Clade), is not supported by a single morphological synapomorphy (Fig. 4.5). However, when the "*Sophronanthe*" Clade is removed from *Gratiola* and treated as a distinct genus, both *Sophronanthe* and *Gratiola* s.s. are each supported by multiple morphological synapomorphies. Based on the phylogeny (Figs. 4.3, 4.4) and the morphological analysis (Figs. 4.5, 4.7-4.10), the "Sophronanthe" Clade stands out both phylogenetically and morphologically compared to the other three clades of *Gratiola* s.s. Pennell (1935) noted that the species of the "*Sophronanthe*" Clade, *G. hispida* and *G. pilosa*, stand sharply distinct from *Gratiola* s.s. He additionally noted that when these two species are excluded from *Gratiola* that *Gratiola* s.s. is "unique in the wide development of the connective of the anthers." Indeed, this morphological study confirms Pennell's (1935) observations that discoid-dilated anther connectives are a morphological synapomorphy for *Gratiola* s.s. (Fig. 4.7). Numerous other morphological differences exist between the "*Sophronanthe*" Clade and the rest of *Gratiola* s.s. All of the species of *Gratiola* s.s. have anther thecae that are oriented perpendicularly to the filament of the stamen, but in the "Sophronanthe" Clade the anther thecae are oriented parallel to the filament (Fig. 4.8). The "*Sophronanthe*" Clade also differs from *Gratiola* s.s. in its conspicuously papillose leaf surfaces (vs. epapillose), revolute leaf margins (vs. plane leaf margins), septate-pilose trichomes on leaf margins (vs. non septate-pilose), sepals conspicuously unequally-sized (vs. sepals ± equally sized), capsules dehiscence primarily loculicidal (vs. primarily septicidal or equally loculicidal and septicidal), capsule walls indurate (vs. capsule walls thinner and more brittle), and capsule valves bifid (vs. valves not bifid) (Fig. 4.9). The members of the "*Sophronanthe*" Clade also possess a caudex-type root system whereas the species in *Gratiola* s.s. have fibrous or rhizomatous root systems (Fig. 4.10). Pennell (1935) considered the differences in corolla form (salverform vs. funnelform) and leaf shape (linear vs. ovate) between *G. hispida* and *G. pilosa* to be sufficiently significant enough to justify placing each species in its own monotypic genus, *Sophronanthe* and *Tragiola*, respectively. The numerous morphological synapomorphies that unite these two species seem to outweigh the differences in corolla form and leaf shape, especially since these two characters are often quite variable within genera and may be influenced by pollinator-type, in the case of corolla shape (Bradshaw and Schemske 2003), or environmental variables (i.e. sun exposure, submergence in water), in the case of leaf shape. In short, the two species of the "*Sophronanthe*" Clade form a monophyletic group that is distinguished by several morphological synapomorphies that when considered as a whole seem sufficiently great enough to justify placing the two species of this clade in the genus *Sophronanthe*, thereby narrowing the circumscription of *Gratiola.*

Morphological Evolution within *Gratiola* s.s.—The three major clades of *Gratiola* s.s. ("*Diandrae*, *Nibora*, *Gratiola*") differ from each other in rather general morphological attributes that are difficult to characterize discretely or quantitatively. For example, the species of the "*Diandrae*" Clade are all annuals with delicate fibrous root systems (Figs. 4.10, 4.11) and reduced or absent staminodia (Fig. 4.14), but a similar root type is found in the species of the "*Nibora*" Clade and absent or reduced staminodia are found in the "*Gratiola aurea* Subclade" of the "*Gratiola*" Clade. The species of "*Nibora*" are annuals or short-lived perennials (in warm temperate regions) with fibrous root systems (similar to those of "*Diandrae*"), often thick fleshy stems, and unique linear-cylindric seeds. The species of "*Gratiola*" are generally somewhat larger plants that are mostly rhizomatous and perennial (Figs. 4.10, 4.11) and mostly have filiform staminodia with subcapitate apices (Fig. 4.14) (except for the "*G. aurea* Subclade" which has reduced staminodia). Pennell (1935) considered the members of sect. *Gratiola* (mostly equals the "*Gratiola*" Clade of this study) to differ from sect. *Nibora* (Pennell's sect. *Nibora* includes the "*Diandrae*" and "*Nibora*" clades of this study) in having leaf bases more strongly clasping and leaf surfaces more frequently glandular-punctate. Although it is true that several of the species of the "*Gratiola*" Clade have strongly clasping leaf bases (e.g. *G. latifolia*, *G. peruviana*, *G. viscidula*), there are also species of "*Diandrae*" (e.g. *G. graniticola*, *G. ebracteata*, *G. heterosepala*) that may have strongly-clasping leaf bases; therefore, this character is not entirely reliable for distinguishing among the major groups of *Gratiola.* Likewise, although several of the species of the "*Gratiola*" Clade may have strongly glandular-dotted leaves (e.g. *G. aurea*, *G. pubescens*) there are many species within this clade that have inconspicuously glandular-dotted leaves like those of species in the "*Nibora*" or "*Diandrae*" clades thus demonstrating that the degree of leaf glandularity is not very reliable for separating the major clades of *Gratiola.*

The results from the morphological analysis indicate that root system type and life history (Figs. 4.10, 4.11) are perhaps the most useful characters for separating the species of *Gratiola* s.s. into the major clades. In terms of life history type, all but one species (*Gratiola* aff. *pubescens*) of the "*Gratiola*" Clade are perennials whereas all species of the "*Diandrae*" and "*Nibora*" clades are annuals (or short-lived perennials in regions with year-round warm climate). The perennial species of "*Gratiola*" also have a rhizomotous root system whereas the annual species of the "*Diandrae*" and "*Nibora*" clades have a delicate fibrous root system. Within *Gratiola* s.s. the annual, fibrous-rooted condition is plesiomorphic and was apparently retained in the "*Diandrae*" and "*Nibora*" clades, whereas the perennial-rhizomatous condition evolved in the lineage that gave rise to the "*Gratiola*" Clade (Figs. 4.10, 4.11). Some of the species with annual, fibrous roots inhabit sites that are seasonally wet and often become severely desiccated during the summer. Such is the case with *G. amphiantha*, *G. flava*, *G. graniticola*, *G. heterosepala*, *G. oresbia*, and *G. quartermaniae*, each of which inhabits ephemeral pools on rock outcrops or vernal pools in grasslands (Estes and Small, Chapter 2, in press; Estes and Small 2007). However, other species with annual, fibrous roots inhabit areas that are more stable hydrologically (i.e. *G. floridana*, *G. japonica*, *G. virginiana*). The perennial-rhizomotous species of the "*Gratiola*" Clade seem to inhabit continually wet sites, but *G.* aff. *pubescens* seems to have adapted to the arid conditions of Western Australia by developing an annual life cycle and fibrous root system.

Pedicel characteristics have often been considered taxonomically important within *Gratiola* s.s. (Bentham 1846; Pennell 1935; Barker 1986, 1992). The evidently pedicellate condition is plesiomorphic (Fig. 4.12) and is shared with the three outgroup genera, *Dopatrium*, *Hydrotriche*, and *Limnophila*, although in *Limnophila* there are many species with pedicellate flowers and some with sessile flowers. In the "*Sophronanthe*" Clade subsessile flowers are derived (Fig. 4.12). Within *Gratiola* s.s., the species of the "*Diandrae*" have evidently pedicellate flowers (Fig. 4.12) except for *G. amphiantha* which has the smallest flowers in the genus that are borne on very short pedicels. *Gratiola oresbia*, the sister species to *G. amphiantha*, also sometimes has nearly sessile flowers but as plants develop and mature these pedicels often elongate and become very distinct. In the "*Nibora*" Clade, *G. japonica* has very short pedicels, but in its sister species, *G. virginiana*, pedicel length is quite variable with some plants having very short pedicels (ca. 1 mm) and others having pedicels up to ca. 1.5 cm in length. *Gratiola* *griffithii*, the third member of *Nibora*, which was not sampled in the molecular analysis, is similar to *G. japonica* in having very short pedicels. The species of the "*Nibora*" Clade also have pedicels that are clavately-thickened distally. Except for its occurrence in *G. fluviatilis*, distally thickened pedicels are otherwise restricted to the "*Nibora*" Clade. In the "*Gratiola*" Clade, the species of the "*Gratiola aurea* Subclade" as well as *G. fluviatilis*, *G. linifolia*, and *G. officinalis* all have conspicuous long pedicels. Bentham (1846) placed most of the pedicellate species of the "*Gratiola*" Clade above in his *Pedunculatae* group. The species of the South American ("*G. peruviana* Subclade") and Australasian subclades (*G. nana* Subclade, *G. pubescens* Subclade, *G. latifolia* Subclade) have subsessile flowers and were included by Bentham in the *Sessiliflorae.* Evidently, the subsessile condition found in the South American and Australasian taxa represents the derived condition (Fig. 4.12). Among the Australasian taxa a reversal back to the plesiomorphic pedicellate condition occurred in the "*Gratiola pedunculata* Subclade."

Pennell (1935) stated that "within each section [of *Gratiola*] it is difficult to see definite stages of evolutionary progress between the species." One character that he thought was a significant "mark of evolutionary advance is the reduction or loss of the bractlets subtending the calyx." Within *Gratiola*, most species have a pair of bractlets (often interchangeably referred to as bracteoles) at the base of the calyx. In some species (e.g. *G. neglecta*) these bractlets are well-developed and extend beyond the sepals. In other species, such as *G. uliginosa*, these bractlets are quite small and shorter than the calyx. The loss of bractlets has apparently occurred three separate times, once in the lineage that gave rise to *G. ebracteata* and *G. heterosepala*, once in the lineage that gave rise to *G. amphiantha* and *G. oresbia*, and once in the lineage that gave rise to *G. ramosa* (Fig. 4.13). Interestingly, although in *G. ramosa* bractlets have been lost for the most part, occasional plants with one or even two very small reduced bractlets are sometimes encountered.

The nature of the sterile staminodia of *Gratiola* was considered by Bentham (1846) to be taxonomically significant whereas Pennell (1935) considered staminodial characters to be less significant and variable. All species of *Gratiola*, including the members of the divergent "*Sophronanthe*" Clade, have two fertile posterior stamens and two sterile anterior staminodia (in some species the anterior staminodia are completely absent). Evidently, *Gratiola* has evolved from an ancestor that had four stamens. Two of the three closest demonstrated relatives of *Gratiola*, *Hydrotriche* and *Dopatrium*, also have just two stamens (Raynal-Roques 1979, Fischer 1997, Fritsch et al. 2007). *Limnophila*, the third close relative of *Gratiola* usually has four stamens but some species have only two (Fischer 1997). Most of the other genera within the Gratioleae have four stamens (Bentham 1846). In addition to a decrease in stamen number, the staminodia in the species of *Gratiola* s.l. have also undergone significant evolutionary modifications (Fig. 4.14). In the "*Sophronanthe*" Clade, the staminodia are either greatly reduced and exist as minute ecapitate projections (remnants of the filaments) or they are absent altogether (Fig. 4.14). The staminodia in the "*Diandrae*" Clade are similar to those of "*Sophronanthe*" (Fig. 4.14). In the "*Nibora*" Clade, the staminodia are long and filiform and have minute subcapitate apices (Fig. 4.14). In the "*Gratiola*" Clade, most of the species have long, filiform, and subcapitate apices except for those in the *"Gratiola aurea* Subclade" which has staminodia that are minute and ecapitate (Fig. 4.14). The findings presented here indicate that staminodial variation is taxonomically important and useful. This conclusion conflicts with Pennell (1935) who noted that the degree of reduction in the staminodia seems nearly the same and equally variable in the different species.

Biogeographical History of *Gratiola*—The origin of *Gratiola*, like most of the genera in the Gratioleae, can ultimately be traced back to the New World, most likely to South America. Albach et al. (2005) proposed a New World origin for the Gratioleae as well as for the sister group to the Gratioleae, the "Angelonieae" Clade (Estes and Small, Chapter 2, in press). Pennell (1935) also speculated that the tribe originated in the Neotropics. Of the ca. 40 genera that comprise the Gratioleae, all but nine are restricted to the New World, with most of the New World taxa found in South and Central America. *Bacopa* and *Scoparia*, two of the nine genera that are not limited to the New World, are most diverse in South America but are represented in the Old World by only one or a few rather weedy Pantropical species, some of which also extend into the warm-temperate and subtropical regions of North America. Only six genera of the tribe are completely restricted to the Old World including *Adenosma* (East Asia), *Deinostema* (East Asia), *Dopatrium* (Africa/East Asia), *Hydrotriche* (Madagascar), *Limnophila* (East Asia, Africa, Australia), and *Morgania* (Australia). At present, it can only be speculated that *Adenosma*, *Deinostema*, and *Morgania* are part of the Gratioleae since none of these genera has been included in any molecular phylogenetic study. Estes and Small (Chapter 2, in press) and Fritsch et al. (2007) showed that *Gratiola* is sister to this Old World clade comprised of *Limnophila* (*Dopatrium* + *Hydrotriche*) and that this clade is derived from within a Neotropical. *Gratiola* is the only genus that, in terms of numbers of species, is represented almost equally in both the New and Old Worlds, thus raising questions about the major region of origin of *Gratiola*.

Based on the biogeographic analysis conducted during this study *Gratiola* originated in the New World, likely in North America (Fig. 4.15). All four major clades evidently originated in North America. The "*Sophronanthe*" Clade, with its two species, is entirely restricted to North America. The nine species of the "Diandrae" Clade are also restricted to North America. Two of the three species of the "*Nibora*" Clade occur in eastern Asia whereas the third occurs in North America. Within the "*Gratiola*" Clade, only the four species of the "*Gratiola aurea* Subclade" occur in North America, the other members of this clade occur in Eurasia, South America, and Australasia.

According to the biogeographic analysis (Fig. 4.15) the Eurasian, South American, and Australasian species of the genus are derived from North American ancestors. *Gratiola* migrated to Eurasia on at least three independent occasions, once in the lineage that gave rise to *G. japonica* ("*Nibora*" Clade), once in the lineage that gave rise to *G. officinalis* and *G. linifolia* ("*Gratiola*" Clade), and once in the lineage that gave rise to *G. fluviatilis* ("*Gratiola*" Clade). Due to the fact that the sister relationship between *G. officinalis* and *G. linifolia*, recognized in most of the equally parsimonious trees (Fig. 4.3B), is not supported in the strict consensus tree from the analysis of the combined molecular data set (Fig. 4.3A) any inferences concerning the biogeographical history of these two species must be interpreted with caution. *Gratiola* also migrated to South America giving rise to the lineage that includes the species of the "*Gratiola peruviana* Subclade." The biogeographic analysis also indicates that the Australasian taxa are derived from a South American ancestor. Unfortunately, the phylogenetic relationships among the South American and Australasian clades are poorly resolved (<50% BS) making it impossible to draw definite conclusions about the biogeographical history of these groups.

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APPENDIX; CHAPTER 4

Appendix 4.1. Accession information for the 34 species and 1 variety of *Gratiola* included in phylogenetic analyses, with voucher data (specimen origin, collector, collection number, and herbarium where specimen is deposited) and GenBank accession number(s) [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. The GenBank accession numbers will be filled in later when these sequences are published.

Gratiola amphiantha D. Estes and R.L. Small nom. nov. ined., USA, Georgia, DeKalb Co., *Wofford et al. s.n.* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Georgia, DeKalb Co., *Estes 06951* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola aurea*** Muhl., USA, South Carolina, Jasper Co., *Bozeman & Radford 11574* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Maryland, [county unknown], *Knapp s.n.* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola bogotensis*** Cortes, Colombia, *Bello 802* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Ecuador, *Holm-Nielsen et al. 4695* (MO), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola brevifolia*** Raf., USA,Tennessee, White Co., *Jones 4819* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Missouri, Howell Co., Summers 10206 (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola concinna*** Colenso, New Zealand, *DeLange AK-251835* (AK), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; New Zealand, *Ecroyd s.n.* (AK). ***Gratiola* aff. *concinna***, New Zealand, DeLange *4808* (AK), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola ebracteata*** Benth., USA, California, Shasta Co., *Estes 06046* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Lassen Co., *Schoolcraft 1664* (UC), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola flava*** Leavenw. ex Pennell, USA, Texas, Lee Co., *Estes 05707* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Grimes Co., *Estes 05703* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola floridana*** Nutt., USA, Tennessee, Bradley Co., *Estes 05056* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Florida, Jackson Co., *Estes 06934*, [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola fluviatilis*** Koidz., Japan, Shikoku, *Matsumoto et al. FOK-067712* (MBK), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; *Matsumoto et al. FOK-060974* (MBK), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola graniticola*** D.Estes, USA, Georgia, DeKalb Co., *Estes 05954* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Butts Co., *Estes 05742* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola heterosepala*** Mason and Bacig., USA, California, Tehama Co., *Estes 06035* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Shasta Co., *Estes 06042* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola hispida*** (Benth. ex Lindl.) Pollard, USA, Florida, Putnam Co., *Beck s.n.* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Bay Co., *Estes 03919* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola japonica*** Miq., Japan, Honshu, *Fugii et al. 8615* (MBK), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Shikoku, *Fukuhara FOK-066346* (MBK). ***Gratiola latifolia*** R.Br., Australia, New South Wales, *Adams 503* (AA), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; New South Wales, *Kodela 398* (NSW), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Tasmania, *Baker* and *Gray 1278* (HO), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola linifolia*** Vahl, Spain, *Delgado LD 963* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola nana*** Benth., Australia, Tasmania, *Ratkowsky 981* (MO), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; *Buchanan 16397* (HO), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola* aff. *nana*-Aust.**, Australia, New South Wales, *Jones 9* (CANB), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; *Crawford 7273* (CANB), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Victoria, *Bates 27109* (AD), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola* aff. *nana*-Tasman.**, Australia, Tasmania, *Orchard 5305* (AD), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola neglecta*** Torr., USA, Tennessee, Rutherford Co., *Estes 06214* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Alabama, Jackson Co., *Beck s.n.* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola officinalis*** L., Bulgaria, Rhodopi, *Frost-Olsen 4356* (MO), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Spain, [collector needed] *MS-632* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola oresbia*** B.L. Robins., Mexico, *Helgeras & Novelo 1078* (MEXU), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; *McDonald and Nesom 2539* (TEX), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola pedunculata*** R.Br., New Zealand, *DeLange s.n.* (AK), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Australia, Queensland, *Fensham4471* (BRI), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola peruviana*** L., Chile, *Leinbach s.n.* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Argentina, *Parodi 8208* (GH), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola pilosa*** Michx. var. ***epilis*** Pennell, USA, Florida, Highlands Co., *Correll & Correll 54015* (NY), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola pilosa*** Michx. var. ***pilosa***, USA, Tennessee, Moore Co., *Estes s.n. 03800* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Florida, Putnam Co., *Beck s.n.* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola pubescens*** R.Br., Australia, South Australia, *W.R. Barker 8364* (AD), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Western Australia, *Carter 477* (PERTH), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola* aff. *pubescens,*** Australia, Western Australia, *Brown* and *Brooks 559* (PERTH), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; *Keighery 11939* (PERTH), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola pumilo*** F. Muell., Australia, New South Wales, *Roberts 785* (CANB),[*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; South Australia, *Taplin 852* (AD), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola quartermaniae*** D.Estes, Canada, Ontario, *Estes 07955* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; USA, Texas, Williamson Co., *Estes 06140* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola ramosa*** Walt., USA, North Carolina, Hoke County, *Sorrie & Schilling s.n.* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Florida, Nassau Co., *Anderson 19217* (FSU), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola sexdentata*** R.Cunn. ex A. Cunn., New Zealand, North Island, *DeLange s.n.* (AK), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; New Zealand, South Island, *DeLange AK-203756* (AK), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola uliginosa*** Phil., Argentina, *Eskuche 2271-2* (SI), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]. ***Gratiola virginiana*** L., USA, North Carolina, Stokes Co., *Estes 06875* (TENN), [*trnS-trnG-trnG* GN; *trnQ-rps16* GN]; Mexico, *McVaugh 24356* (MICH), [*trnS*-*trnG*-*trnG* GN; *trnQ*-*rps16* GN]. ***Gratiola viscidula*** Pennell, USA, North Carolina, Stokes Co., *Estes 06870* (TENN), [*trnS*-*trnG*-*trnG* GN; *trnQ*-*rps16* GN]; Tennessee, Carter Co., *Somers 856* (TENN), [*trnQ*-*rps16* GN]; Kentucky**,** Rowan Co., *Cusick 28320* (MU), [*trnQ*-*rps16* GN]. ***Hydrotriche hottoniaeflora*** Zucc., USA, cultivated at University of Tennessee greenhouse, not vouchered.

Table 4.1. Results of phylogenetic analyses of the individual and combined datasets using *Gratiola pilosa* and *G. hispida* as outgroup taxa.

	trnS-trnG-trnG	***trnQ-rps16***	***trnS-trnG-trnG + rps16***
Maxtrees Setting	1000	1000	5000
No. Taxa	35	35	35
No. Accessions	65	66	68
% Missing Data	1%	<<1%	<1%
No. Excluded Regions	1	2	3
No. Excluded Characters	27	203	230
No. Coded Indels	53	25	78
No. Base Pairs	1832	1126	2980
Total No. Included Characters	1885	1161	3058
No. Parsimony Informative Characters (PICS)	344	227	572
No. Equally Parsimonious Trees	338	1,000 (stopped prematurely)	234

Table 4.1. continued

	trnS-trnG-trnG	***trnQ-rps16***	***trnS-trnG-trnG + rps16***
Tree Length	565	377	952
CI	0.789	0.806	0.793
RI	0.934	0.938	0.936

Table 4.2. Morphological characters and character states of *Gratiola* and outgroups examined during this study.

Character	Character States
1. Duration:	perrenial (0); annual (1)
2. Root type:	caudex (0); rhizomatous (1); fibrous (2)
3. Leaf surfaces papillose?	no (0); yes (1)
4. Leaf edge shape:	revolute-thickened (0); plane, not thickened (1)
5. Leaf edge pubescence type:	glabrous, glandular puberulent, or short pubescent (0); septate-pilose or hispid (1)
6. Pedicel type:	subsessile, < 2 mm long (0); evidently pedicellate, ≥ 5 mm long (1)
7. Bracteoles present?	yes (0); no (1)
8. Degree of sepal fusion:	free (0); partially connate (1)
9. Sepals equal in size?	no (0); yes (1)
10. Corolla shape:	salverform (0); funnelform (1)
11. Stamen number:	2 (0); 4 (1)
12. Stamen reduction:	abaxial reduced or absent (0); adaxial reduced or absent (1)
13. Staminode type:	absent or reduced, ecapitate (0); filiform and capitate (1)
14. Anther thecae orientation:	parallel with filament (0); transverse to filament (1)
15. Anther thecae disjunct?	no (0); yes (1)
16. Anther connective type:	not discoid-dilated (0); connective discoid-dilated (1)
17. Capsule dehiscence:	primarily septicidal (0); both septicidal and loculicidal (1); primarily loculicidal (2)
18. Capsule wall texture:	indurate (0); not indurate (1)
19. Capsule valve shape:	bifid (0); not bifid (1)
20. Seed shape:	ovoid (0); narrowly cylindrical (1)

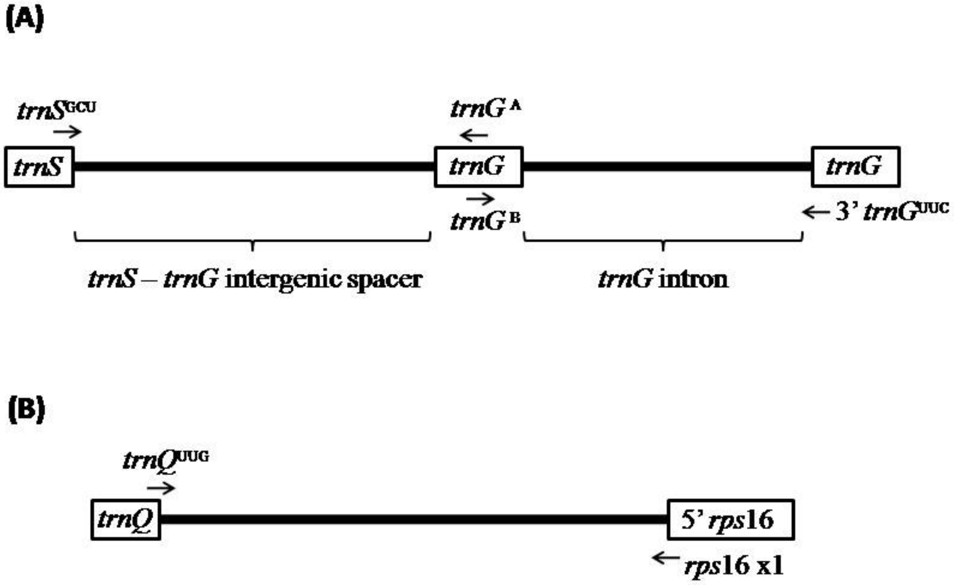


Fig. 4.1. Map of the chloroplast DNA regions surveyed in this investigation with arrows showing relative location of primer annealing sites. White boxes represent coding regions (exons); heavy black bars represent non-coding regions (intergenic spacers or introns). **A.** *trnS*-*trnG* intergenic spacer and *trnG* intron. **B.** *trnQ-rps16* intergenic spacer.

Fig. 4.2. Comparison of the strict consensus trees from the individual analyses of *trnQ-rps16* **(A)** and *trnS-trnG-trnG* **(B)**. Numbers above branches equal bootstrap percentages (BS). Asterisks represent branches with less than 50% BS. Dashed lines represent accessions not sampled for that particular molecular region. Clade 1 = "*Sophronanthe*" Clade. Clade 2 = "*Diandrae*" Clade. Clade 3 = "*Nibora*" Clade. Clade 4 = "*Gratiola*" Clade.

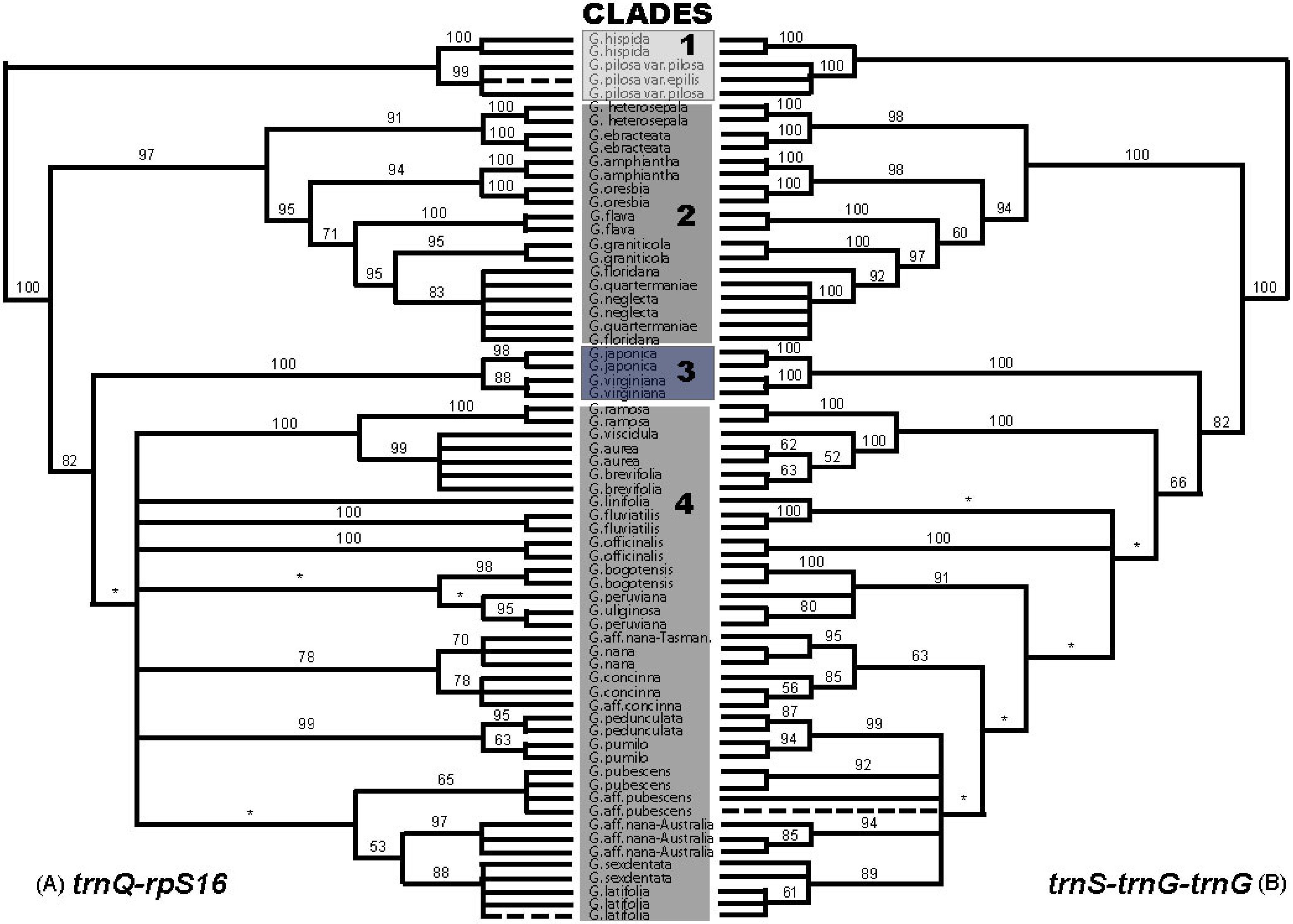
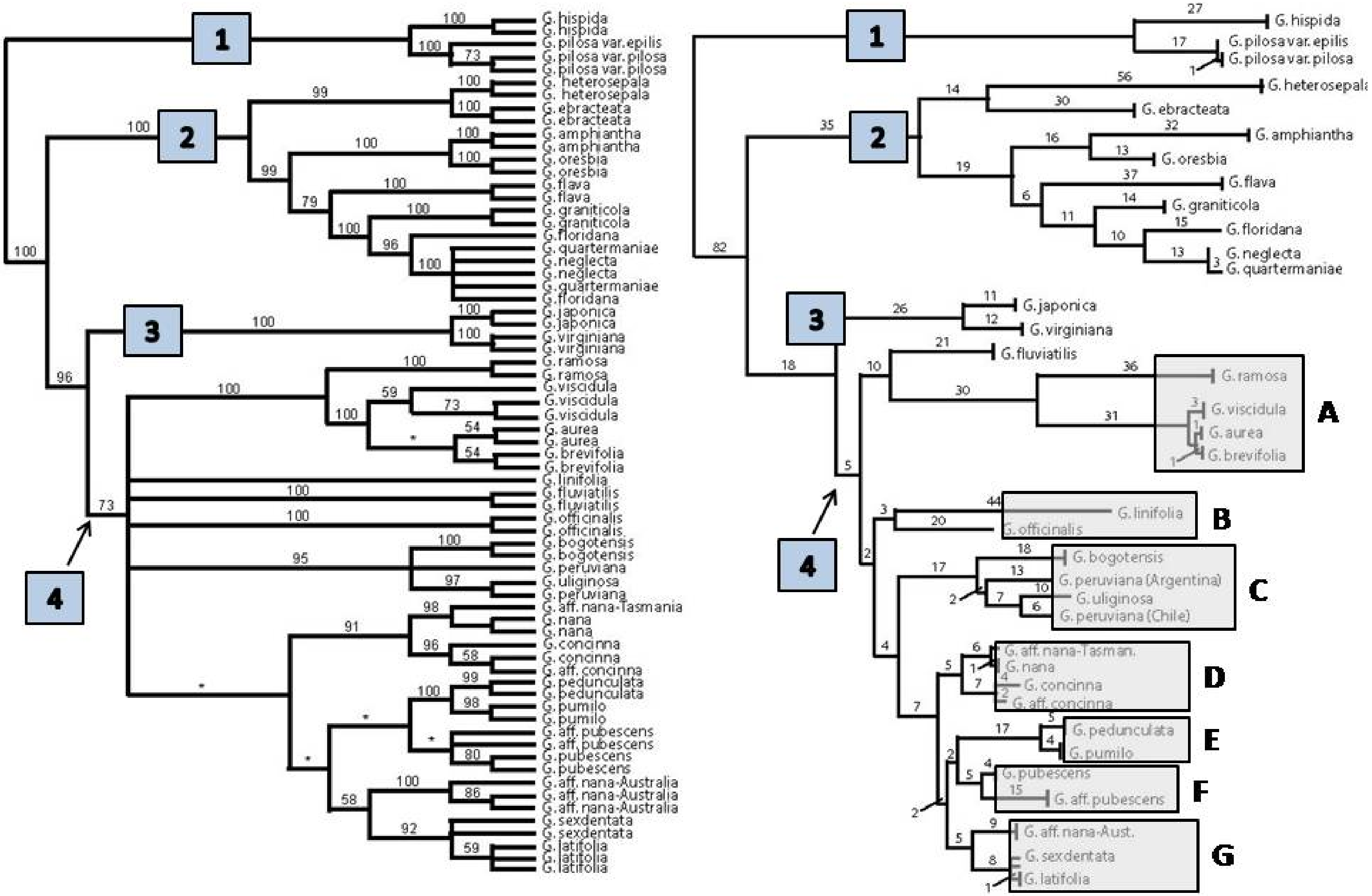

CLADES
1
2
3
4
G.hispida
G.hispida
G.pilosa var.pilosa
G.pilosa var.epilis
G.pilosa var.pilosa
G. heterosepala
G. heterosepala
G.ebracteata
G.ebracteata
G.amphiantha
G.amphiantha
G.oresbia
G.oresbia
G.flava
G.flava
G.graniticola
G.graniticola
G.floridana
G.quartermaniae
G.neglecta
G.neglecta
G.quartermaniae
G.floridana
G.japonica
G.japonica
G.virginiana
G.virginiana
G.ramosa
G.ramosa
G.viscidula
G.aurea
G.aurea
G.brevifolia
G.brevifolia
G.linifolia
G.fluviatilis
G.fluviatilis
G.officinalis
G.officinalis
G.bogotensis
G.bogotensis
G.peruviana
G.uliginosa
G.peruviana
G.aff.nana-Tasman.
G.nana
G.nana
G.concinna
G.concinna
G.aff.concinna
G.pedunculata
G.pedunculata
G.pumilo
G.pumilo
G.pubescens
G.pubescens
G.aff.pubescens
G.aff.pubescens
G.aff.nana-Australia
G.aff.nana-Australia
G.aff.nana-Australia
G.sexdentata
G.sexdentata
G.latifolia
G.latifolia
G.latifolia
(A) trnQ-rpS16
trnS-trnG-trnG (B)
100
99
91
97
100
100
94
100
100
95
71
100
95
95
83
100
98
88
100
100
100
99
100
100
82
*
*
98
*
95
78
70
78
99
95
63
65
*
97
53
88
100
100
100
100
98
100
100
98
94
100
60
100
97
92
100
100
100
100
100
100
82
62
52
63
66
100
*
100
*
100
91
80
95
63
85
56
*
87
99
94
92
*
94
85
89
61

Fig. 4.3. **A**. Strict consensus tree from the combined analysis of the *trnQ-rps16* and *trnS-trnG-trnG.* Numbers above branches represent bootstrap percentages (BS). Asterisks represent clades that are supported by less than 50% BS. **B**. A phylogram representing one of the equally parsimony trees from the combined analysis of *trnQ-rps16* and *trnS-trnG-trnG.* Numbers above branches represent branch lengths. In both trees, numbered boxes represent the major clades of *Gratiola* s.l.: 1 = "*Sophronanthe*" Clade; 2 = "*Diandrae*" Clade; 3 = "*Nibora*" Clade; 4 = "*Gratiola*" Clade. In **B,** lettered boxes represent the seven recognized subclades of the "*Gratiola*" Clade: A = *Gratiola aurea* Subclade; B = *Gratiola officinalis* Subclade; C = *Gratiola peruviana* Subclade; D = *Gratiola nana* Subclade; E = *Gratiola pedunculata* Subclade; F = *Gratiola pubescens* Subclade; G = *Gratiola latifolia* Subclade.



1
2
3
4
A
B
C
D
E
F
G
G. hispida
G. pilosa var. epilis
G. pilosa var. pilosa
G. heterosepala
G. ebracteata
G. amphiantha
G. oresbia
G. flava
G. graniticola
G. floridana
G. quartermaniae
G. neglecta
G. japonica
G. virginiana
G. ramosa
G. viscidula
G. aurea
G. brevifolia
G. linifolia
G. fluviatilis
G. officinalis
G. bogotensis
G. peruviana
G. uliginosa
G. aff. nana-Tasmania
G. nana
G. concinna
G. aff. concinna
G. pedunculata
G. pumilo
G. aff. pubescens
G. pubescens
G. aff. nana-Australia
G. sexdentata
G. latifolia
G. peruviana (Argentina)
G. peruviana (Chile)
G. aff. nana-Tasman.
G. aff. nana-Aust.

Fig. 4.4. Phylogeny of *Gratiola* and major clades identified in this study compared to the classifications of Bentham (1846) and Pennell (1935).

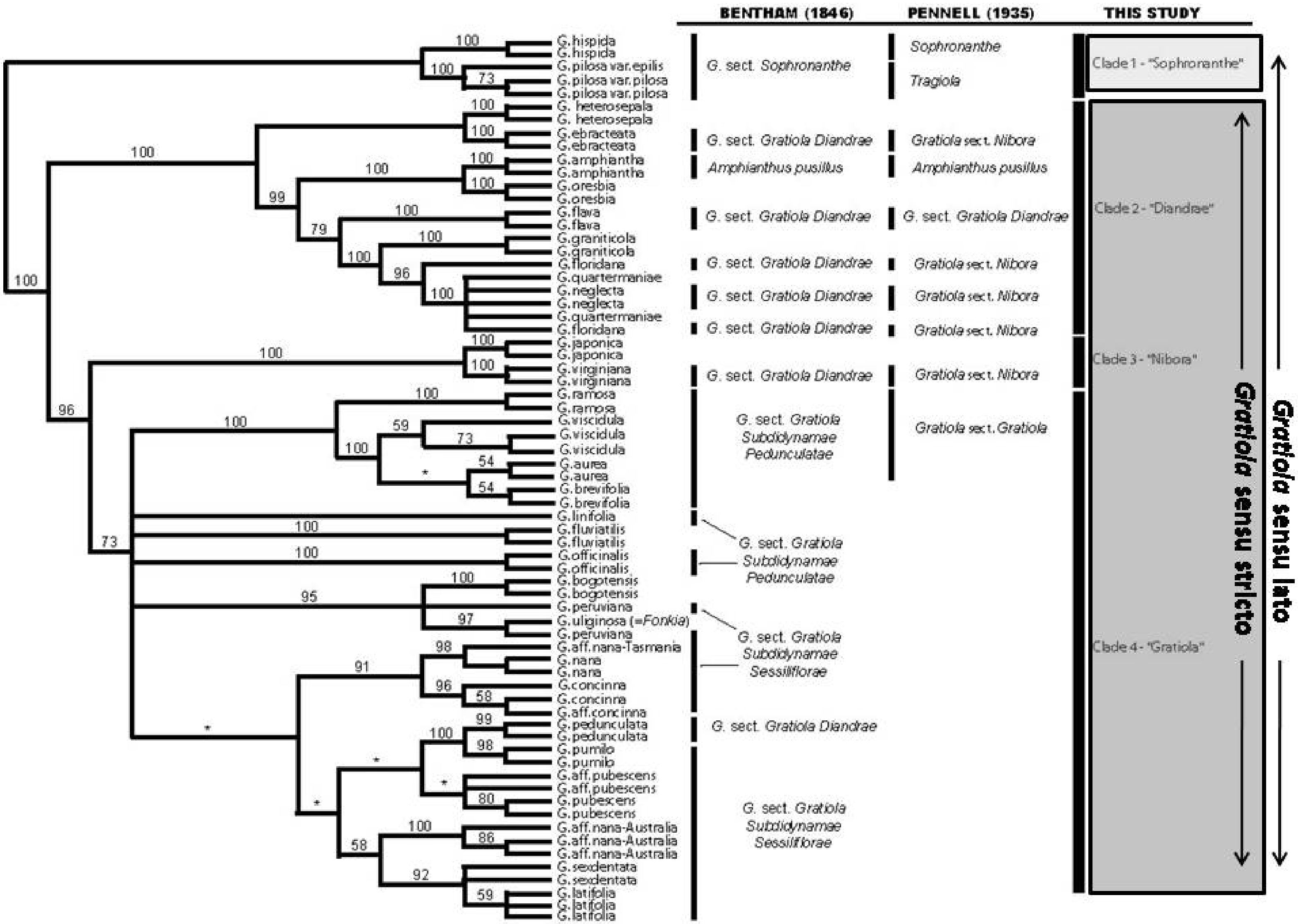
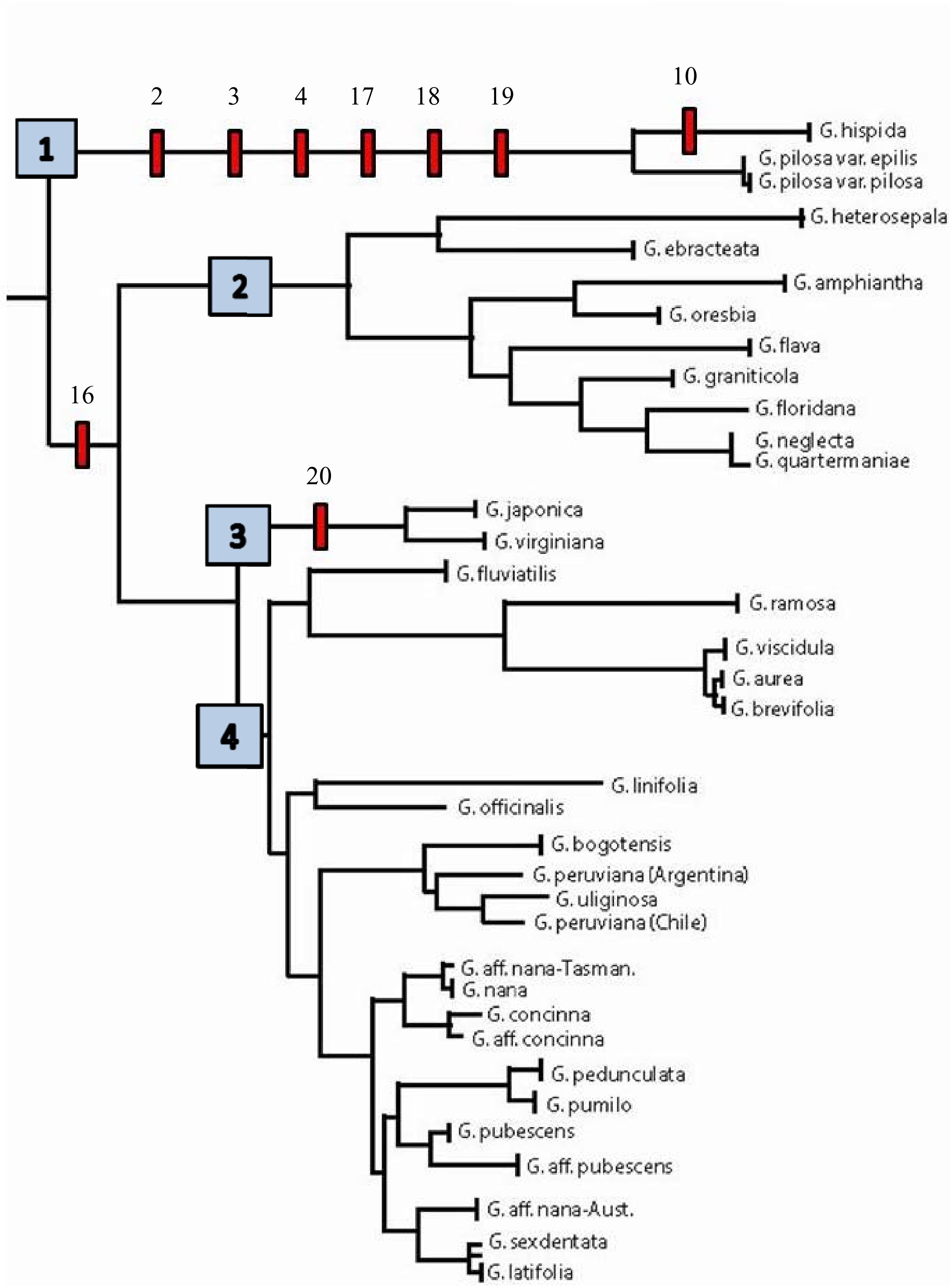

BENTHAM (1846)
PENNELL (1935)
THIS STUDY
G.hispida
G.hispida
G.pilosa var.epilis
G.pilosa var.pilosa
G.pilosa var.pilosa
G. heterosepala
G. heterosepala
G.ebracteata
G.ebracteata
G.amphiantha
G.amphiantha
G.oresbia
G.oresbia
G.flava
G.flava
G.graniticola
G.graniticola
G.floridana
G.quartermaniae
G.neglecta
G.neglecta
G.quartermaniae
G.floridana
G.japonica
G.japonica
G.virginiana
G.virginiana
G.ramosa
G.ramosa
G.viscidula
G.viscidula
G.viscidula
G.aurea
G.aurea
G.brevifolia
G.brevifolia
G.linifolia
G.fluviatilis
G.fluviatilis
G.officinalis
G.officinalis
G.bogotensis
G.bogotensis
G.peruviana
G.uliginosa (=Fonkia)
G.peruviana
G.aff.nana-Tasmania
G.nana
G.nana
G.concinna
G.concinna
G.aff.concinna
G.pedunculata
G.pedunculata
G.pumilo
G.pumilo
G.aff.pubescens
G.aff.pubescens
G.pubescens
G.pubescens
G.aff.nana-Australia
G.aff.nana-Australia
G.aff.nana-Australia
G.sexdentata
G.sexdentata
G.latifolia
G.latifolia
G.latifolia
G. sect. Sophronanthe
G. sect. Gratiola Diandrae
Amphianthus pusillus
G. sect. Gratiola Diandrae
G. sect. Gratiola Diandrae
G. sect. Gratiola Diandrae
G. sect. Gratiola Diandrae
G. sect. Gratiola Diandrae
G. sect. Gratiola Subdidynamae Pedunculatae
G. sect. Gratiola Subdidynamae Pedunculatae
G. sect. Gratiola Subdidynamae Sessiliflorae
G. sect. Gratiola Diandrae
G. sect. Gratiola Subdidynamae Sessiliflorae
Sophronanthe
Tragiola
Gratiola sect. Nibora
Amphianthus pusillus
G. sect. Gratiola Diandrae
Gratiola sect. Nibora
Gratiola sect. Nibora
Gratiola sect. Nibora
Gratiola sect. Nibora
Gratiola sect. Gratiola
Clade 1 - "Sophronanthe"
Clade 2 - "Diandrae"
Clade 3 - "Nibora"
Clade 4 - "Gratiola"
Gratiola sensu stricto
Gratiola sensu lato

Fig. 4.5. One of 234 equally parsimonious trees from the combined molecular analysis. Numbered red boxes represent morphological synapomorphies (2 = caudex root type; 3 = papillose leaf surfaces; 4 = revolute leaf margins; 10 = salverform corollas; 16 = anther connective discoid-dilated; 17 = capsules primarily loculicidally dehiscent; 18 = indurate capsules; 19 = bifid capsule valves; 20 = narrowly cylindrical seeds. Numbered gray boxes represent the four major clades of *Gratiola* sensu lato: 1 = "*Sophronanthe*" Clade; 2 = "*Diandrae*" Clade; 3 = "*Nibora*" Clade; 4 = "*Gratiola*" Clade.

10
2
3
4
17
18
19
1
G. hispida
G. pilosa var. epilis
G. pilosa var. pilosa
G. heterosepala
G. ebracteata
2
G. amphiantha
G. oresbia
G. flava
G. graniticola
16
G. floridana
G. neglecta
G. quartermaniae
20
3
G. japonica
G. virginiana
G. fluviatilis
G. ramosa
G. viscidula
G. aurea
G. brevifolia
4
G. linifolia
G. officinalis
G. bogotensis
G. peruviana (Argentina)
G. uliginosa
G. peruviana (Chile)
G. aff. nana-Tasman.
G. nana
G. concinna
G. aff. concinna
G. pedunculata
G. pumilo
G. pubescens
G. aff. pubescens
G. aff. nana-Aust.
G. sexdentata
G. latifolia

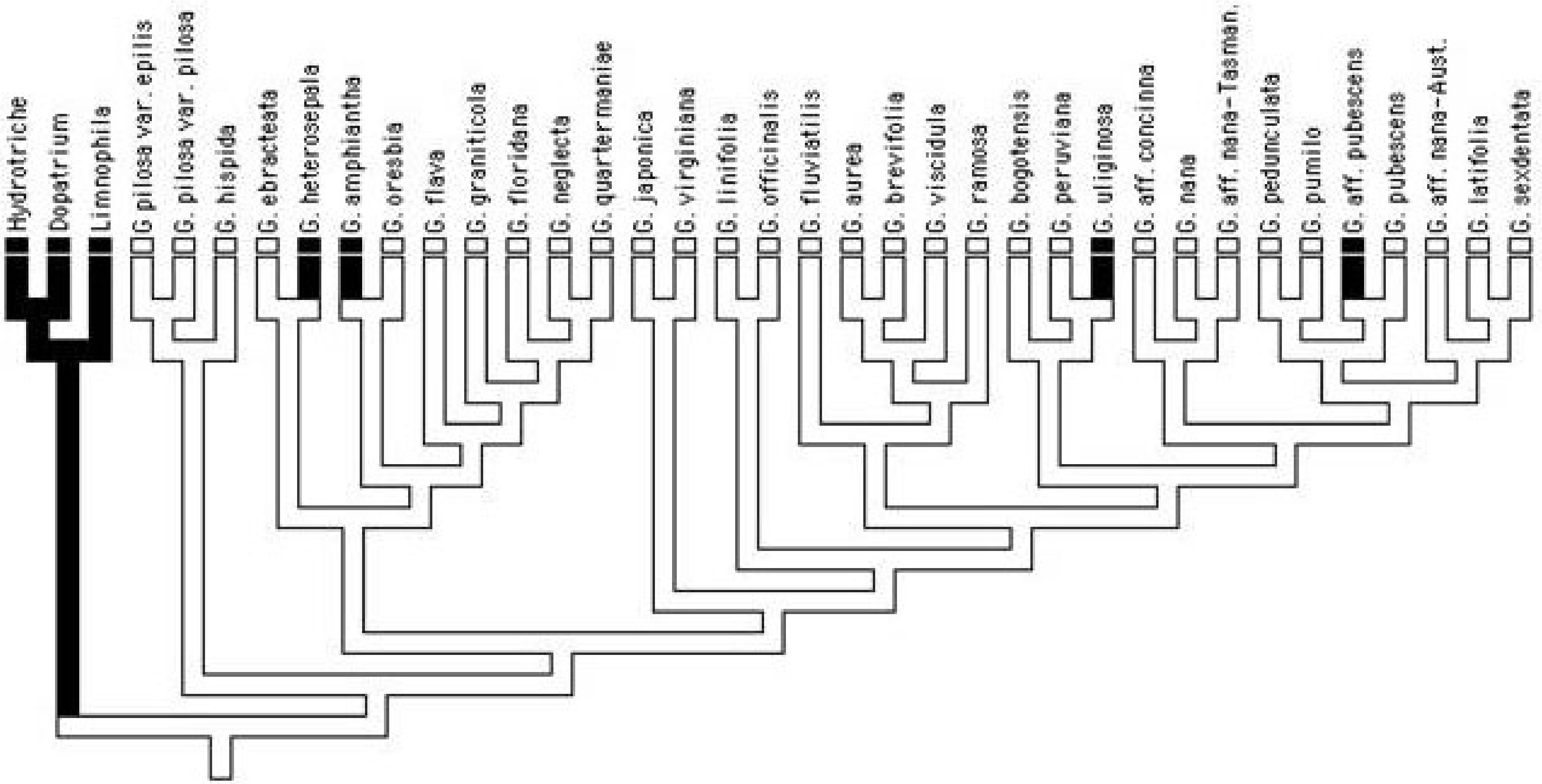


Fig. 4.6. Ancestral character state reconstruction for degree of sepal fusion. Black branches = sepals fused at least 25% of length; white branches = sepals free to base.

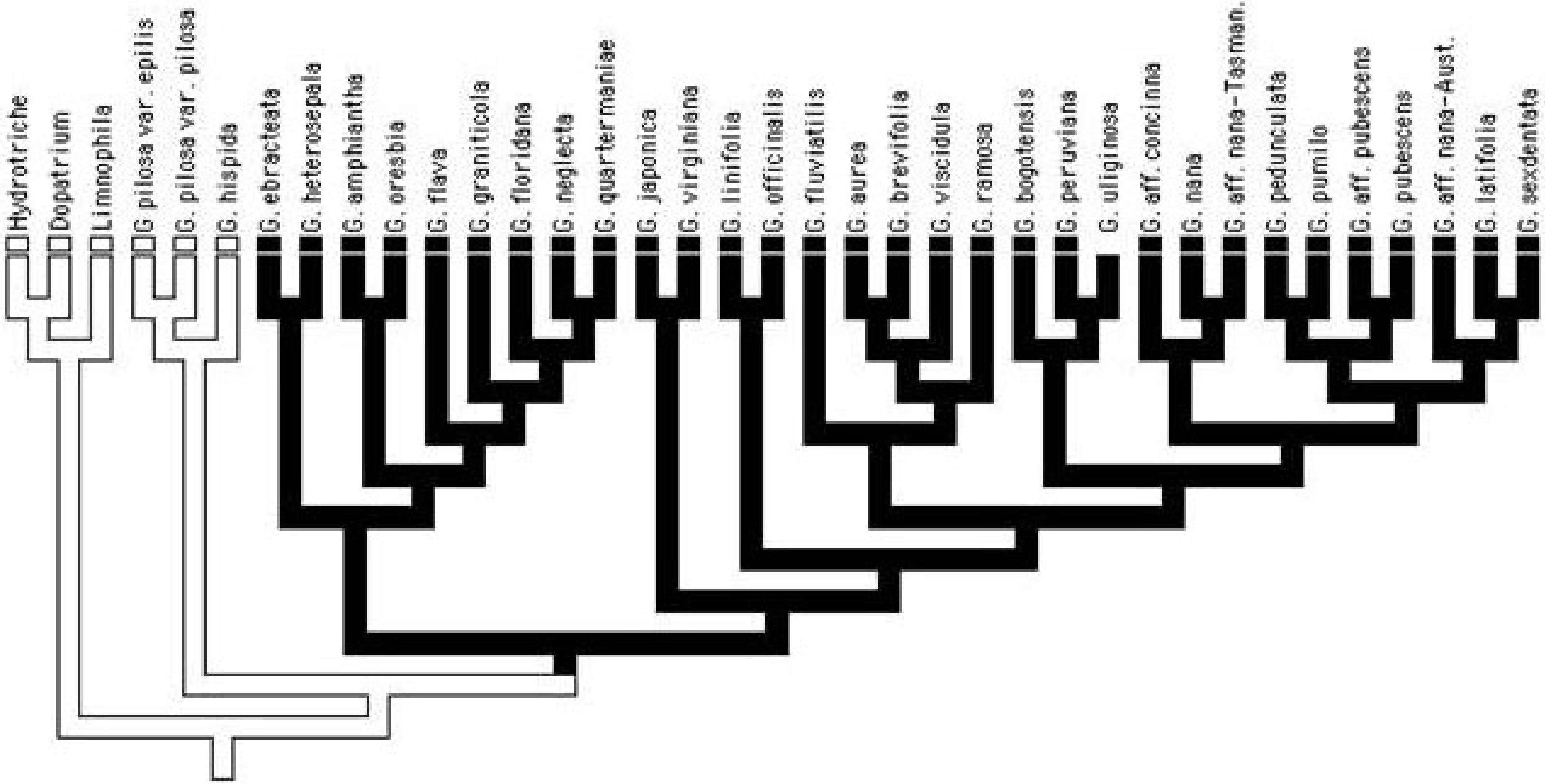


Fig. 4.7. Anther connective type: solid black branches = discoid dilated connectives; white branches = non-dilated connectives.

Fig. 4.8. Ancestral state reconstructions for anther morphology in *Gratiola.* A. Anther thecae orientation: solid black branches = anther thecae transversely oriented to filament; white branches = anther thecae oriented parallel to filament; lined branches = thecae orientation unknown.

Fig. 4.9. Ancestral character state reconstructions for leaf papillosity, leaf margin type, leaf pubescence, capsule texture, and capsule valve shape. White branches represent plants with papillose leaf surfaces, revolute leaf margins, indurate capsule walls, and bifid capsule valves. Black branches represent plants with smooth leaf surfaces, planar leaf margins, relatively thin-walled capsules, and non-bifid capsule valves.

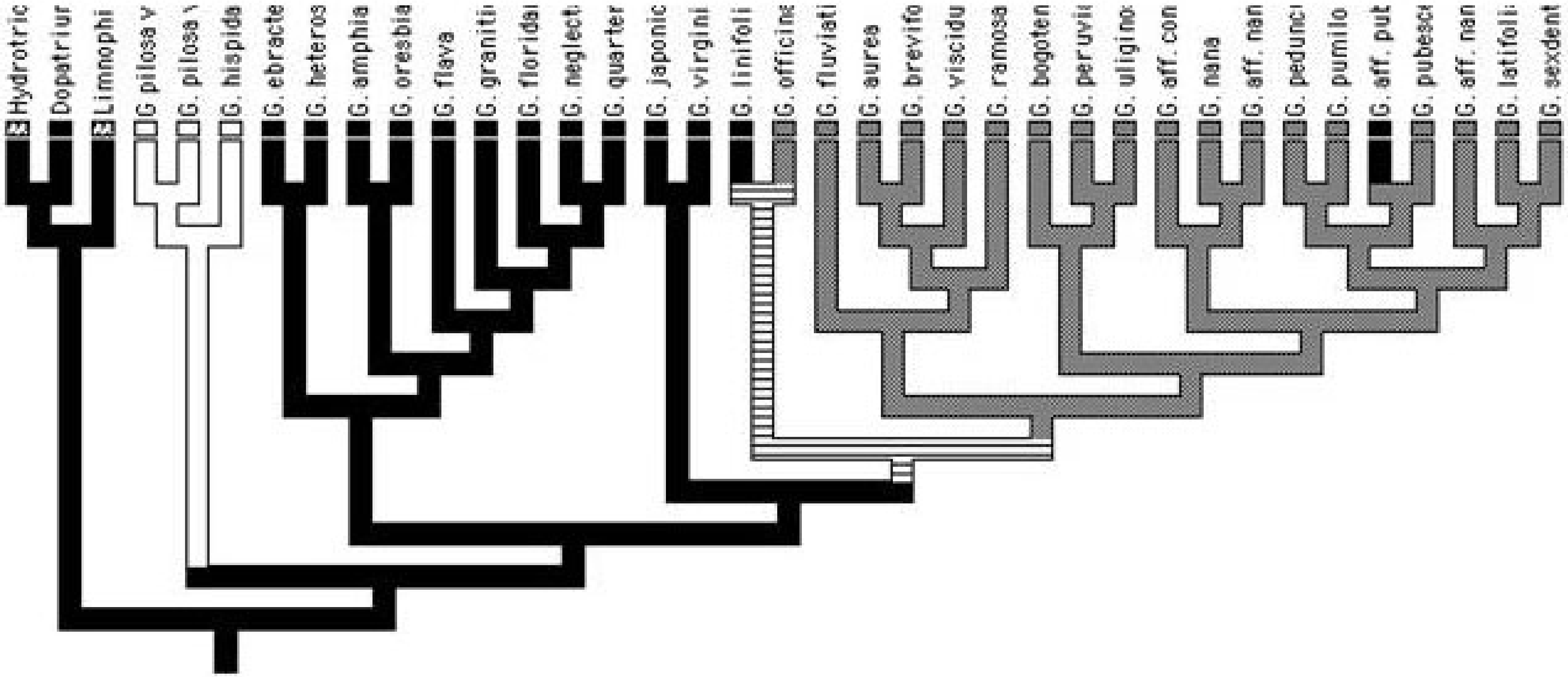


Fig. 4.10. Ancestral state reconstruction for root system type. Black branches = fleshy fibrous root system; gray branches = perennials with cord-like rhizomes; lined branches = equivocal; white branches = caudex type root system.

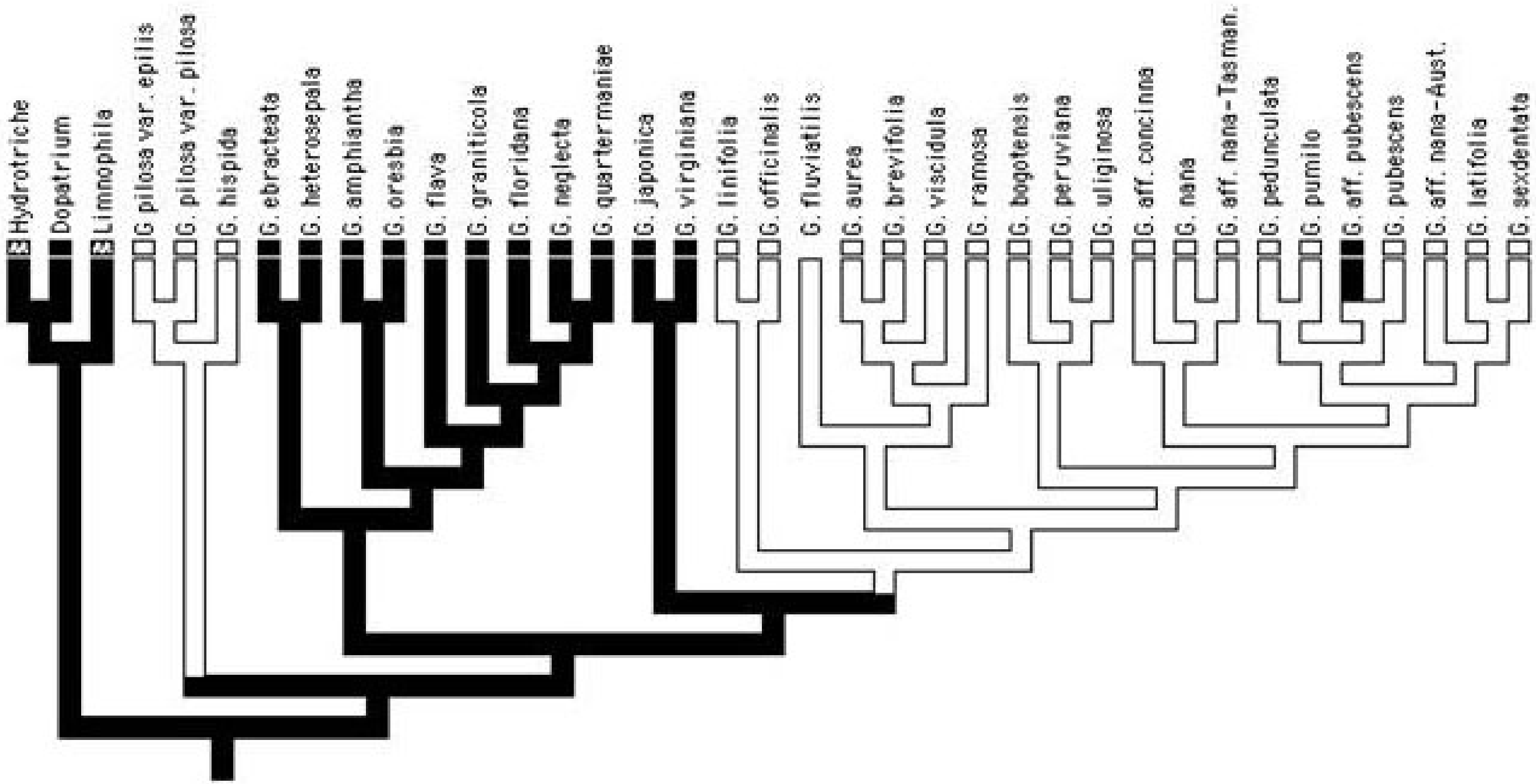


Fig. 4.11. Ancestral state reconstruction for life history. Black branches = annual or biennial life history; white branches = perennial life history.

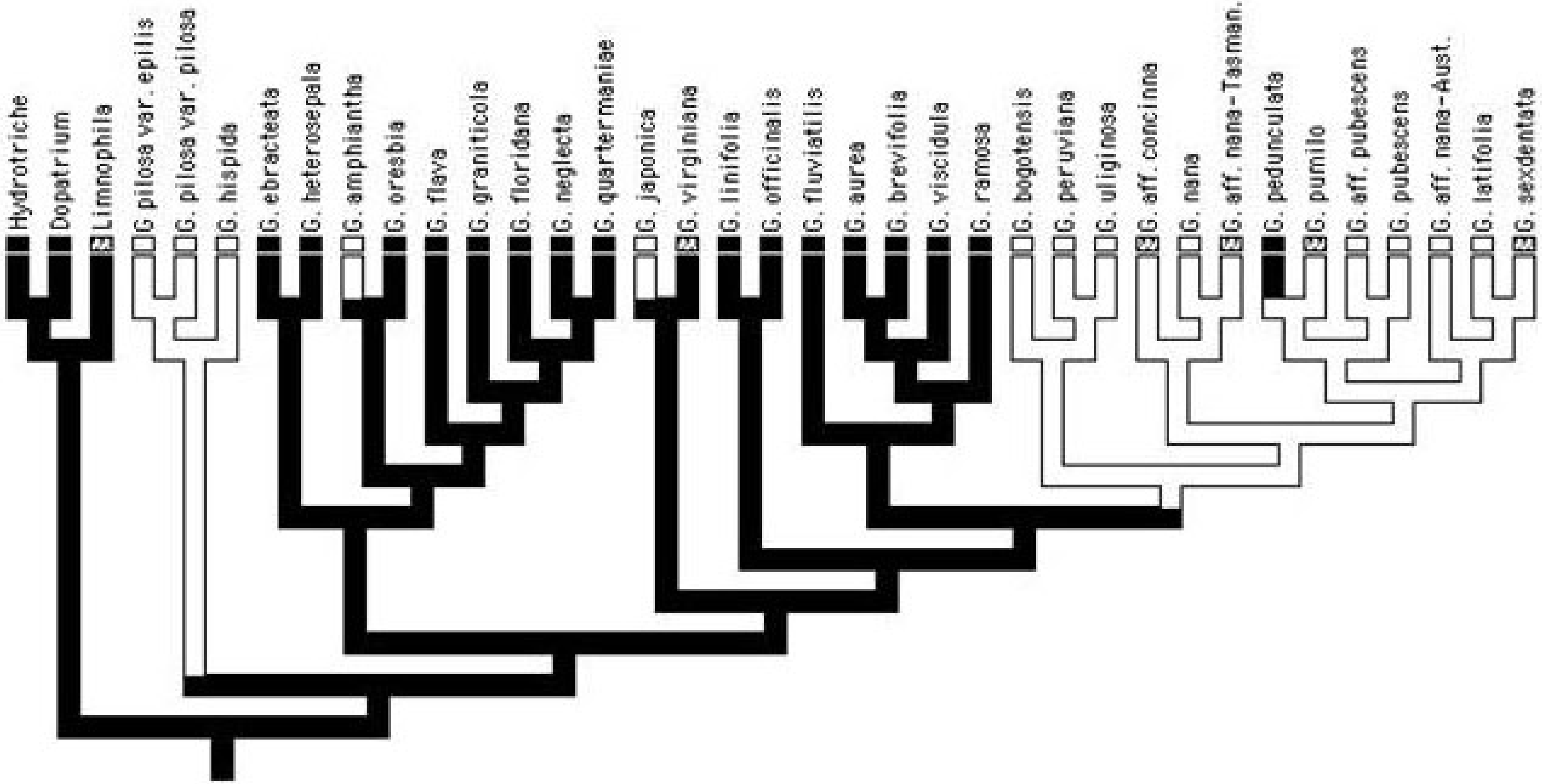


Fig. 4.12. Ancestral state reconstruction for pedicel type. Black branches = pedicels conspicuous and elongate; white branches = pedicels subsessile and < 2 mm long.

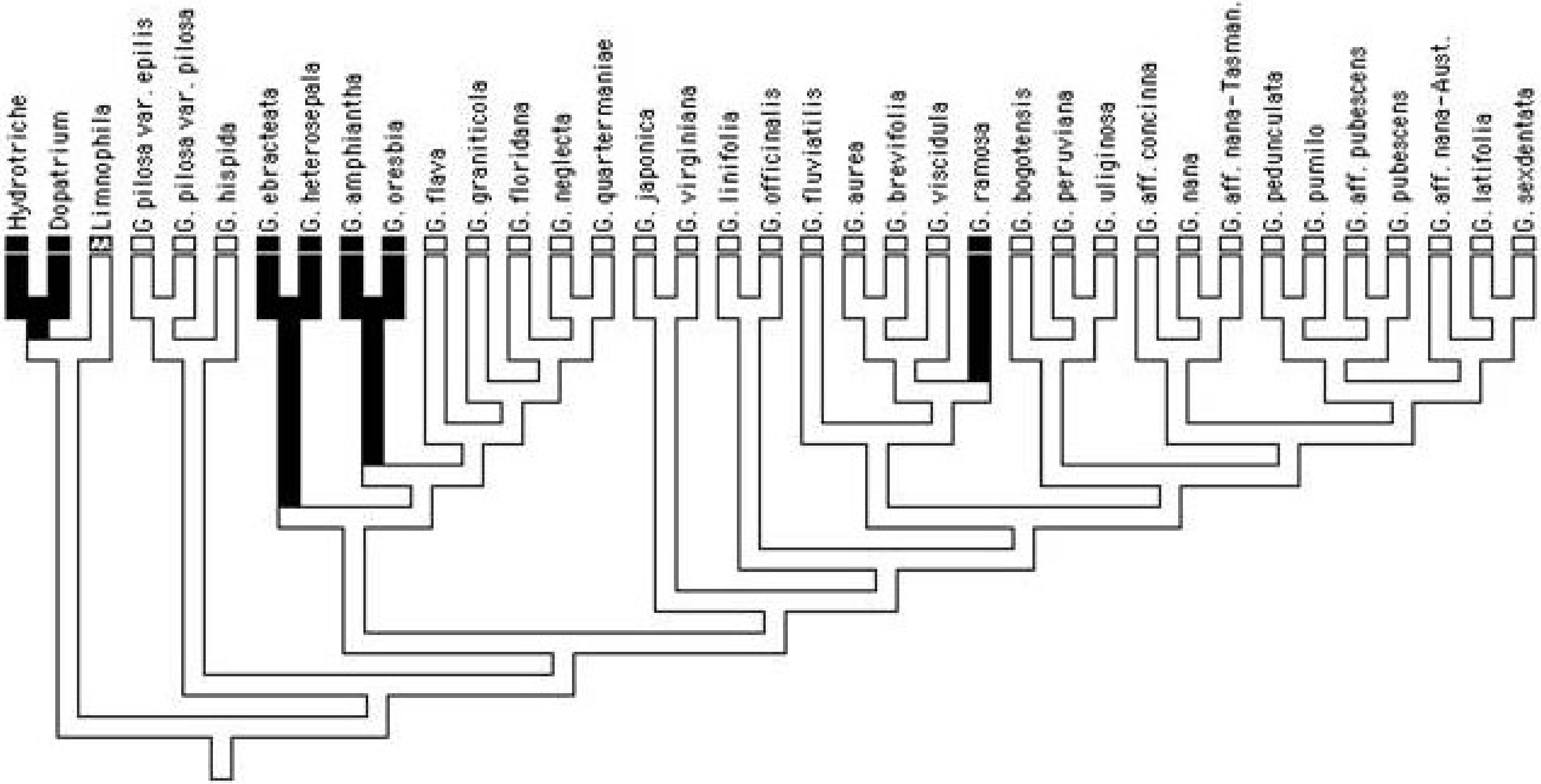


Fig. 4.13. Ancestral state reconstruction for presence/absence of bracteoles beneath the calyx. Black branches = bracteoles absent; white branches = bracteoles present.

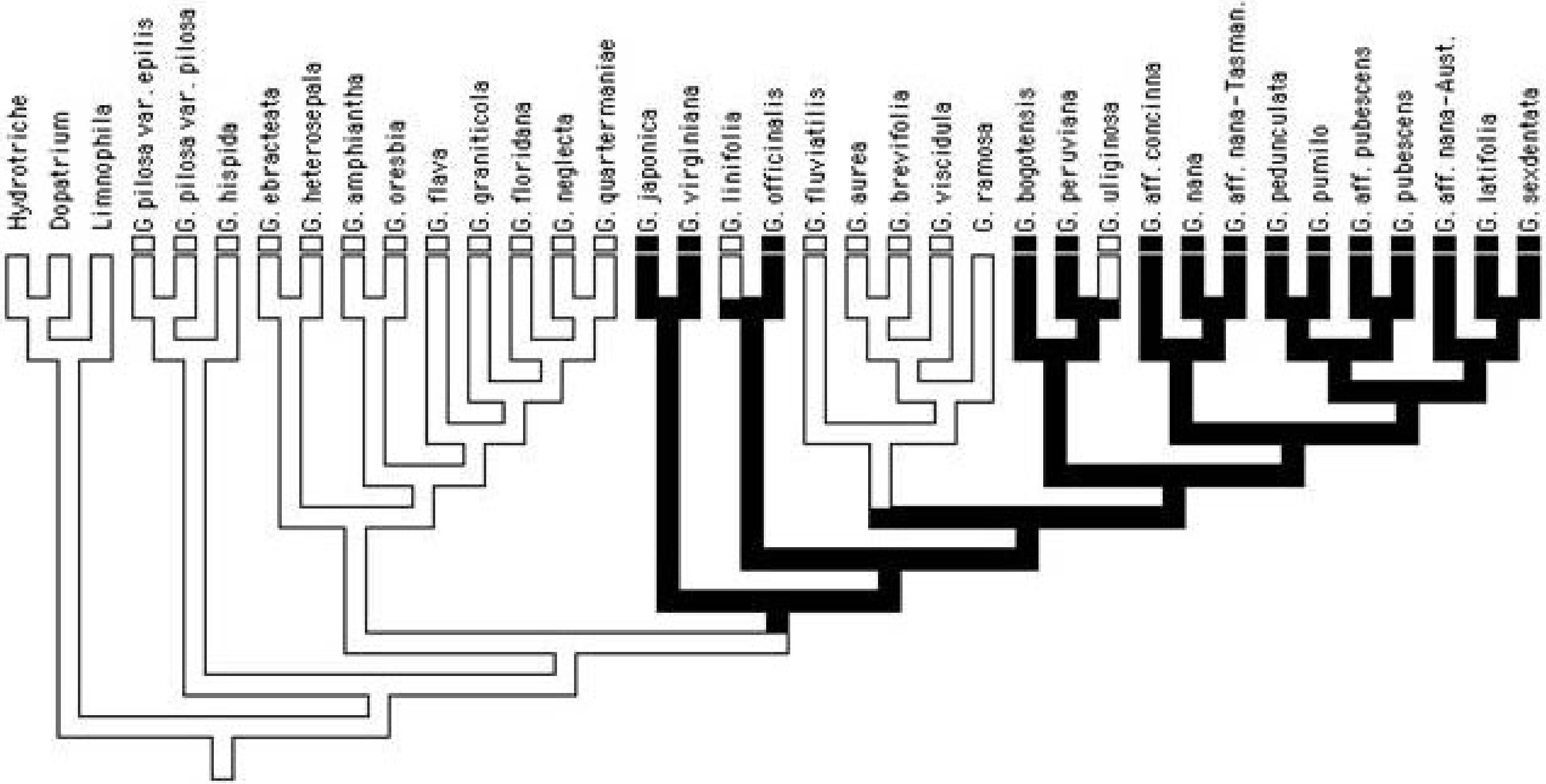


Fig. 4.14. Ancestral state reconstruction for staminode type. White branches = staminodia absent or poorly developed, short, and lacking capitates apices; black branches = staminodia well developed, filiform and with capitates apices.

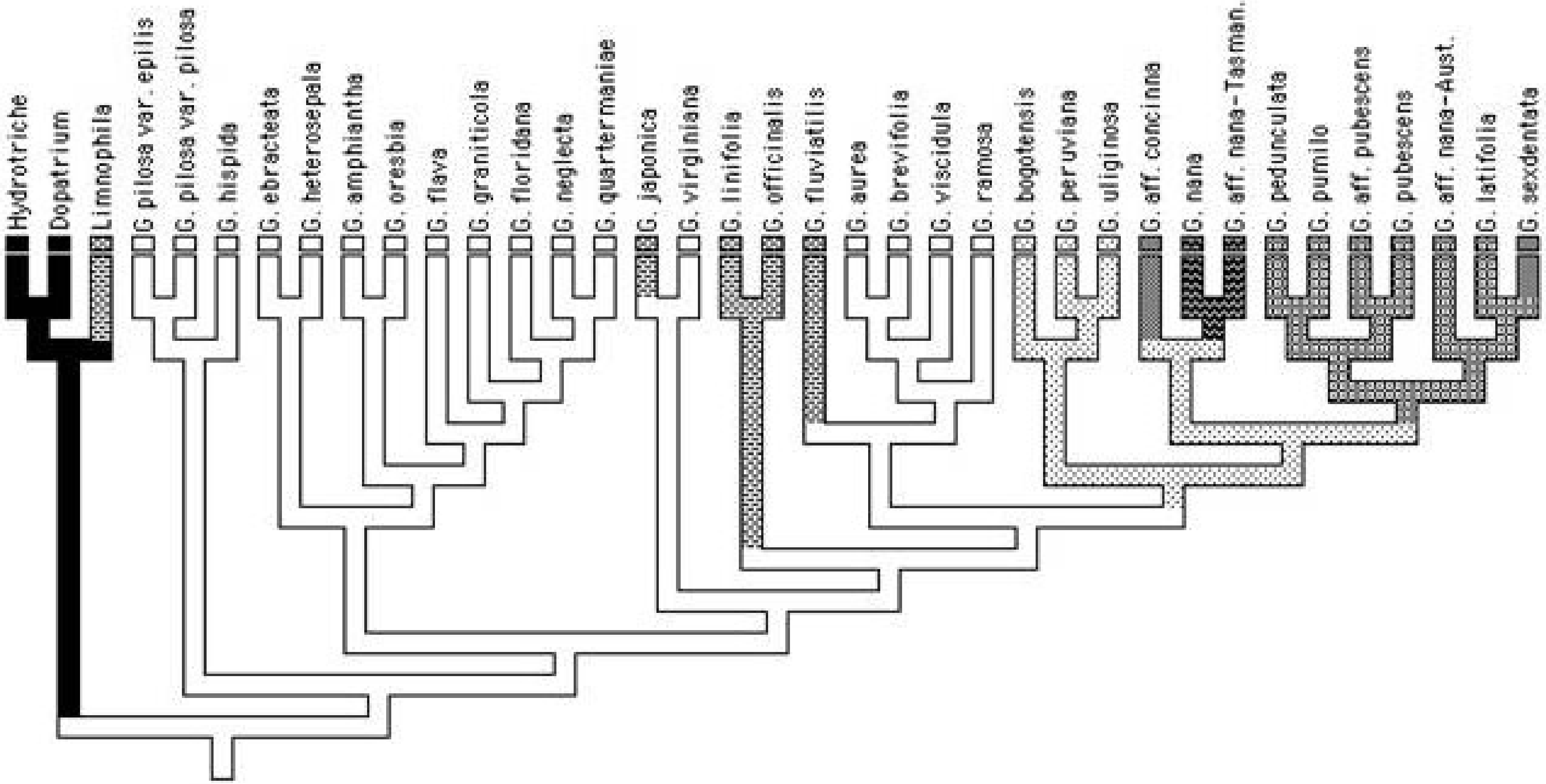


Fig. 4.15. Reconstruction of the geographic distribution of *Gratiola*. Black branches = Africa; bricked pattern = Eurasia; white = North America; cross-hatched = South America; wavy pattern = Tasmania; gray = New Zealand; patchwork pattern = Australia.

Chapter 5

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

General Conclusions—The three major goals of this dissertation were to determine the phylogenetic placement of *Gratiola* within Plantaginaceae tribe Gratioleae, to conduct a taxonomic study of the *Gratiola neglecta* complex, and to assess the phylogenetic relationships, morphological character evolution, and biogeographical history of the species within *Gratiola*. This dissertation is important because it (1) helps to clarify the relationships of the Gratioleae and its genera, a predominantly Neotropical group that has long been poorly understood; (2) adds to a growing body of phylogenetic data that can be used by future systematists to continue to resolve the phylogeny of Plantaginaceae; (3) provides a better understanding of a taxonomically difficult group for academic reasons; (4) adds to a growing body of literature regarding biogeographic connections between eastern Asia / eastern North America/ Mexico and South America / Australasia; (5) resulted in the identification of six new species to science, two of which were described during this study and an additional four which will be described in the near future.

This work represents the accumulation of three original research papers and a Doctoral Dissertation Improvement Grant proposal submitted to the National Science Foundation. Chapter 2 is in press and will be published in *Systematic Botany* in the spring of 2008. Chapter 3 was recently published in the *Journal of the Botanical Research Institute of Texas*. Chapter 4 will be submitted for publication to *Systematic Botany* in the spring of 2008.

Chapter 2 (Preliminary Phylogenetic Relationships of Plantaginaceae tribe Gratioleae with emphasis on the Monotypic Genus *Amphianthus*) addresses the phylogenetic relationships of Plantaginaceae tribe Gratioleae and specifically examines the placement of *Gratiola* and *Amphianthus* in the tribe. Before this study, only six of the ca. 40 genera from tribe Gratioleae had been included in published phylogenetic studies making it one of the least understood groups within the Plantaginaceae. *Amphianthus* and *Gratiola* were considered to be distinct genera, and *Amphianthus* was even considered to be a paleoendemic without any extant close relatives. Chloroplast DNA sequence data from the gene *ndhF* and the *trnS-trnG* intergenic spacer and *trnG* intron were used to explore relationships among 11 genera of the Gratioleae, including *Amphianthus* and six species from the major clades of *Gratiola*. This study is important in that it represents the most inclusive phylogeny of the tribe to-date. Four main conclusions can be drawn from this investigation. First, the Gratioleae is composed of six major clades. Second, a clade of *Limnophila* + *Hydrotriche* was identified as the sister group to *Gratiola* (including *Amphianthus*). Third, this work demonstrates that *Amphianthus* is phylogenetically embedded within a paraphyletic *Gratiola*, a finding that is supported by morphology. Finally, we formally proposed the transfer of *Amphianthus pusillus* to *Gratiola* resulting in *Gratiola amphiantha* D. Estes and R. Small nom. nov. ined.

Chapter 3 (Two New Species of *Gratiola* from Eastern North America and an Updated Circumscription for *Gratiola neglecta*) addresses the taxonomy of the *Gratiola neglecta* complex. Prior to this study, *G. neglecta* was considered to be a morphologically variable and wide-ranging species of temperate North America. This investigation employed an extensive amount of herbarium specimen examination, field work, and morphometric analyses to study this variable species. The major finding of this study is that *G. neglecta* actually includes two undescribed species, *G. quartermaniae* and *G. graniticola*, both of which were formally described as new-to-science during this study. The first of these, *G. quartermaniae*, is endemic to limestone cedar glades and dolomite prairies of eastern North America where it has a highly disjunct range including portions of middle Tennessee, northern Alabama, central Texas, northeastern Illinois, and southeastern Ontario, Canada. The second new species, *G. graniticola*, is endemic to granite outcrops of central Georgia. In addition to describing these two new species as new members of the *G. neglecta* complex, an updated circumscription was provided for *G. neglecta*, including a revised description for this species. Finally, a dichotomous key differentiating the species of the *G. neglecta* complex was included.

Chapter 4 (Molecular Phylogenetic Relationships, Morphological Evolution, and Biogeography of *Gratiola* with New Insights on the Status of *Fonkia*, *Sophronanthe*, and *Tragiola*) addresses the phylogenetic relationships between the species of *Gratiola* as well as the evolution of morphological characters and biogeographical history of the genus. To conduct this investigation, chloroplast DNA sequence data from the *trnS-trnG* intergenic spacer and *trnG* intron and the *trnQ-rps16* intergenic spacer were sampled from 34 of the 36 species of *Gratiola* including *G. uliginosa* (=*Fonkia uliginosa*), *G. hispida* (=*Sophronanthe hispida*), and *G. pilosa* (=*Tragiola pilosa*). The results from the phylogenetic analysis show that *Gratiola* is monophyletic (when *Fonkia* is treated as a *Gratiola*). The species of *Gratiola* were found to group into four major clades provisionally referred to as the "*Sophronanthe*" Clade, the "*Diandrae*" Clade, the "*Nibora*" Clade, and the "*Gratiola*" Clade. The "Sophronanthe" Clade includes just two eastern North American species and is sister to all remaining *Gratiola.* The "Diandrae" Clade contains nine North American species and is sister to the "*Nibora*" + "*Gratiola*" Clade. The "*Nibora*" Clade contains three species (one not sampled), one of North America and two in eastern Asia, and is sister to the "*Gratiola*" Clade. The fourth and largest clade in the genus is the "Gratiola" Clade with 22 species (one not sampled). Within the *"Gratiola*" Clade there are seven major subclades, each referred to by informal names associated with one of the major species of each of these groups (*Gratiola aurea* Subclade, *G. officinalis* Subclade, *G. peruviana* Subclade, *G. nana* Subclade, *G. latifolia* Subclade, *G. pedunculata* Subclade, *G. pubescens* Subclade). The results of the morphological analysis indicate that *Gratiola* s.l., as currently circumscribed, is not diagnosable by even a single morphological synapomorphy.

However, of the four major clades of *Gratiola*, "*Sophronanthe*" is morphologically quite different and is supported by numerous morphological synapomorphies suggesting that "*Sophronanthe*" possibly needs to be segregated from *Gratiola* s.s. as a distinct genus, in which case *Gratiola* s.s. would then be supported by two morphological synapomorphies. Many of the other characters investigated that have been considered important by previous workers (i.e. connate sepals, absence of bracteoles, pedicellate vs. subsessile flowers) were shown to be homoplasious in this study when considered across the whole genus, but still appear to have taxonomic utility, at least in distinguishing species. As for the biogeographical analysis, *Gratiola* likely originated in the Northern Hemisphere perhaps in North America. Evidence for this stems from the fact that all major clades of *Gratiola* are represented in North America and the phylogenetic data indicate that the Eurasian taxa and the Southern Hemisphere (South America and Australasia) taxa ultimately are derived from North American ancestors. Finally, results from this study indicate that *Fonkia* should be merged with *Gratiola* because the sole species of this genus is strongly supported as sister to *Gratiola peruviana* of the "*Gratiola*" Clade.

Future Directions—Although this study provided an important phylogenetic framework for the tribe Gratioleae and a thorough phylogeny of *Gratiola*, there is still much more work that needs to be done. The Gratioleae have been considered by some systematists to be a distinct family, the Gratiolaceae. Our work in Chapter 2 corroborates recent studies that demonstrate the Gratioleae are sister to the "Angelonieae" Clade and form an early diverging lineage within the Plantaginaceae sensu lato, and it shows that the Gratioleae are strongly supported phylogenetically. However, at this point, we feel that more data (molecular phylogenetic, morphological, cytological, and anatomical) from a variety of sources needs to be accumulated before it can be accurately determined whether the Gratioleae should be treated as a tribe within the Plantaginaceae or as a distinct family. In this study we were only able to sample 11 genera from the tribe, leaving 16 mostly Neotropical genera unsampled. In addition we were only able to sample a few species from the relatively large and somewhat heterogeneous genera *Bacopa*, *Limnophila*, and *Stemodia*, each of which has been segregated in the past into multiple smaller genera. In this study *Stemodia* was clearly shown to be polyphyletic. Future phylogenetic studies of the Gratioleae should concentrate on sampling the remaining members of the Gratioleae including multiple species from the larger genera. In addition to a more detailed phylogenetic investigation, much more work needs to be done to better characterize and clarify many of the genera within the Gratioleae. Only a few of the genera have been monographed recently meaning that most are poorly known taxonomically. Most of these genera are in need of a detailed taxonomic revision.

In our taxonomic study of the *Gratiola neglecta* complex (Chapter 3), we showed that *G. neglecta*, *G. quartermaniae*, and *G. graniticola* are morphologically distinct, tend to grow in different geographic regions, and generally inhabit different plant communities. However, in our phylogeny of *Gratiola* (Chapter 4), using non-coding chloroplast DNA sequence data we discovered that although *G. graniticola* is phylogenetically distinct and sister to the rest of the complex, the relationships between *G. neglecta*, *G. quartermaniae*, and *G. floridana* could not be resolved. More work is needed to clarify the relationships of these taxa. In particular, a molecular marker that evolves more quickly and therefore is likely to harbor more molecular variation (i.e. low-copy nuclear DNA sequences) should be employed to study the phylogenetic relationships of this complex. A study using such a molecular marker, in combination with a thorough amount of geographic sampling, could address not only the origin and relationships of these taxa but also the biogeographical history and haplotype diversity of the group. In addition, it would be helpful to obtain chromosome counts from *G. graniticola*, *G. quartermaniae*, and *G. floridana* in order to better understand the relationships of these taxa. Future investigations into this complex should also include a hybridization study to determine whether the taxa are reproductively isolated. Any study that attempts to resolve this complex, should also consider include the other members of the "Diandrae" Clade (*G. amphiantha*, *G. ebracteata*, *G. flava*, *G. heterosepala*, *G. oresbia*).

In the phylogenetic study of *Gratiola* (Chapter 4), we showed that *Gratiola* is monophyletic when circumscribed to include *Fonkia*. We also presented a phylogeny depicting the relationships of 34 of the 36 species of the genus, and discussed the evolution of the major morphological characters that have been used by previous taxonomists to differentiate the sections and species of *Gratiola.* Future work on this problem is needed in several areas. First, the support for the "*Gratiola*" Clade was quite low and the relationships of the major subclades within this clade were either poorly resolved or poorly supported. It could be beneficial to gather additional sequence data from other molecular regions, including possibly other chloroplast non-coding sequences as well as nuclear sequences. Several species groups (i.e. *G. aurea* Subclade, *G. nana* Subclade, *G. peruviana* Subclade, *G. pubescens* Subclade, *G. latifolila* Subclade) were discovered during this study whose relationships either could not be resolved with cpDNA sequence data or whose relationships were poorly supported. Given the wide divergence of chromosome numbers within the genus, polyploidy has certainly played a role in the evolution of *Gratiola.* Therefore, attempts to further resolve these groups should likely employ a biparental marker (i.e. low copy nuclear DNA) that evolves fast enough to harbor enough molecular variation to resolve these groups. Regarding the morphology of *Gratiola*, more work is needed to identify possible synapomorhies for *Gratiola* s.l., *Gratiola* s.s., and the major clades and subclades within the genus. This should include additional morphological characters as well as anatomical characters and chromosome counts for all species. In terms of the biogeography of *Gratiola*, this study was only able to address major biogeographical concepts such as the origin of *Gratiola* and the origin of its major clades. More work is needed to examine the biogeographical patterns of the subclades and species complexes. Ideally, future biogeographical analyses should employ a DIVA analysis and should consider employing a molecular clock to identify possible dates of clade divergence.

VITA

Dwayne was born June 7, 1978 and was raised by his mother, Shirley, in rural Giles County, Tennessee. His passion for studying plants began in 1992 during his sixth grade year at Bridgeforth Middle School in the town of Pulaski. Dwayne spent the next six years of his life roaming the hills and hollers near his home in the small rural community of Bunker Hill studying the local flora. In 1996 he graduated from Giles County High School but early attempts to attend college were unsuccessful and so he worked several jobs until finally, in 1999, he committed himself to earning a college degree and enrolled at Columbia State Community College (CSCC). After receiving an A.S. degree in pre-biology in 2001 from CSCC, he enrolled in the biology program at Middle Tennessee State University where he graduated with a B.S. in Plant Biology in 2003. Upon graduating from MTSU, Dwayne was awarded a graduate assistantship at the University of Tennessee. He earned his Doctor of Philosophy degree in Ecology and Evolutionary Biology in January 2008. As of spring 2007, Dwayne has accepted an Assistant Professorship in the Department of Biology at Austin Peay State University in Clarksville, Tennessee.

Currently, he, Shawna, Madison, and Josie reside in Clarksville and are living happily ever after....