Diversification of *Galium* within Tribe Rubieae (Rubiaceae): Evolution of Breeding Systems, Species Complexes, and Gene Duplication

Valerie L. Soza

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2010

Program Authorized to Offer Degree:
Department of Biology

University of Washington
Graduate School

This is to certify that I have examined this copy of a doctoral dissertation by

Valerie L. Soza

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Chair of the Supervisory Committee:

_____
Richard G. Olmstead

Reading Committee:

_____
Richard G. Olmstead

_____
Veronica Di Stilio

_____
George J. Kenagy

Date:_____

In presenting this dissertation in partial fulfillment of the requirements for the doctoral degree at the University of Washington, I agree that the Library shall make its copies freely available for inspection. I further agree that extensive copying of the dissertation is allowable only for scholarly purposes, consistent with “fair use” as prescribed in the U.S. Copyright Law. Requests for copying or reproduction of this dissertation may be referred to ProQuest Information and Learning, 300 North Zeeb Road, Ann Arbor, MI 48106-1346, 1-800-521-0600, or to the author.

Signature_____

Date_____

University of Washington

Abstract

Diversification of *Galium* within Tribe Rubieae (Rubiaceae): Evolution of Breeding Systems, Species Complexes, and Gene Duplication

Valerie L. Soza

Chair of the Supervisory Committee:
Professor Richard G. Olmstead
Department of Biology

Tribe Rubieae is unique within Rubiaceae with its herbaceous habit, temperate distribution, and whorls of leaf-like structures. This dissertation examines the evolution of the tribe, evolution a clade within the tribe (*Cruciata*-*Galium*-*Valantia* [CGV] clade), and finally a section of the genus *Galium* (sect. *Baccogalium*). A molecular phylogeny of the tribe, based on three chloroplast (cp) regions, strongly supports seven major clades within the tribe. The resulting phylogeny is used to examine geographic distribution patterns and evolution of leaf-like whorls in the tribe. An Old World origin of the tribe is inferred, followed by at least eight dispersal events into North America. The ancestral whorl morphology of the tribe is inferred as composed of six organs, from which whorls of four organs are derived. Polygamy, dioecy, and hermaphroditism all occur within the CGV clade, in which dioecy is hypothesized to have evolved from hermaphroditism via polygamy. A molecular phylogeny of the CGV clade, based on cp and nuclear ribosomal data, strongly supports nine lineages of New World *Galium* taxa. The resulting phylogeny is used to examine evolution of breeding systems, fruit types, and fruit hairs. Dioecy is inferred to have arisen at least three times from hermaphroditism; polygamy is

inferred to have arisen at least twice from dioecy and at least six times from hermaphroditism. Polygamy appears to be a terminal condition in the CGV clade and not a pathway to dioecy. Fruit characters traditionally used in the taxonomy of this group have arisen multiple times within this clade and are not reliable indicators of shared evolutionary history. Approximately 30 *Galium* taxa are designated rare by the California Native Plant Society, ten of which occur within *G.* sect. *Baccogalium*. Within *G.* sect. *Baccogalium,* relationships among taxa are not well resolved with either cp or nuclear data. A molecular phylogeny of the section, based on cp data, indicates that subspecies from three species complexes do not form respective monophyletic groups, which will have implications for management of rare infraspecific taxa. A molecular phylogeny based on nuclear *RPB2* indicates that *Galium* taxa examined lack the *I* copy and contain a duplicated *D* copy.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to thank my advisor Richard Olmstead and my graduate committee members: Toby Bradshaw, Veronica Di Stilio, Jim Kenagy, and Gabrielle Rocap. I would like to thank Friedrich Ehrendorfer for his continued discourse, comments, knowledge, and information provided about the tribe, and review of results produced during this research; and to Ben Hall for his suggestions and guidance. I am grateful to Birgitta Bremer, Jean-François Manen, and Sylvain Razafimandimbison for providing DNA; to Steve Boyd, Pilar Catalán, Andrew Eckhert, Robert Lauri, Naomi Fraga, Jaime Pacheco, Nancy Refulio, Lars Gunnar Reinhammar, and Johan Rova for tissue samples; to J. Travis Columbus, Saruhén Avila Moreno, Gilberto Ocampo, Jose Delgadillo Rodriguez, Yuan Yaowu, and the Instituto de Botánica Darwinion for assistance in field collection of samples; to Rachel Meyer for lab assistance; to Veronica Di Stilio, Friedrich Ehrendorfer, Jim Kenagy, Rolf Rutishauser, and two anonymous reviewers for valuable comments on this dissertation; and to the following herbaria for use of specimens - RSA, LL/TEX, MO, and WTU. I thank the following funding sources: American Society of Plant Taxonomists, Botanical Society of America, University of Washington (UW) Department of Biology Giles Graduate Student Field Research Award, Denton Writing Fellowship, and Molecular Phylogenetics and Systematics Graduate Fellowship, UW Graduate School Stroum Endowed Minority Dissertation Fellowship, and NSF grants DEB-0542493 and EF-0431184 to Richard Olmstead.

DEDICATION

For my mom, Patricia C. Soza, and dad, Victor T. Soza.

CHAPTER I

MOLECULAR SYSTEMATICS OF TRIBE RUBIEAE (RUBIACEAE): EVOLUTION OF MAJOR CLADES, DEVELOPMENT OF LEAF-LIKE WHORLS, AND BIOGEOGRAPHY[1]

SUMMARY

Rubieae are centered in temperate regions and characterized by whorls of leaf-like structures on their stems. Previous studies that primarily included Old World taxa identified seven major clades with no resolution between and within clades. In this study, a molecular phylogeny of the tribe, based on three chloroplast regions (*rpoB-trnC, trnC-psbM, trnL-trnF-ndhJ*) from 126 Old and New World taxa, is estimated using parsimony and Bayesian analyses. Seven major clades are strongly supported within the tribe, confirming previous studies. Relationships within and between these seven major clades are also strongly supported. In addition, the position of *Callipeltis,* a previously unsampled genus, is identified. The resulting phylogeny is used to examine geographic distribution patterns and evolution of leaf-like whorls in the tribe. An Old World origin of the tribe is inferred from parsimony and likelihood ancestral state reconstructions. At least eight subsequent dispersal events into North America occurred from Old World ancestors. From one of these dispersal events, a radiation into North America, followed by subsequent diversification in South America, occurred. Parsimony and likelihood ancestral state

[1] This chapter was first published in *Taxon*: Soza, V. L. and R. G. Olmstead. 2010. Molecular systematics of tribe Rubieae (Rubiaceae): evolution of major clades, development of leaf-like whorls, and biogeography. *Taxon* 59: 755—771 (http://www.ingentaconnect.com/content/iapt/tax/2010/00000059/00000003/art00008).

reconstructions infer the ancestral whorl morphology of the tribe as composed of six organs. Whorls composed of four organs are derived from whorls with six or more organs. Transitions between four and six or more organs per whorl are common within the tribe, whereas reduction to two leaves at a node is derived and rare.

INTRODUCTION

Rubiaceae (coffee family) is the fourth-largest angiosperm family, comprising approximately 660 genera and 11,500 species and classified into 42 tribes (Robbrecht and Manen, 2006). Most of the family is tropical and woody. Rubieae is the only tribe centered in temperate regions, but obtains a cosmopolitan distribution. Most of its members are herbaceous and adapted to xeric habitats (Robbrecht, 1988; Jansen et al., 2000). Rubieae are a monophyletic group, sharing both morphological and molecular synapomorphies (Manen et al., 1994; Natali et al., 1995; Bremer, 1996; Natali et al., 1996; Andersson and Rova, 1999; Bremer and Manen, 2000; Nie et al., 2005; Backlund et al., 2007; Bremer and Eriksson, 2009; Rydin et al., 2009a). In addition to their herbaceous habit and temperate distribution, leaf-like whorls, a rudimentary calyx, a two-locular ovary with one ovule per locule, and pluricolpate pollen differentiate Rubieae morphologically from other Rubiaceae. Several pollen characteristics also are cited as synapomorphies for the tribe, including perforate and microechinate tectum, absence of endoapertures, a coarse nexine beneath the ectocolpi, and absence of orbicules (Huysman et al., 2003).

However, classification and identification within Rubieae have been problematic, especially for the larger genera *Asperula* and *Galium*. A number of taxa within *Asperula* appear morphologically similar to *Galium*, differing only in corolla tube length, and these have been transferred from *Asperula* to *Galium* (Ehrendorfer, 1958; Natali et al., 1995; Ehrendorfer et al., 2005). *Galium* itself is problematic taxonomically, because taxa from different sections exhibit similar habit, many species are widely distributed and polymorphic, and species groups often are poorly differentiated both morphologically and geographically (Schischkin, 2000). *Relbunium* presents another problem of generic delimitation; some taxonomists treat this group as a section of *Galium*, while others recognize it as a genus (Natali et al., 1996).

Opposite leaves characterize most Rubiaceae. However, the presence of four or more leaf-like organs at a node differentiates Rubieae as having “leaf-like whorls”. The development of leaf-like whorls in Rubieae has been hypothesized as originating from a pair of opposite leaves and associated, independent leaf-like stipules (Takeda, 1916). This hypothesis has been supported by the observation that only two leaves in a whorl are associated with axillary buds and obtain their vasculature from the stele, whereas leaf-like stipules obtain their vasculature from girdling leaf traces (Rutishauser, 1999). In addition, scanning electron microscopy (SEM) has shown an asynchronous development of whorls within the tribe; two decussate leaf primordia develop first, followed by primordia of leaf-like stipules (Rutishauser, 1999). However, whorls within Rubieae vary with respect to number of

leaf-like organs, from four to twelve or more. This variation is interpreted as due to fusion or fission of stipular organs (Takeda, 1916). Recent SEM studies do not show any evidence of fusion of stipules during development (Rutishauser, 1999). Although, it has been assumed that fusion occurs before the appearance of primordia (Schoute, 1938), as evidenced by forked leaf-like stipules in some taxa (Takeda, 1916).

The presence of two stipules per leaf is considered ancestral and rarely occurs in Rubiaceae (Robbrecht, 1988). Stipules in Rubiaceae are generally fused into one interpetiolar structure on either side of the stem. Whorls in Rubieae may develop from a pair of opposite leaves and two interpetiolar structures. Subsequent expansion of the interpetiolar stipules produces whorls with greater than four leaf-like organs (Robbrecht, 1988). This hypothesis of whorl development coincides with Takeda's original idea that whorls of six leaf-like organs have been derived from a four-organ whorl (Takeda, 1916). Cronquist (1968) also hypothesized that six or eight organs at a node were derived from species with four-organ whorls, but did not provide a detailed description of this change. On the other hand, whorls in Rubieae may have originated from a pair of opposite leaves and paired stipules associated with each leaf, as seen in *Didymaea*. This arrangement of organs would give rise to whorls of six leaf-like organs, which have subsequently been expanded or reduced in number.

A history of the classification of Rubieae is summarized in Table 1.1. No comprehensive global treatment of the tribe has been published in over a century (Schumann, 1897).

In a recent classification of Rubieae (Robbrecht and Manen, 2006), the tribe is expanded to include *Kelloggia* and *Theligonum* (Table 1.1). The tribe Rubieae is proposed to comprise three subtribes: *Kellogginae*, *Theligoninae*, and *Rubiinae* Robbr. & Manen. Members of subtribe *Rubiinae* belong to the former Rubieae s.str.: *Asperula, Crucianella, Cruciata, Didymaea, Galium, Phuopsis, Rubia, Sherardia,* and *Valantia* (Robbrecht and Manen, 2006). Two remaining genera, *Callipeltis* and *Mericarpaea,* previously included in Rubieae based upon morphology (Ehrendorfer et al., 2005), remain to be sampled in a molecular phylogenetics context and, therefore, were not included in Robbrecht and Manen's (2006) classification.

We define Rubieae as comprised of eleven genera: nine genera included in *Rubiinae* (Robbrecht and Manen, 2006), plus two genera previously included in Ehrendorfer et al. (2005), and excluding *Kelloggia* and *Theligonum* (Table 1.1). Other recent studies have also supported the exclusion of *Kelloggia* and *Theligonum* from Rubieae (Nie et al., 2005; Backlund et al., 2007). Recent checklists of the tribe (Govaerts, 2006) show a total of approximately 975 species distributed as follows: *Asperula* (183 spp.), *Callipeltis* (3 spp.), *Crucianella* (31 spp.), *Cruciata* (9 spp.), *Didymaea* (7 spp.), *Galium* (655 spp.), *Mericarpaea* (1 sp.), *Phuopsis* (1 sp.), *Rubia* (77 spp.), *Sherardia* (1 sp.), and *Valantia* (7 spp.).

Molecular systematic studies on tribe Rubieae, based on the *atpB-rbcL* intergenic region, included 70 species across nine genera (Manen et al., 1994; Natali et al., 1995, 1996). This work confirmed the monophyly of the tribe, characterized by a 50-bp deletion, and revealed the problematic classification within the tribe. All prior studies confirm that the large genera *Asperula* and *Galium* are not monophyletic. Seven major clades have been identified from these studies: (1) a clade comprising the genus *Didymaea*; (2) a clade comprising the genus *Rubia*; (3) a clade comprising five sections of *Galium*; (4) a clade comprising several sections of *Asperula* and the genera *Crucianella*, *Phuopsis*, and *Sherardia*; (5) a clade comprising *Asperula* sect. *Glabella* and *Galium* sect. *Aparinoides*; (6) a clade comprising several sections of *Asperula*, and (7) a clade comprising several sections of *Galium* and the genera *Cruciata* and *Valantia* (Manen et al., 1994; Natali et al., 1995, 1996). However, relationships between and within these clades were not well resolved and taxon sampling was limited.

With its temperate distribution, predominant herbaceousness, and leaf-like whorls, Rubieae are considered a derived member of Rubiaceae. The tribe is considered to have radiated relatively recently from a tropical/subtropical ancestor (Manen and Natali, 1995), and has obtained a worldwide distribution, with a center of diversity in the Mediterranean and Asia (Ehrendorfer et al., 2005). Prior molecular studies have sampled predominantly European taxa (Manen et al., 1994; Natali et al., 1995) or encompassed more Old World taxa (Natali et al., 1996). In these studies, Rubieae are hypothesized to have originated from a subtropical ancestor in

common with the Paederieae s.l. in the course of adaptation to temperate regions (Natali et al., 1995). A molecular phylogeny that incorporates extensive sampling from New World taxa, from both North America and South America, is needed to determine the ancestral distribution of the tribe and how its worldwide distribution was obtained.

Because prior molecular systematic work within Rubieae identified the polyphyly of *Galium* and *Asperula,* the goal of the present research is to identify the major clades and their relationships within the tribe to provide the basis for revised classification. We seek to (1) resolve relationships within and among clades; (2) place unsampled genera; (3) identify traits to diagnose these clades; (4) determine the ancestral whorl-type for the tribe and examine the evolution of whorls; and (5) determine worldwide, geographic distribution patterns for the tribe. To achieve these goals we increased sampling within Rubieae to represent the breadth of current classification at generic and subgeneric levels, and increased the number of DNA regions sequenced for phylogenetic analyses. Data from three rapidly-evolving, non-coding cp regions (*rpoB-trnC, trnC-psbM, trnL-trnF-ndhJ*), recently identified as particularly variable and appropriate for phylogenetic studies at this level (Shaw et al., 2005; Mort et al., 2007; Shaw et al., 2007), are used. Parsimony and Bayesian phylogenetic analyses are conducted to obtain a phylogeny, which is subsequently used for reconstruction of ancestral states of whorl morphology and geographic distribution.

MATERIALS AND METHODS

Sampling methods—A total of 126 taxa was sampled, representing 13% of all species in the tribe. Ten of the eleven genera within Rubieae (excluding *Mericarpaea* because of difficulty in obtaining material) were sampled: *Asperula*, *Callipeltis*, *Crucianella*, *Cruciata*, *Didymaea*, *Galium*, *Phuopsis*, *Rubia*, *Sherardia*, and *Valantia* (Table 1.2). For *Asperula* and *Galium*, we sampled widely among the sections currently recognized for each genus, as a guideline for sampling the breadth of these larger genera. For *Galium*, 13 of the 15 sections were sampled (Table 1.2). For *Asperula*, six of the eleven sections were sampled (Table 1.2). In addition, four outgroup taxa were sampled: *Kelloggia galioides*, previously identified as sister genus to Rubieae (Andersson and Rova, 1999; Backlund, 2005; Nie et al., 2005; Robbrecht and Manen, 2006; Backlund et al., 2007), and *Galianthe brasiliensis, Spermacoce brachystemonoides*, and *Staelia thymoides* from the Rubiidinae II clade, sister to the Rubiidinae I clade that includes Rubieae (Robbrecht and Manen, 2006).

Molecular methods—DNA samples were obtained from field-collected silica-gel dried tissue, herbarium specimens, or other Rubiaceae researchers (Appendix A). DNA was extracted using the 2% hexadecyltrimethylammonium bromide (CTAB) procedure (Doyle and Doyle, 1987). DNA from field-collected silica-gel dried tissue, was purified using Wizard SV minicolumns (Promega Corporation, Madison, Wisconsin, U.S.A.). DNA from herbarium specimens was purified by precipitating with an equal volume of 100% isopropanol overnight at –20°C, followed by an

additional precipitation with 2x volume of 100% ethanol and 1/10 volume of 3M pH 5.2 sodium acetate overnight at –20°C, as outlined in Sambrook et al. (1989).

The *rpoB-trnC* region was amplified using the rpoB and trnC^{GCA}R primers published in Shaw et al. (2005). This region was amplified in two adjacent fragments for DNA of lower quality using the rpoB and rpoBdR primers, and rpoBd and trnC^{GCA}R primers (Table 1.3). The *trnC-psbM* region was amplified using the trnC^{GCA}F and psbMR primers (Shaw et al., 2005). This region was amplified in two adjacent fragments for DNA of lower quality using the trnC^{GCA}F and ycf6R primers, and the ycf6F and psbMR primers (Shaw et al., 2005). The *trnL-trnF-ndhJ* region was amplified using the “c” primer of Taberlet et al. (1991) and the ndhJ primer of Shaw et al. (2007). The region was amplified in two overlapping fragments for DNA of lower quality using the c and “f” primers designed by Taberlet et al. (1991), and the “e” primer (Taberlet et al., 1991) and ndhJ primer.

Polymerase chain reactions (PCR) were conducted in a MJ Research PTC-100 Peltier thermal cylcer in 25 µL volumes: 2.5 µL 10x 30 mM $MgCl_2$ reaction buffer, 2.5 µL 10x *Taq* diluent, 2.5 µL DNTPs (10 mM), 1.25 µL each primer (5 µM), 0.125 µL *Taq*, 0.5—1 µL template, and remaining volume of H_20. PCR conditions were an initial denaturation of 94°C for 2 min, followed by 35 cycles of 94°C denaturation for 15 s, 48°C—55°C annealing for 15 s, 72°C extension for 1--2 min, and a final extension at 72°C for 5 min. PCR products were purified by a 20% polyethylene glycol precipitation prior to sequencing.

Sequencing was performed with the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Buckinghamshire, U.K.). Both strands of each region were sequenced using the same amplification primers as above and several internal primers (Table 1.3). Sequencing reactions were done in 5 µL volumes: 2 µL five-fold diluted dRhodamine sequencing reagent premix, 0.25 µL primer (5 µM), 0.5--2.75 µL template, and remaining volume of H_20. Sequencing conditions were an initial denaturation of 94°C for 2 min, followed by 35 cycles of 92°C denaturation for 10 s, 50°C or 55°C annealing for 5 s, and 60°C extension for 2.5 min. Sequencing products were purified with a sodium acetate/EDTA and ethanol precipitation and analyzed on an ABI Prism 377 DNA Sequencer.

Analytical methods—Sequences were initially aligned in ClustalW (Chenna et al., 2003) and then manually adjusted in MacClade v.4.08 (Maddison and Maddison, 2000). Ambiguously aligned regions were excluded from the analyses. All chloroplast regions were combined and analyzed using maximum parsimony and Bayesian analyses.

Maximum parsimony (MP) analyses were conducted in PAUP* v.4.0b10 (Swofford, 2002). Heuristic searches were performed with 1000 stepwise random taxon addition replicates and tree bisection-reconnection (TBR) branch swapping. MULTREES and steepest descent options were not used due to computational time. The strict consensus tree obtained from this first analysis was used as an inverse constraint to do a second analysis to determine whether there were other trees of the same or shorter length not compatible with the strict consensus (Catalán et al.,

1997). The second analysis was performed with 1000 stepwise random taxon addition replicates, TBR branch swapping, MULTREES on, but saving only two trees per replicate with length greater than or equal to 5, and steepest descent not in effect. No additional trees equal in length or shorter were found with the inverse constraint analysis. Support for individual clades was estimated with bootstrap (bs) values (Felsenstein, 1985) from 500 replicates, each with 20 stepwise random taxon addition replicates, and TBR branch swapping (DeBry and Olmstead, 2000), with MULTREES off and steepest descent not in effect.

For Bayesian analyses, models of evolution for the combined dataset were determined by Modeltest v.3.7 (Posada and Crandall, 1998). The model selected under all three criteria---likelihood ratio test, Akaike Information Criterion, and Bayesian Information Criterion---was GTR + I + G. Bayesian analyses were conducted in MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) under the GTR + I + G model, with the default priors of no prior knowledge for the parameters of this model. The prior probability density for the six nucleotide substitution rates and four stationary nucleotide frequencies was a flat Dirichlet, with all values set to 1.0. The prior for the proportion of invariable sites and gamma shape parameter for among-site rate variation was a uniform distribution, from 0 to 1 and 0 to 200 for α, respectively. The default prior probability for topology was uniform, with all possible trees equally probable a priori. The default prior probability distribution on branch lengths was unconstrained and exponential with a parameter of 10.

Bayesian analyses (MB) were conducted with three independent Markov Chain Monte Carlo analyses of 4,000,000 generations, with a sampling frequency of every 100th generation. Metropolis coupling for each analysis was conducted with the default of four chains started from different random trees, three heated and one cold chain, temperature of 0.2, and one swap of states tried between chains every generation. Convergence was determined when the average standard deviation of split frequencies became less than 0.01. To verify convergence, all three runs were examined with AWTY's "compare", "cumulative", and "var" analyses (Nylander et al., 2008) to compare split frequencies between runs, examine cumulative split frequencies for runs, and compare the symmetric tree-difference scores within and among runs, respectively. The first 15,000 trees (37.5%) were discarded and the remaining 25,000 trees were used from each run, and pooled to construct a consensus tree with all compatible groups to obtain posterior probabilities (pp).

For ancestral state reconstruction, distribution and whorl morphology of each terminal taxon was gleaned from the literature, personal observations, and/or herbarium specimens (Appendix A).

For distribution, native distribution (i.e., pre-European settlement) was used. Old World was not divided into smaller distribution categories because (1) many Old World taxa are widespread; (2) we were mainly interested in distribution of New World taxa; and (3) for ease of visual representation. Distribution categories were North America (NA), including taxa centered in northern Central America, South America (SA), and Old World (OW).

Unlike our outgroup *Kelloggia galioides*, all other members of the Rubiidinae I clade are primarily Old World and tropical in distribution, with the exception of the pantropical genus *Paederia* (Rydin et al., 2009b). The sister Rubiidinae II clade is pantropical, with members in both Old and New Worlds (Kårehed et al., 2008); however, only South American taxa were used as outgroups in our phylogenetic analyses due to availability of fresh material. In a biogeographic analysis of *Kelloggia*, Nie et al. (2005) included more Old World outgroups from the Rubiidinae I clade to examine historical biogeography and determined the ancestor of *Kelloggia* and Rubieae as Eurasian. To account for our outgroups' distributions, we pruned taxa from the Rubiidinae II clade from our analyses of geographic distribution. We also assigned *Kelloggia* to OW in an attempt to fix the ancestral node of Rubieae + *Kelloggia* to OW. (States cannot be fixed at nodes in Mesquite.)

For whorl morphology, whorls were categorized as composed of two, four, four to six (for several variable taxa), or six or more (6+) organs (including leaves and stipules). Number of whorl organs usually varies along the stem with fewer organs occurring at lower and upper nodes, and maximum number of organs occurring along most of the length of the stem towards middle nodes. The predominant number of organs/whorl along most of the length of the stem recorded for each species was used for state assignment.

Character matrices were comprised of categorical data with multiple states and analyzed in Mesquite v.2.6 (Maddison and Maddison, 2009). Ancestral states were reconstructed using parsimony (unordered model) and likelihood under the

Markov k-state one-parameter model (MK1), where all changes are equally probable. Because Mesquite cannot do likelihood calculations on trees with polytomies, only the fully resolved topology from MB analyses was used. Polymorphic taxa are not allowed under likelihood reconstructions in Mesquite. Therefore, three taxa (*Galium kamtschaticum, G.trifidum, G. triflorum*) that are distributed in both North America and Old World were assigned to either NA or OW in all eight possible optimizations. In addition, nine variable taxa that exhibit four to six organs per whorl among or within individuals were assigned a fourth state in whorl optimizations (Appendix A).

RESULTS

Molecular results—A quantitative summary of sequences is found in Table 1.4.

Phylogenetic results—In both the MB and MP analyses (Figs. 1.1—1.2), the tribe Rubieae contains seven major clades, which are strongly supported with parsimony bootstrap values ≥ 85% and Bayesian posterior probabilities > 0.95. Relationships among these major clades include *Didymaea* (Clade I) and *Rubia* (Clade II) as sister genera, which together form a clade as sister to the remaining Rubieae (Clades III—VII). Similarly, the relationships among the remaining five clades are strongly supported (Figs. 1.1—1.2).

Species of *Asperula* and *Galium* occur in three of the major clades (Clades IV—VI, and III, V, VII, respectively). Of the remaining genera sampled, *Crucianella,*

Cruciata, Didymaea, and *Rubia* are strongly supported as monophyletic. Only one sample each of *Callipeltis* and *Valantia* were included in our analyses (Figs. 1.1—1.2). *Phuopsis* and *Sherardia* are monotypic genera.

Clade III consists entirely of *Galium* species. We identified four main subclades (Figs. 1.1—1.2): Clade A, comprising members of *Galium* sect. *Galium,* sect. *Hylaea* and sect. *Kolgyda;* Clade B, comprising members of *G.* sect. *Kolgyda*, sect. *Leptogalium*, and sect. *Orientigalium;* Clade C, comprising members of *G.* sect. *Trachygalium;* and Clade D, comprising members of *G.* sect. *Galium,* sect. *Leiogalium,* and sect. *Trachygalium.*

Clade IV consists of two strongly supported subclades (Clades E and F, Figs. 1.1—1.2). Clade E comprises *Sherardia* and *Asperula* sect. *Cynanchicae,* sect. *Hexaphylla,* and sect. *Thliphthisa*. Clade F comprises species representing four genera: *Asperula* (sect. *Cruciana*)*, Callipeltis, Crucianella,* and *Phuopsis. Callipeltis* is sister to the remaining members of Clade F in MP analyses, whereas *Crucianella* is sister to the remaining members in MB analyses.

Clade V consists of two strongly supported subclades (Clades G and H, Figs. 1.1—1.2). Clade G comprises *Galium* sect. *Depauperata.* Clade H comprises members of *Asperula* sect. *Glabella*, which form a paraphyletic grade, from which *Galium* sect. *Aparinoides* is derived.

Clade VI comprises members of *Asperula* sect. *Asperula* and sect. *Glabella* (Figs. 1.1—1.2).

Clade VII includes members of three genera: *Cruciata, Galium,* and *Valantia* (Figs. 1.1—1.2). *Cruciata* and *Valantia* are sister groups in both analyses, but without strong support. A well-supported clade of *Galium* taxa includes *G.* sect. *Baccogalium,* sect. *Lophogalium,* sect. *Platygalium,* and sect. *Relbunium,* however, relationships within this clade are not well supported.

Ancestral state reconstructions—Parsimony reconstruction of geographic distribution ranged from eight to ten steps over all eight optimizations. Similarly, likelihood reconstruction of distribution ranged from likelihood scores of -ln L = 42.123 to 50.106 over all eight optimizations. All parsimony optimizations reconstructed an unequivocal origin of the tribe in the Old World. All likelihood reconstructions gave proportional likelihoods from 0.975 to 0.986 for an Old World origin of the tribe. The most parsimonious and highest likelihood reconstruction is shown in Figure 1.3.

Parsimony reconstruction of whorl morphology was 13 steps (Fig. 1.4). Parsimony reconstructed an unequivocal origin of the tribe with whorls of six or more organs. Likelihood reconstruction of ancestral whorl morphology (-ln L = 62.750) for the tribe gave a proportional likelihood of 0.988 for whorls of six or more organs.

DISCUSSION

Phylogeny—All seven previously identified clades (Manen et al., 1994; Natali et al., 1995, 1996) are strongly supported by our analyses, and relationships between these clades are fully resolved (Figs. 1.1—1.2). Natali et al. (1996) were

unable to resolve relationships among *Didymaea*, *Rubia*, and the rest of the tribe. We show that *Didymaea* and *Rubia* are sister genera and form a clade sister to the remaining Rubieae. *Didymaea* (Clade I) consists of perennial herbs from Mexico and Central America with opposite leaves, each with a pair of scale-like stipules, and 4-parted, campanulate flowers. *Rubia* (Clade II) consists of herbaceous to woody perennials from Eurasia and Africa with whorls of generally four or more leaf-like organs, and mostly 5-parted, rotate flowers (Fig. 1.1). Both *Didymaea* and *Rubia* have fleshy fruits (Fig. 1.1), which are a synapomorphy for this group. Fleshy fruits are derived from dry fruits in the common ancestor of these two genera (Bremer and Eriksson, 1992; Bremer, 1996).

Manen et al. (1994) and Natali et al. (1995, 1996) were unable to resolve relationships among the remaining five clades. We show a split between Clade III and the remaining four clades. Clade III previously included representatives of five sections of *Galium*: *G.* sect. *Galium,* sect. *Hylaea,* sect. *Kolgyda,* sect. *Leiogalium,* and sect. *Leptogalium.* We show that two additional *Galium* sections also belong to this clade: *G.* sect. *Orientigalium* and sect. *Trachygalium.* None of these sections are monophyletic. This clade comprises mostly perennials of Old World origin, with annuals arising in Clades A (e.g., *G. aparine, G. tricornutum, G. verrucosum*) and B (e.g., *G. divaricatum, G. intricatum, G. murale, G. parisiense*), six or more leaf-like organs per whorl, and 4-parted, rotate flowers. All of these characteristics are plesiomorphic and no synapomorphies have been identified for this clade, or Clades A--D. Clade III contains *G. verum*, the type species for *Galium.*

Clade IV previously included representatives of *Crucianella*, *Phuopsis*, *Sherardia*, and *Asperula* sect. *Cynanchicae,* sect. *Hexaphylla,* and sect. *Thliphthisa* (Manen et al., 1994; Natali et al., 1995, 1996). We show that *Callipeltis* and *Asperula* sect. *Cruciana* also belong to this clade. Prior studies showed a close relationship between *Crucianella* and *Phuopsis* (Manen et al., 1994; Natali et al., 1995, 1996). However, our analyses support *Phuopsis* as sister to *Asperula* sect. *Cruciana* (not sampled in prior studies), and together forming a larger clade with *Callipeltis* and *Crucianella* (Clade F). *Sherardia* forms another clade with the other sections of *Asperula* (Clade E). Clade IV comprises mostly perennials of Old World origin, with annuals arising in Clades E (e.g., *Sherardia*) and F (e.g., *Callipeltis* and *Crucianella,* excluding *C. sintenisii*), and whorls of four or more leaf-like organs. Tubular flowers appear to be a synapomorphy for this clade (Fig. 1.1). Four-parted flowers are inferred to be ancestral in this clade. However, 5-parted flowers arise in Clade F, in *Asperula* sect. *Cruciana, Phuopsis,* and several species of *Crucianella* (*C. filifolia, C. sintenisii*).

Clade V was represented previously by a paraphyletic grade of *Asperula* sect. *Glabella*, from which *Galium* sect. *Aparinoides* was derived (Manen et al., 1994; Natali et al., 1995, 1996). We show that *Galium* sect. *Depauperata* (Clade G) also belongs to this clade as sister to the clade with *Asperula* sect. *Glabella* and *Galium* sect. *Aparinoides* (Clade H). Clade V is of Old World origin (Fig. 1.3). Members are characterized by whorls of mostly four leaf-like organs and generally 4-parted, rotate flowers. Within Clade V, Clade G comprises annuals with unequal leaves and leaf-

like stipules. Clade H comprises perennials with four to six, equal whorl organs that exhibit much variation in number of organs per whorl among and within individuals. Tubular flowers have arisen in *Asperula* species that are members of Clade H. Three-parted flowers have arisen in Clade H, in *Asperula tinctoria* and several species of *Galium* sect. *Aparinoides* (*G. tinctorium, G. trifidum*).

Clade VI previously included representatives of *Asperula* sect. *Asperula* and sect. *Cynanchicae* (Natali et al., 1996). We show that Clade VI also includes members of *Asperula* sect. *Glabella*. We did not sample any members of sect. *Cynanchicae* from this clade to confirm membership. Clade VI contains *A. arvensis,* the type species for *Asperula.* Clade VI is of Old World origin (Fig. 1.3), comprising annuals with whorls of six or more leaf-like organs (*A.* sect. *Asperula*) and perennials with whorls of four leaf-like organs (*A.* sect. *Glabella*). Tubular flowers and capitate inflorescences appear to be synapomorphies for this clade (Fig. 1.1).

Clade VII previously included representatives of *Cruciata, Valantia,* and *Galium* sect. *Baccogalium,* sect. *Platygalium,* and sect. *Relbunium* (Manen et al., 1994; Natali et al., 1995, 1996). We show that *Galium* sect. *Lophogalium* also belongs to this clade. This clade is of Old World origin (Fig. 1.3), but includes a large clade with New World distribution. Members have whorls of strictly four leaf-like organs, and 4-parted, rotate flowers; most are perennials. These characteristics, however, are plesiomorphic, and no synapomorphies for this clade have been identified. A diversity of sexual systems occurs in this clade, including hermaphroditism, andromonoecy, polygamy and dioecy.

Geographic distribution—Given the distribution of extant members of Rubieae, our sampling of ingroup taxa, and prior knowledge of historical biogeography of the Rubiidinae I clade (Nie et al., 2005; Rydin et al., 2009b), the origin of Rubieae is Old World (Fig. 1.3), regardless of how widespread species are scored. The previous study of *Kelloggia* (Nie et al., 2005) indicated Eurasia and Eurasia plus North America as possible ancestral areas for Rubieae, but included only nine taxa from the tribe. Our analyses confirm an Old World origin of the tribe, and more specifically, possibly Eurasian, as indicated by Nie et al. (2005). In addition, Old World ancestors have been inferred for six of the seven major clades (Fig. 1.3), excluding *Didymaea*. At least eight North American lineages have arisen from Old World ancestors in the tribe, including three widespread taxa (*Galium kamtschaticum, G.trifidum, G. triflorum*).

A recent estimate of 28.6 Ma (20.2—37.6 Ma) as the minimum divergence time of the tribe (Bremer and Eriksson, 2009) is inconsistent (unless estimates are off by a factor of 2—3) with a report of a fossil *Galium* fruit from Greenland dating to the Paleocene, ~65—55 Ma (Graham, 2009). However, the first fossil record of the tribe in North America is from *Galium* (pollen) in Alaska during the middle Miocene, ~15 Ma (White and Ager, 1994; Graham, 2009). This distribution may have originated from dispersal over the Bering bridge from Europe to North America. The Bering bridge originated in the late Cretaceous, 100—65 Ma, and remained present throughout most of the Cenozoic, 65—0.013 Ma (Cox and Moore, 2000). The Bering bridge was the only link from North America to Europe after the end of the Eocene,

~55--34 Ma (Cox and Moore, 2000). The climate began cooling in the late Eocene, restricting the dispersal of plants over the Bering bridge, and culminating in the ice ages of the Pliocene/Pleistocene, ~5—0.011 Ma (Cox and Moore, 2000; Graham, 2009). The climate began to warm again about 21 ka, terminating the Bering bridge 13--14 ka (Cox and Moore, 2000). Therefore, it is not surprising that more fossils of the tribe have been reported from western North America and South America during the Quaternary, between 40 and 10 ka (Thompson, 1990; Behling, 1997; Lozano-García et al., 2002; Latorre et al., 2003; Graham, 2009), suggesting a recent diversification in the New World. A land bridge between North America and South America was not formed until the beginning of the Quaternary (Cox and Moore, 2000), and diversification into South America likely occurred after that. From our results, it appears that there has been at least one relatively recent diversification in South America from North American ancestors in Clade VII (Fig. 1.3).

Most of the reported older fossil records of the tribe are pollen assigned to *Galium*. Use of these fossils as calibration points in estimations of divergence times is challenging, because *Galium* is not monophyletic. In addition, pollen morphology is useful for identification at the tribal level in Rubieae, but uninformative in identifying genera or groups within the tribe (Huysmans et al., 2003). This implies that pollen fossils identified as *Galium* may belong to other genera within the tribe and should only be assigned to Rubieae.

At least two dispersal events into North America have occurred in Clade III (Figs. 1.3), as evidenced by *Galium mexicanum* and *G. triflorum*. The potential origin

of *G. mexicanum* in western North America is unclear, but its closely related species are primarily European in distribution. *Galium triflorum*, however, diverged early in the evolution of this clade and has a circumboreal distribution in the Northern Hemisphere. It may have obtained its distribution from land connections between North America and Europe via Greenland sometime before the end of the Eocene, or via the Bering bridge much later.

At least two dispersal events into North America have occurred in Clade V (Fig. 1.3), as evidenced by *Galium bifolium* and *G. trifidum* + *G. tinctorium*. *Galium bifolium* is distributed in western North America, sister to a northeast Asian taxon (*G. songaricum*), and may have originated via connections through the Bering bridge. *Galium trifidum* is circumboreal, distributed in Europe and North America, and may have achieved this distribution through land connections between North America and Europe. *Galium tinctorium* is distributed in eastern North America, and is derived from an ancestor it shared with *G. trifidum*.

In Clade VII, a close relationship between *G. kamtschaticum* and *G. oreganum* (previously treated as a variety of *G. kamtschaticum*) has been suggested by morphological similarities and overlapping geographic distributions. However, this relationship is not supported in our analyses, but not strongly in conflict either. These taxa represent one or two dispersal events into North America before the larger, more recent radiation in North America (Fig. 1.3). *Galium kamtschaticum* is distributed in northeast Asia and western North America, and may have obtained its distribution via the Bering bridge. *Galium oreganum*, distributed in western North

America, may be derived from an ancestor it shared with *G. kamtschaticum*. A large, recent radiation of the tribe in North America as evidenced in Clade VII, with subsequent diversification in South America, is consistent with the fossil record. A paraphyletic grade of Eurasian species gave rise to this radiation in western North America, although relationships in this region of the tree are not well supported.

Evolution of leaf-like whorls—Leaf-like whorls in Rubieae originated from whorls of six or more organs (Fig. 1.4). This supports the hypothesis that whorls were derived from opposite leaves, each associated with two stipules. Whorls of four organs are derived later in the tribe. In addition, whorl morphology appears to be evolutionarily labile. Whorls of four organs have evolved from whorls of six or more organs at least five times (excluding variable taxa). Whorls of six or more organs have arisen from whorls of four organs at least once, and possibly twice (excluding variable taxa). Complete loss of leaf-like stipules to a single pair of leaves is rare and derived at least twice within the tribe: once in Clade VII from whorls of four organs, and once in Clade II, possibly from whorls of six or more organs. Within Rubieae, having whorls of six or more organs is ancestral, with whorls of fewer organs being derived in the tribe.

Whorl type is more stable in Clades III and VII. In Clade III, whorls of six or more organs occur consistently throughout, except for *Galium murale*. In Clade VII, whorls of four organs occur consistently throughout, except for *Galium lilloi*. Previous studies of several members from Clade VII (*Galium kinuta, G. rubioides*) show these taxa as unusual in that all four organs obtain vascular traces directly from the stele

(Fukuda, 1988; Rutishauser, 1999). In addition, forked leaf-like stipules are rarely seen in members of Clade VII (Rutishauser, 1999). In contrast, whorl type is unstable in the remaining four clades, excluding *Didymaea*. Studies on several members from Clade II (*Rubia fruticosa*) and Clade IV (*Phuopsis stylosa*) have shown the number of primary leaves to sometimes be three instead of two (Rutishauser, 1999), which may reflect the instability of whorl types in these clades.

Leaf whorls have evolved multiple times in ferns and fern allies, gymnosperms, and across angiosperms (Rutishauser, 1999). In addition to evolving multiple times, at least eight different ways of leaf whorl development have been documented, from decussate and tricussate phyllotaxy (i.e., Rubieae) to helical phyllotaxy (Rutishauser, 1999). Whorls have been shown to maximize light harvesting in preliminary computer simulations (Niklas, 1998), and may have been advantageous in parallel cases of whorl development (Rutishauser, 1999).

Because of the evolutionary lability of leaf-like whorls in Rubieae and the variation of leaf-like stipules from zero to ten at a whorl, these organs may be more like leaves than stipules, as Bremekamp (1966) had suggested. In a study done on *Galium elongatum* (Jeune, 1980), no morphogenetic difference was observed in the growth of the two leaves and two leaf-like stipules, except for the slightly larger size of the leaves, presence of axillary buds and vascular traces from the stele. Similarly, this was observed in two species of *Rubia* with four organs per whorl (Fukuda, 1988). These differences may be due primarily to the later initiation of leaf-like stipules.

Another study on *Galium aparine* (Pötter and Klopfer, 1987) confirmed the initial development of decussate leaf primordia, and the development of two meristems at a node on either side of the leaf primordia at the same time. This meristem later divides into one to four primordia, resulting in what has been termed "leaf-like stipules." In this study, the only difference between leaves and leaf-like stipules is the initiation of the leaves from the shoot apical meristem and their slightly advanced growth. Because the leaves and leaf-like stipules develop from different meristems and develop independently, Pötter and Klopfer (1987) suggest that these leaf-like stipules could be termed differently.

In Rubieae, the leaf-like stipules are independent structures, not part of the leaf, and have the capacity to develop into functional leaves. Recent work on *Pisum sativum cochleata* mutants also confirms that stipules have the capacity to develop into leaf blades (Kumar et al., 2009). From this work, it appears that one or two genes are involved in switching stipules to leaf blades in *Pisum*, and this may be similar in Rubieae. *COCHLEATA* is a master regulator that inhibits the leaf blade developmental pathway in stipules by repressing genes that are involved in producing the *Pisum* leaf blade. *COCHLEATA* is also essential for stipule initiation, growth, and development. Another gene also required for growth of the stipule and involved in the maintenance and/or proliferation of meristematic cells is *STIPULE-REDUCED* (Kumar et al., 2009).

***Conclusions*—**Seven major clades are well supported within Rubieae, as well as relationships among and within these clades. Some of these clades exhibit plesiomorphic characters and do not have obvious synapomorphies.

An Old World origin of the tribe is inferred from both parsimony and likelihood ancestral state reconstructions, with at least eight subsequent dispersal events into North America. A radiation in North America, followed by subsequent diversification in South America, has occurred in Clade VII.

Both parsimony and likelihood ancestral state reconstructions infer the ancestral whorl morphology of the tribe to be composed of six organs. Whorls composed of four organs are derived from whorls with six or more organs. Transitions between four and six or more organs per whorl are common within the tribe. Reduction to two leaves at a node is derived and rare within the tribe. The instability of number of whorl organs in the tribe, along with developmental studies of leaves and leaf-like stipules in Rubieae and *Pisum* suggest that leaves and leaf-like stipules have similar developmental capacities.

***Future perspectives*—**Several sections of *Asperula* remain to be sampled: *A.* sect. *Crucianelloides,* sect. *Dioicae,* sect. *Oppositifolia,* sect. *Trichodes,* and sect. *Tricostella.* In addition, one section of *Galium* (sect. *Jubogalium*), one section of *Crucianella* (sect. *Maritimae*), and the genus *Mericarpaea* remain to be sampled. A formal revision of the tribe will be the subject of a future paper. All clades thus far sampled appear to be strictly hermaphroditic, with the exception of Clade VII. Future work will involve sampling this clade more extensively to resolve relationships, as

well as to determine evolution of sexual systems. Now with an improved understanding of the relationships within Rubieae, taking an evolutionary development approach to examine whorl development within the group will be a fascinating area of future research. One such area would be to examine whorl development from various representative taxa from the major clades to determine whether whorl development is indicative of the lability or constancy of whorl morphology within certain clades. Another area of research would be to examine expression of certain candidate genes like *COCHLEATA* in stipules and leaves of *Didymaea* and its sister genus *Rubia* to determine if stipules in Rubieae do have the capacity to develop into leaves.

Table 1.1. History of classification of Rubieae.

Tribal name	Author (date)	Genera included
----	De Jussieu (1789)	*Anthospermum* L.*, Asperula, Crucianella, Galium, Rubia, Sherardia, Valantia*
Asperuleae	Richard (1829)	*Asperula, Crucianella, Galium, Rubia, Sherardia, Valantia*
Stellatae	De Candolle (1830)	*Asperula, Callipeltis, Crucianella, Galium, Rubia, Sherardia, Valantia*
Galieae	Hooker (1873)	*Asperula, Callipeltis, Crucianella, Didymaea, Galium, Mericarpaea, Phuopsis, Relbunium, Rubia, Sherardia, Valantia*
Rubieae	Baillon (1880)	*Asperula, Rubia*
Galieae	Schumann (1897)	*Asperula, Callipeltis, Crucianella, Didymaea, Galium, Mericarpaea, Phuopsis, Relbunium, Rubia, Sherardia, Valantia*
Rubieae	Robbrecht (1988)	*Asperula, Bataprine* Nieuwl.*, Callipeltis, Crucianella, Cruciata, Didymaea, Galium, Mericarpaea, Microphysa* Schrenk*, Phuopsis, Relbunium, Rubia, Sherardia, Valantia, Warburgina* Eig
Rubieae	Bremer and Manen (2000)	*Asperula, Callipeltis, Cruciata, Didymaea, Galium, Mericarpaea, Microphysa, Phuopsis, Relbunium, Rubia, Sherardia, Valantia, Warburgina*
Rubieae	Ehrendorfer et al. (2005)	*Asperula, Callipeltis, Crucianella, Cruciata, Galium, Mericarpaea, Phuopsis, Relbunium, Rubia, Sherardia, Valantia*
Rubieae	Robbrecht and Manen (2006)	*Asperula, Crucianella, Cruciata, Didymaea, Galium, Kelloggia* Torr. ex Hook. f.*, Phuopsis, Rubia, Sherardia, Theligonum* L.*, Valantia*
Rubieae	Soza and Olmstead (this study)	*Asperula, Callipeltis, Crucianella, Cruciata, Didymaea, Galium, Mericarpaea, Phuopsis, Rubia, Sherardia, Valantia*

Table 1.2. Sections and representative taxa sampled among genera of Rubieae (classification follows Ehrendorfer et al., 2005; Ehrendorfer pers.comm.).

Genus	***Section***	**Taxa sampled**
Asperula L.	*Asperula*	*A. arvensis*
		A. orientalis
		A. setosa
	Cruciana Griseb.	*A. albovii*
		A. glomerata
		A. glomerata subsp. *turcomanica*
		A. molluginoides
	Crucianelloides Boiss.	--
	Cynanchicae DC. ex Boiss.	*A. cynanchica*
		A. gussonei
		A. sp.
	Dioicae Shaw & Turrill	--
	Glabella Griseb.	*A. laevigata*
		A. taurina
		A. taurina
		A. tinctoria
		A. tinctoria
	Hexaphylla Ehrend.	*A. hirta*
	Oppositifoliae Schischk. ex E. Schönb.-Tem	--
	Thliphthisa (Griseb.) Ehrend.	*A. chlorantha*
		A. purpurea
	Trichodes Boiss.	--
	Tricostella Schönb.-Tem. & Ehrend.	--
Calllipeltis Steven		*C. cucullaris*
Crucianella L.	*Crucianella*	*C. angustifolia*
		C. chlorostachys
		C. filifolia
	Maritimae Bornm.	--
	Roseae Bornm.	*C. sintenisii*
Cruciata Mill.		*C. glabra*
		C. laevipes
		C. pedemontana
		C. taurica
Didymaea Hook. f.		*D. alsinoides*
		D. floribunda

Table 1.2 continued

Galium L.	*Aparinoides* (Jordan) Gren.	*G. elongatum*
		G. palustre
		G. tinctorium
		G. trifidum
	"*Baccogalium*"	*G. ambiguum* subsp. *siskiyouense*
		G. andrewsii subsp. *andrewsii*
		G. bolanderi
		G. martirense
		G. porrigens
	Bataprine Nwd.	--
	Depauperata Pobed.	*G. bifolium*
		G. songaricum
	Galium	*G. ossirwaense*
		G. perralderii
		G. sp.
		G. tomentosum
		G. verum
	Hylaea (Griseb.) Ehrend.	*G. odoratum*
		G. triflorum
	Jubogalium Ehrend.	--
	Kolgyda Dumort.	*G. aparine*
		G. divaricatum
		G. intricatum
		G. murale
		G. parisiense
		G. tricornutum
		G. verrucosum
	Leiogalium Ledeb.	*G. aetnicum*
		G. album
		G. corrudifolium
		G. friedrichii
		G. fruticescens
		G. lucidum
		G. mollugo
		G. productum
		G. sylvaticum
	Leptogalium Lange	*G. cespitosum*
		G. corsicum
		G. estebani
		G. pumilum

Table 1.2 continued

	G. saxatile
	G. suecicum
	G. valdepilosum
Lophogalium K. Schum.	*G. angustifolium* subsp. *angustifolium*
	G. argense
	G. collomiae
	G. coloradoense
	G. fendleri
	G. gilliesii subsp. *gilliesii*
	G. glabrescens subsp. *modocense*
	G. gracilicaule
	G. grayanum
	G. hallii
	G. hilendiae subsp. *carneum*
	G. hypotrichium subsp. *inyoense*
	G. hystricocarpum
	G. jepsonii
	G. juniperinum
	G. moranii subsp. *aculeolatum*
	G. multiflorum
	G. parishii
	G. stellatum
	G. volcanense
	G. wrightii
Orientigalium Ehrend.	*G. cometerhizon*
	G. pyrenaicum
Platygalium W. Koch	*G. bailloni*
	G. boreale
	G. circaezans
	G. kamtschaticum
	G. oreganum
	G. pilosum
	G. rotundifolium
	G. rubioides
	G. scabrum
	G. uncinulatum
Relbunium Endl.	*G. bigeminum*
	G. hirtum
	G. hypocarpium

Table 1.2 continued

		G. megapotamicum
		G. richardianum
	Trachygalium K. Schum.	*G. mexicanum* subsp. *asperrimum*
		G. rivale
		G. uliginosum
	Miscellaneous	*G. hintoniorum*
		G. latoramosum
		G. lilloi
		G. proliferum
		G. texense
		G. virgatum
Mericarpaea Boiss.		--
Phuopsis Benth. & Hook. f.		*P. stylosa*
Rubia L.	*Campylanthera* Pojark.	*R. florida*
	Oligoneura Pojark.	*R. cordifolia*
		R. horrida
		R. oncotricha
	Rubia	*R.* sp.
		R. tinctorum
Sherardia L.		*S. arvensis*
Valantia L.		*V. muralis*

Table 1.3. Internal primers designed for sequencing cp regions.

Region	Primer	Sequence (5'--3')
rpoB-trnC	rpoBb	CGGATATTAATAKMTACATACG
	rpoBbR	CGTATGTAKMTATTAATATCCG
	rpoBd	GTTGGGGTTTACATATACT
	rpoBdR	AGTATATGTAAACCCCAAC
trnC-psbM	psbMa	GACATCRTGGTTGTCKAACGAG
	psbMb	GGTAAGAACCYRTTGATTGAAATAG
	psbMc	CGAATRCATAACCCTTTTCRA
trnL-trnF	d	(see Taberlet, 1991)
	e	(see Taberlet, 1991)
trnF-ndhJ	ndhJa	GATTTCTTYRTTTCKCTTA
	ndhJb	AATCTCTAATTGTAYTATCTT
	ndhJbR	AAGATARTACAATTAGAGATT
	trnFF	CTCGTGTCACCAGTTCAAATC

Table 1.4. Molecular results of cp regions sequenced in this study.

	rpoB-trnC	***trnC-psbM***	***trnL-trnF-ndhJ***	**Combined**
Unaligned length (bp)	1012—1200	1247—1712	1591—1819	3850—4731
Aligned length (bp)	2357	3043	2762	8162
Excluded regions (bp)	45	217	30	292
Number of base pairs (bp) analyzed	2312	2826	2732	7870
Parsimony informative characters	316	472	434	1222
Number of taxa completed	119	128	130	117
Number of taxa partially sequenced	9	1	0	13

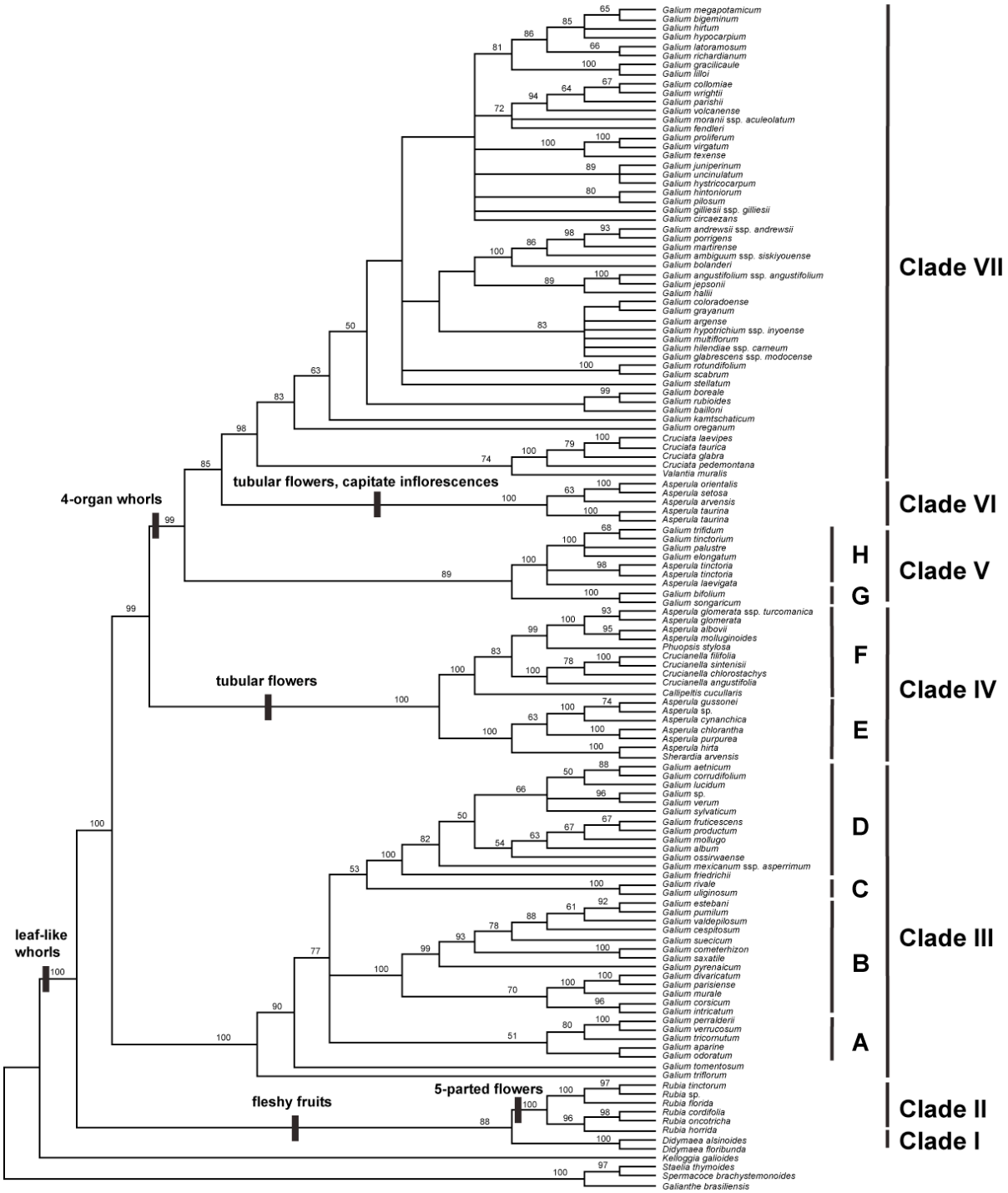

Figure 1.1. Phylogeny of Rubieae, estimated from the strict consensus tree of 185 most parsimonious trees (length = 3474) based on three combined cp regions (*rpoB-trnC, trnC-psbM, trnL-trnF-ndhJ*). Bootstrap values ≥ 50% displayed above branches. Summary of known synapomorphies or diagnostic characters for clades mapped onto phylogeny. I—VII and A—H, clades.

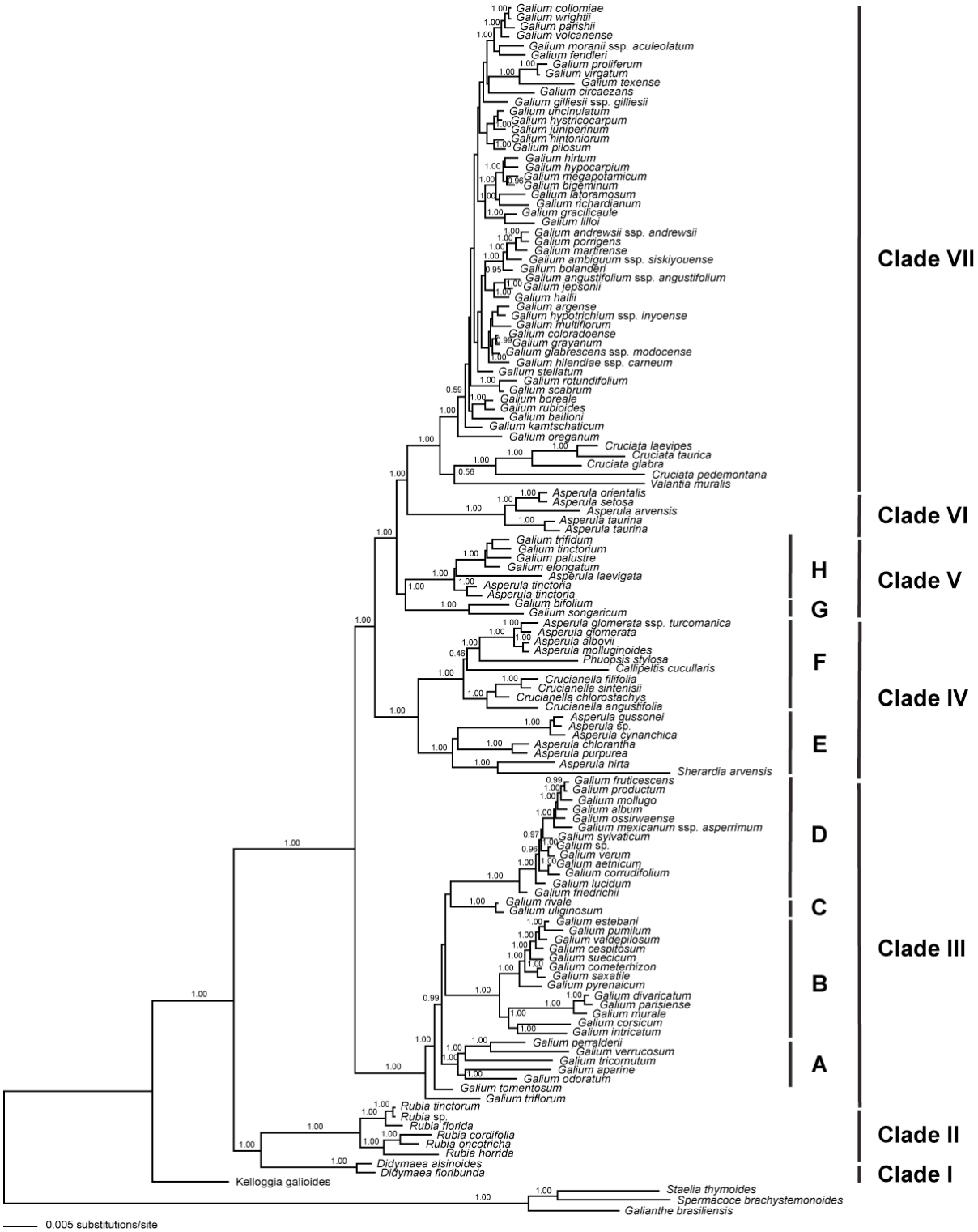

Figure 1.2. Phylogeny of Rubieae, estimated from the Bayesian consensus tree based on three combined cp regions (*rpoB-trnC, trnC-psbM, trnL-trnF-ndhJ*). Posterior probabilities (pp) ≥ 0.95 displayed above branches, including lower pp for clades showing placement of *Callipeltis*, *Galium kamtschaticum*, and *Valantia*. I—VII and A—H, clades.

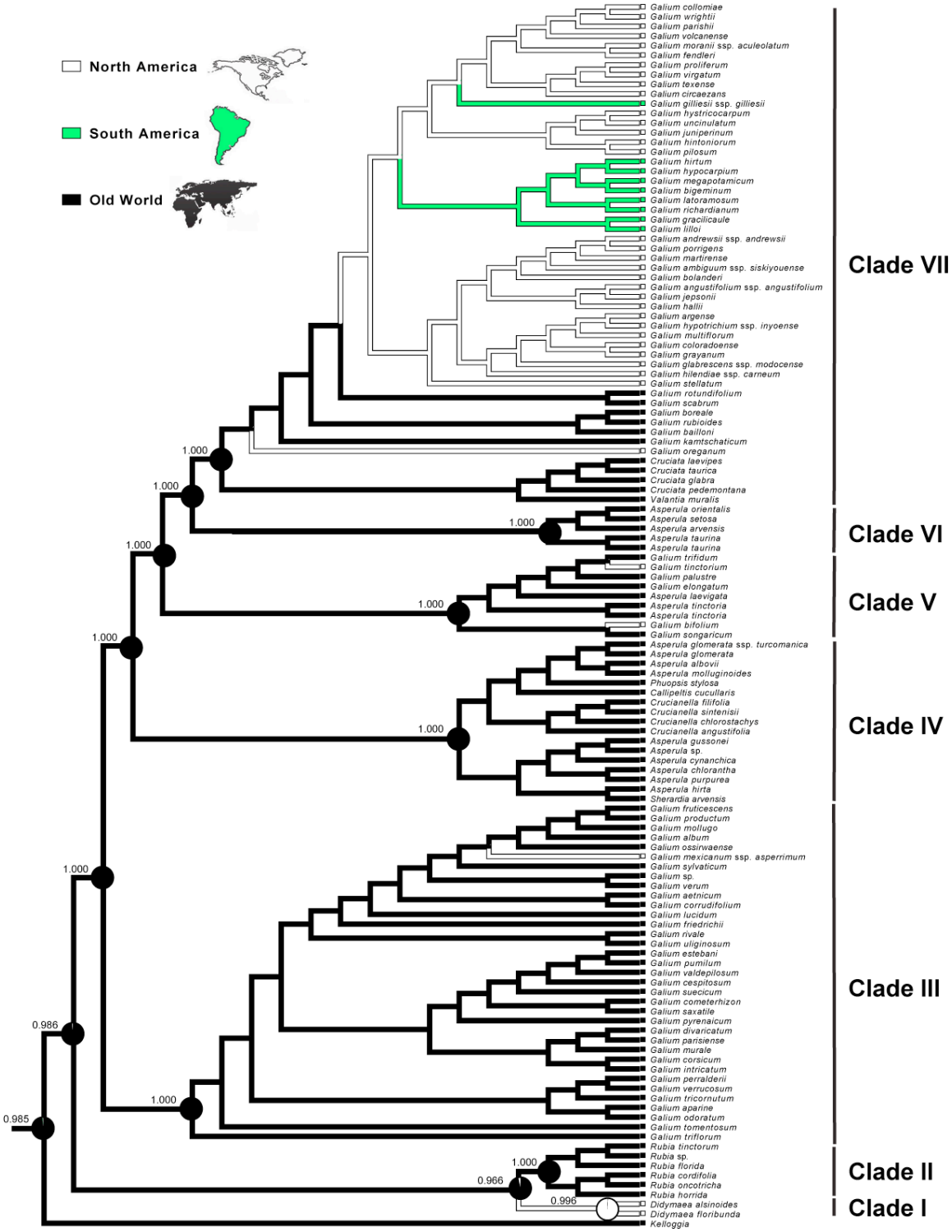

Figure 1.3. Bayesian consensus tree with most parsimonious and highest likelihood reconstruction of ancestral states of geographic distribution. Most parsimonious and highest likelihood optimization out of eight possible = all polymorphic taxa (*Galium kamtschaticum, G. trifidum, G. triflorum*) coded as Old World. Proportional likelihoods of most likely state shown at ancestral nodes for backbone of tribe and seven major clades. Maps modified from world map by Studio7Designs 2008. I—VII, clades.

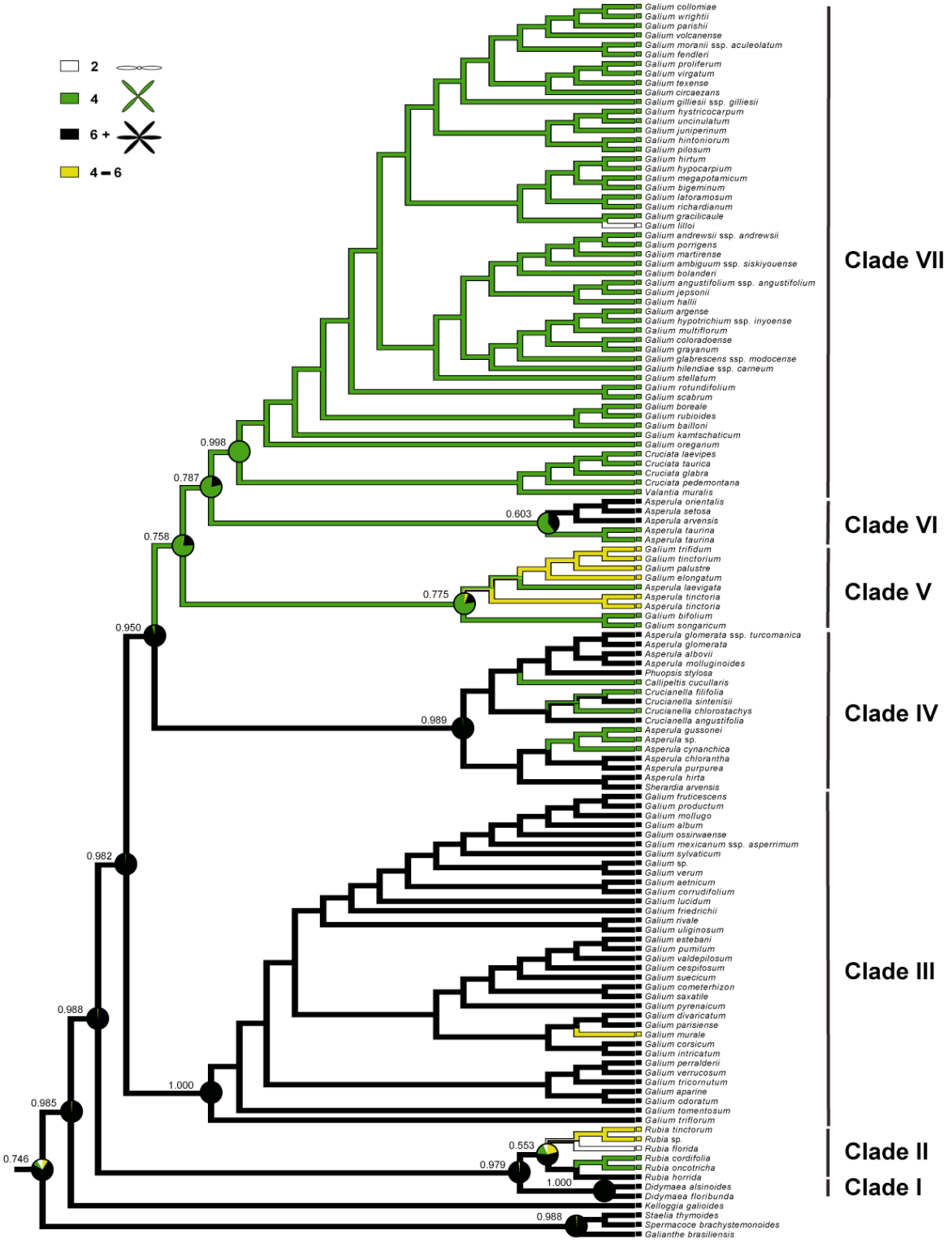

Figure 1.4. Bayesian consensus tree with parsimony and likelihood reconstruction of ancestral states of number of whorl organs (including leaves and stipules). Proportional likelihoods of most likely state shown at ancestral nodes for backbone of tribe, seven major clades, and outgroup. I—VII, clades.

CHAPTER II

EVOLUTION OF BREEDING SYSTEMS AND FRUITS IN NEW WORLD *GALIUM* AND RELATIVES (RUBIACEAE)[2]

SUMMARY

Dioecy occurs in only about 6% of angiosperms, yet it has evolved many times from hermaphroditism. Polygamy is an even more uncommon condition within angiosperms, in which both unisexual and bisexual flowers occur within a species. Polygamy, dioecy, and hermaphroditism all occur within a New World clade of *Galium* (Rubiaceae), in which dioecy is hypothesized to have evolved from hermaphroditism via polygamy. At least five sections of *Galium* as traditionallly defined by fruit morphology occur within this group. We seek to test the monophyly of sections defined by fruit morphology and sought to determine origins and pathways of breeding systems within this group. We obtained chloroplast (*rpoB-trnC, trnC-psbM, trnL-ndhJ*) and nuclear ribosomal (external transcribed spacer) DNA sequences for 89 taxa from the *Cruciata-Galium-Valantia* (CGV) clade to estimate the phylogeny. Ancestral states for breeding systems, fruit types, and fruit hairs were reconstructed using parsimony and likelihood analyses. We identified nine well-supported lineages of New World *Galium* taxa. However, none of the sections traditionally defined by fruit morphology are monophyletic. Dioecy is inferred to have arisen at least three times from hermaphroditism; polygamy is inferred to have

---

[2] This chapter was first published in *American Journal of Botany*: Soza, V. L. and R. G. Olmstead. 2010. Evolution of breeding systems and fruits in New World *Galium* and relatives (Rubiaceae). *American Journal of Botany* 97: 1630—1646 (http://www.amjbot.org/cgi/content/short/97/10/1630).

arisen at least twice from dioecy and at least six times from hermaphroditism. Polygamy appears to be a terminal condition in the CGV clade and not a pathway to dioecy. Fruit characters traditionally used in the taxonomy of this group have arisen multiple times within this clade of *Galium* and are not reliable indicators of shared evolutionary history.

INTRODUCTION

The majority of angiosperm species are hermaphroditic, having both functional male and female organs within a flower. Separate male and female plants within a species, a condition termed dioecy, occurs in only about 6% of angiosperms (Renner and Ricklefs, 1995). Yet, dioecy occurs in approximately 160 of 430 plant families, representing well over 100 independent evolutionary origins within families (Renner and Ricklefs, 1995; Charlesworth and Guttman, 1999). In addition, within certain plant families, dioecy has arisen multiple times (Charlesworth and Guttman, 1999; Mitchell and Diggle, 2005). Dioecy has evolved many times from hermaphroditic ancestors because of such factors as avoidance of inbreeding, sexual selection, and resource allocation (Mitchell and Diggle, 2005). In addition, dioecy has evolved from hermaphroditism via different pathways throughout angiosperms. At least five evolutionary pathways to dioecy from hermaphroditism have been proposed and examined in the literature: (1) directly, (2) via gynodioecy, (3) via androdioecy, (4) via monoecy, and (5) via heterostyly (Bawa, 1980; Ross, 1982; Ainsworth, 2000; Barrett, 2002; Delph and Wolf, 2005; Mitchell and Diggle,

2005). The two pathways most frequently associated with dioecy are gynodioecy (Lloyd, 1980; Hart, 1985; Ainsworth et al., 1998; Weller and Sakai, 1999; Weiblen et al., 2000; Barrett, 2002) and monoecy (Lewis, 1942; Lloyd, 1980; Renner and Ricklefs, 1995; Ainsworth et al., 1998; Renner, 1998; Renner and Won, 2001; Barrett, 2002).

Polygamy is another intermediate form between hermaphroditism and dioecy that occurs infrequently, and is often considered the result of environmentally labile sex expression, rather than a pathway to dioecy (Richards, 1997). Polygamy refers to populations that may exhibit a mix of unisexual, hermaphroditic, andromonoecious, or gynomonoecious individuals (Richards, 1997).

A clade of New World *Galium* (Rubiaceae) includes approximately 30 species characterized as polygamous, in addition to dioecious and hermaphroditic species within the clade. Dempster (1973) used the term *polygamous* to refer to hermaphroditic and unisexual flowers on the same or on different individuals of the same species. Within polygamous *Galium* species*,* individuals range from entirely pistillate with all stamens abortive and all ovaries fertile, to pistillate with many sterile flowers, to mostly pistillate with some hermaphroditic or staminate flowers, to plants with both hermaphroditic and staminate flowers, to mostly staminate with some hermaphroditic flowers, to purely staminate with fertile stamens and no fertile ovaries (Dempster, 1973).

Most individuals in polygamous *Galium* species are either predominantly pistillate or predominantly staminate, and species appear to be functionally

dioecious (Dempster, 1973). Therefore, Dempster considered polygamous species ancestral to dioecious species, having evolved from a state of hermaphroditism. As further justification for her hypothesis, she noted that dioecious species exhibit a high degree of polyploidy, whereas polygamous species are mostly diploid (Dempster, 1973). Thus, she considered polyploid, dioecious species to be derived from diploid, polygamous ancestors.

The occurrence of polygamy, alongside several other breeding systems, adds an interesting evolutionary question to the taxonomic and phylogenetic problems present within the *Cruciata-Galium-Valantia* (CGV) clade of tribe Rubieae. Members of *Cruciata* are either hermaphroditic or andromonoecious. Members of *Valantia* are andromonoecious. Among the *Galium* species within this clade, members are hermaphroditic, dioecious, or polygamous. Dioecy in *Galium* is exhibited by unisexual flowers with rudimentary organs of the sterile sex. These are type I unisexual flowers, in which unisexuality does not exist from inception but instead is caused by abortion relatively late in development (Mitchell and Diggle, 2005). No sex chromosomes have been observed in cytological investigations of dioecious *Galium* species (Ehrendorfer, 1961), and no other genetic basis of sex determimation has been identified for the group.

The CGV clade comprises mostly perennial taxa with whorls of strictly four leaves to a node, and four-parted, rotate flowers. *Cruciata* and *Valantia* are small genera, containing nine and seven species, respectively (Bisby et al., 2009), distributed in the Mediterranean and in Eurasia (Manen et al., 1994; Natali et al.,

1995). In prior molecular studies, *Valantia* has been shown to be sister to *Cruciata* and *Galium*, without strong support (Manen et al., 1994; Natali et al., 1995), and to be unresolved with *Cruciata* and *Galium* (Natali et al., 1996). More recently, *Valantia* has been shown to be sister to *Cruciata*, without strong support (Soza and Olmstead, 2010a).

The clade containing members of *Galium* is much more species rich. Four *Galium* sections are restricted to the CGV clade: *Baccogalium, Lophogalium, Platygalium,* and *Relbunium* (Manen et al., 1994; Natali et al., 1995, 1996; Ehrendorfer et al., 2005; Soza and Olmstead, 2010a), representing at least 166 species. These sections have been distinguished from one another mainly on the basis of fruit type (i.e., dry vs. fleshy) and hairiness of fruit, as well as breeding system.

Galium section *Baccogalium* has never been typified nor validly published, but has been characterized as dioecious and bearing fleshy fruits lacking specialized hairs (Fig. 2.1A, B); it is distributed from Oregon to Baja California (Dempster and Stebbins, 1965, 1968). Thirteen species constitute the group (Dempster and Stebbins, 1965). Dempster also considered these species to form a monophyletic group with three other fleshy-fruited species from southeastern USA and Mexico (Dempster, 1978).

Galium section *Lophogalium* (Schumann, 1897) is characterized as polygamous or dioecious and as bearing dry fruits with long-straight hairs (Fig. 2.1C, D). Section *Lophogalium* comprises about 50 species distributed in mountain ranges

of western North America and the Andes of South America (Dempster and Ehrendorfer, 1965; Dempster and Stebbins, 1971; Dempster 1978, 1980), with one disjunct species in Asia (Ehrendorfer, 1956; Dempster, 1978, 1980).

Galium section *Platygalium* is a large and morphologically diverse group. No comprehensive, worldwide treatment exists for this section (Ehrendorfer et al., 2005). However, from a review of various floras, the section comprises at least 70 species (Ehrendorfer et al., 1976; Dempster, 1978, 1981, 1982; Ehrendorfer and Schönbeck-Temesy, 1982; Yamazaki, 1993; Pobedimova, 2000; Ehrendorfer et al., 2005; Tao and Hua, in preparation). The section is distributed worldwide with centers of diversity in eastern Asia, eastern North America, the Mediterranean and the Caucasus (Ehrendorfer et al., 2005). Taxa are hermaphroditic and bear dry fruits that are glabrous or with hooked (Fig. 2.1E, F) or curved hairs (Ehrendorfer et al., 1976; Ehrendorfer and Schönbeck-Temesy, 1982; Pobedimova, 2000; Ehrendorfer et al., 2005).

Galium section *Relbunium* (Endlicher, 1839), which also has been treated at the generic level (Ehrendorfer, 1955), comprises about 33 species centered in South America and extending into southwestern North America and the Caribbean (Porto et al., 1977). Section *Relbunium* is characterized by a two- or four-leaved involucre subtending the flowers. Members are mostly hermaphroditic, bearing fruits that are generally fleshy and glabrous (Fig. 2.1G, H; Ehrendorfer, 1955).

Another section suspected to belong to the CGV clade is *Galium* section *Bataprine,* which also has been treated at the generic level (Niewland, 1910).

Section *Bataprine* is composed of two hermaphroditic species, bearing fleshy fruits lacking specialized hairs, and is distributed in southeastern USA (Niewland, 1910).

The goals of the current study were to resolve relations within the CGV clade and to examine the evolution of breeding systems within this group. We sought to (1) identify additional members of the CGV clade, (2) resolve relations among *Galium* members, (3) test the monophyly of *Galium* sections, (4) determine whether fruit morphology is indicative of monophyletic groups, (5) determine origins of dioecy and andromonoecy within the CGV clade, and (6) determine whether polygamy is a pathway from hermaphroditism to dioecy.

To achieve these goals, we increased sampling within the CGV clade, particularly among *Galium* sections, and included other New World species suspected of belonging to this clade. Data were used from three chloroplast (cp) regions (*rpoB-trnC, trnC-psbM, trnL-ndhJ*) and a nuclear ribosomal (nr) region (ETS). We conducted Bayesian MCMC (Yang and Rannala, 1997) phylogenetic analyses to estimate the CGV clade phylogeny, which was subsequently used for reconstruction of ancestral states for breeding system and fruit morphology.

MATERIALS AND METHODS

Sampling—Ninety accessions, representing 89 taxa, were sampled (Appendix B), including all three genera (*Cruciata*, *Galium*, and *Valantia*) and approximately 49% of all species in the CGV clade. For *Galium*, we sampled from five sections and unassigned taxa previously shown, or suggested, to belong to the

CGV clade (Table 2.1; Manen et al., 1994; Natali et al. 1995, 1996; Soza and Olmstead, 2010a). For outgroups, we sampled four species from its sister clade ("*Asperula* sect. *Asperula* clade" in Natali et al., 1996; "Clade VI" in Soza and Olmstead, 2010a) and *Galium obtusum,* representing the next more distant clade ("*Asperula* sect. *Glabella* clade" in Manen et al., 1994; Natali et al. 1995, 1996; "Clade V" in Soza and Olmstead, 2010a).

Molecular methods—DNA samples were obtained from field-collected, silica gel-dried tissue; herbarium specimens; or other Rubiaceae researchers (Appendix B). We extracted DNA using the 2% CTAB procedure (Doyle and Doyle, 1987). DNA from field-collected, silica gel-dried tissue was purified using Wizard SV Minicolumns (Promega Corporation, Madison, Wisconsin, USA). DNA from herbarium specimens was purified by precipitating with an equal volume of 100% isopropanol overnight at –20°C, followed by an additional precipitation with 2x volume of 100% ethanol and 1/10 volume of 3M pH 5.2 sodium acetate overnight at –20°C, as outlined in Sambrook et al. (1989).

We amplified the cp *rpoB-trnC* region with the rpoB and trnC^{GCA}R primers (Shaw et al., 2005). For DNA of lower quality, we amplified this region in two adjacent fragments using the rpoB and rpoBdR primers and the rpoBd and trnC^{GCA}R primers (Table 2.2). The *trnC-psbM* region was amplified by using the trnC^{GCA}F and psbMR primers (Shaw et al., 2005). For DNA of lower quality, this region was amplified in two adjacent fragments by using the trnC^{GCA}F and ycf6R primers and the ycf6F and psbMR primers (Shaw et al., 2005). The *trnL-trnF-ndhJ* region was

amplified with use of the “c” (Taberlet et al., 1991) and ndhJ (Shaw et al., 2007) primers. For DNA of lower quality, we amplified the region in two overlapping fragments using the “c” and “f” primers (Taberlet et al., 1991) and the “e” (Taberlet et al., 1991) and ndhJ primers. The 3’ end of the nr external transcribed spacer (ETS) was amplified with use of the ETS-9 (Wright et al., 2001) and 18S-IGS (Baldwin and Markos, 1998) primers.

Polymerase chain reactions (PCR) were conducted in an MJ Research PTC-100 Peltier thermal cycler (Biorad, Hercules, California, USA) in 25-µL volumes: 2.5 µL 10x 30 mM $MgCl_2$ reaction buffer, 2.5 µL 10x *Taq* diluent, 2.5 µL dNTPs (10 mM), 1.25 µL each primer (5 µM), 0.125 µL *Taq*, 0.5—1 µL template, and remaining volume of H_20. PCR conditions were an initial denaturation of 94°C for 2 min, followed by 35 cycles of 94°C denaturation for 15 s, 48—55°C annealing for 15 s, 72°C extension for 1--2 min for cp regions, or 30 s for ETS region, and a final extension at 72°C for 10 min. PCR products were purified by a 20% polyethylene glycol precipitation (Sambrook et al., 1989) before sequencing.

For taxa that could not be sequenced directly from initial PCR ETS products, we reamplified the region with a high-fidelity enzyme, PfuUltra II fusion HS DNA polymerase (Strategene, La Jolla, California, USA), for subsequent cloning. PCR was conducted in 25-µL volumes: 2.5 µL PfuUltra II reaction buffer, 2.5 µL dNTPs (10 mM), 1.25 µL each primer (5 µM), 0.5 µL polymerase, 0.5—1 µL template, and remaining volume of H_20. PCR conditions were as outlined above. Addition of 3’ A-overhangs to PCR products was performed as outlined in TOPO TA Cloning Kit for

Sequencing (Invitrogen, Carlsbad, California, USA), with 0.1 µL *Taq* per reaction for 10 minutes at 72°C before purification.

TOPO cloning reactions and One Shot (Invitrogen) chemical transformation were performed following the manufacturer's instructions in quarter reactions. Sixteen or 32 colonies, depending on ploidy level, were picked from each PCR product and were screened and amplified by PCR with "M13*F" (5'-GTAAAACGACGGCCAGTGAAT-3') and "M13*R" (5'-CAGGAAACAGCTATGACCATG-3'; primers modified by K. Karol, New York Botanical Garden) in 20-µL volumes: 2.0 µL 10x 30 mM $MgCl_2$ reaction buffer, 2.0 µL 10x *Taq* diluent, 2.0 µL dNTPs (10 mM), 1.2 µL each primer (5 µM), 0.1 µL *Taq*, and 11.5 µL H_20. PCR conditions were an initial denaturation of 94°C for 2 min, followed by 30 cycles of 94°C denaturation for 15 s, 55°C annealing for 15 s, 72°C extension for 45 s, and a final extension at 72°C for 10 min. Cloned PCR products were purified as outlined above, and 9—16 positive clones per accession were sequenced as described below.

Sequencing was performed with the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Buckinghamshire, UK). Both strands of each region were sequenced with the same amplification primers above and several internal primers (Table 2.2). For direct sequencing of ETS, only the ETS-9 amplification primer and the 18S-E primer were used. For sequencing of cloned ETS products, only the T3 and T7 primers (Invitrogen) were used. Sequencing reactions were done in 5-µL volumes: 2 µL 5-fold diluted dRhodamine sequencing reagent premix, 0.25

μL primer (5 μM), 0.5--2.75 μL template, and remaining volume of H_20. Sequencing conditions were an initial denaturation of 94°C for 2 min, followed by 35 cycles of 92°C denaturation for 10 s, 50 or 55°C annealing for 5 s, and 60°C extension for 2.5 min. Sequencing products were purified with a sodium acetate/EDTA and ethanol precipitation and then analyzed on an ABI Prism 377 DNA Sequencer (Applied Biosystems, Carlsbad, California, USA).

Phylogenetic methods—Sequences were initially aligned in ClustalW (Chenna et al., 2003) and then manually adjusted in MacClade 4.08 (Maddison and Maddison, 2000) on the basis of phylogenetic weighting and similarity criteria (Mindell, 1991; Simmons, 2004). Unambiguously aligned gaps that were phylogenetically informative for ingroup were coded as presence/absence characters (Graham et al., 2000; Simmons and Ochoterena, 2000). Regions in which the alignment was ambiguous were excluded from the analyses.

To determine whether conflicting phylogenetic signal existed between cp and nr data sets, all cp regions were combined and analyzed separately from the ETS region with the use of Bayesian analyses. The majority rule consensus tree based on the combined cp data set was compared with the majority rule consensus tree based on the ETS region to determine whether and where conflicting phylogenetic signal existed.

A third analysis combined and analyzed all cp and nr regions with Bayesian analyses. For taxa with various clonal ETS sequences, one sequence was selected from each monophyletic group of sequences representing a given taxon. All other

clonal sequences not forming monophyletic groups with other clones from the same accession were included in analyses. For accessions with more than one included ETS clone, the corresponding cpDNA sequences were duplicated for use in the combined nr and cp data set. The combined cp and nr data set is available through TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S10364).

For Bayesian analyses, models of evolution for the combined cp data set and nr data set were determined separately by Modeltest 3.7 (Posada and Crandall, 1998). The models selected under the Akaike information criterion (Akaike, 1974) were GTR + I + Γ and GTR + Γ, respectively. In addition, the binary model was used for gap data, with ascertainment coding bias set to variable, in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Bayesian analyses were conducted with data partitioned under the selected models, and default priors of no prior knowledge were used for the parameters of these models.

The prior probability density for the six nucleotide substitution rates and four stationary nucleotide frequencies was a flat Dirichlet, with all values set to 1.0. The prior for the proportion of invariable sites and gamma shape parameter for among-site rate variation was a uniform distribution, from 0 to 1 and 0 to 200 for α, respectively. The default prior probability for topology was uniform, with all possible trees equally probable a priori. The default prior probability distribution on branch lengths was unconstrained and exponential with a parameter of 10. Parameters for nucleotide frequencies (statefreq), substitution rates (revmat), and gamma shape (shape) were unlinked across both data partitions. All partitions were allowed to

evolve under different rates, and site-specific rates were allowed to vary under a flat Dirichlet prior across partitions.

Bayesian analyses were conducted with three independent Markov Chain Monte Carlo analyses of 15 million generations for the combined cp and cp + nr data sets, and 40 million generations for the nr data set, with a sampling frequency of every 1000th generation. Metropolis coupling (Yang and Rannala, 1997) for each analysis was conducted with the default of four chains started from different random trees, three heated and one cold chain, temperature of 0.2, and one swap of states tried between chains every generation. Convergence was determined when the average standard deviation of split frequencies remained less than 0.01. To verify convergence, all three runs were examined with AWTY's "compare" and "cumulative" analyses (Nylander et al., 2008) to compare split frequencies between runs and examine cumulative split frequencies for runs, respectively.

For the combined cp data set, the first 3380 trees were discarded before convergence. For the nr data set, the first 30 338 trees were discarded before convergence. For each analysis, the remaining trees from each run were pooled to construct a 50% majority rule consensus tree. For the combined cp + nr data set, the first 6262 trees were discarded, and the remaining trees from each run were pooled to construct a consensus tree with all compatible groups to obtain posterior probabilities (pp).

Ancestral state reconstructions—For ancestral state reconstruction, breeding system, fruit type, and fruit hairs of each terminal taxon were gleaned from

the literature, personal observations, and/or herbarium specimens (Appendix B). Breeding systems were categorized as andromonoecious, dioecious, hermaphroditic, or polygamous. Fruit types were categorized as nutlets (i.e., dry; Fig. 2.1C--F) or berries (i.e., fleshy; Fig. 2.1A, B, G, H). Fruit hairs were categorized as none (Fig. 2.1A, H), long-straight (Fig. 2.1C, D), hooked (including curved; Fig. 2.1E, F), or pubescent (i.e., minute; Fig. 2.1B, G).

Character matrices were composed of categorical data with multiple states and analyzed in Mesquite 2.72 (Maddison and Maddison, 2009). We reconstructed ancestral states on the fully resolved combined tree using equally weighted parsimony (unordered states; Fitch, 1971) and likelihood, under the Markov k-state one-parameter (MK1) model, in which all changes are equally probable.

Polymorphic taxa are not allowed under likelihood reconstructions in Mesquite. Therefore, five taxa with both glabrous and pubescent fruits (Appendix B) were assigned to either state in all 32 possible combinations to determine the most likely and parsimonious reconstructions. One taxon with extremely variable fruit hairs (*Cruciata taurica*) was not coded.

Topology testing—To positively accept or reject Dempster's (1973) hypothesis of polygamy as an intermediate state between hermaphroditism and dioecy, alternative topologies were constructed in MacClade 4.08 to test against the Bayesian consensus tree (Appendix C). Five alternative topologies were constructed in which polygamous taxa were constrained as ancestral or sister to dioecious taxa

from hermaphroditic ancestors. Such constraints were placed on either of the two main clades of *Galium* and on both of the main clades at the same time.

Site-wise log-likelihoods for all six trees were obtained from PAUP* version 4.0b10 (Swofford, 2002) under the GTR + I + Γ model, with model parameters estimated by GARLI 0.960 (Zwickl, 2006) from CIPRES Portal 2.0 (Miller et al., 2010). Site-wise log-likelihoods were imported into CONSEL (Shimodaira and Hasegawa, 2001) to assess the confidence set of trees. One hundred thousand bootstrap replicates of log-likelihoods were generated in CONSEL to obtain *P* values for the six topologies under the approximately unbiased (AU) test (Shimodaira, 2002). Topologies with *P* values less than 0.05 were rejected as candidate trees.

RESULTS

Sampling and molecular results— Summarized in Table 2.3 are the unaligned and aligned lengths, excluded regions, total base pairs analyzed, uncorrected pairwise distances, and gaps scored, and number of completely or partially sequenced accessions for each region.

Phylogenetic results—We examined the majority rule consensus trees from the cp and nr datasets (Figs. 2.2—2.3) and found many of the relations among taxa were not resolved or were weakly supported with the individual ETS data set, in contrast to the cp data set. In addition, the phylogenetic signal in our ETS data set was weak. Therefore, only analyses from the combined cp and nr datasets were

used to estimate the phylogeny of the CGV clade and in ancestral state reconstructions.

In the Bayesian analyses of the combined cp and nr datasets (Fig. 2.4), the CGV clade was found to contain two major clades that were strongly supported with pp = 1.00. One clade corresponded to *Cruciata* and the other to *Galium* species included in the CGV clade. These two clades are sister groups, and together are sister to *Valantia,* here represented by a single species (*V. muralis*).

Within this clade of *Galium*, members of sect. *Platygalium* form a paraphyletic grade at the base (Fig. 2.4). Two strongly supported groups are derived from this grade of sect. *Platygalium*: clades 1—3 and clades 4—9 (Fig. 2.4). These two groups represent nine well-supported branches of strictly New World taxa. However, relations among these clades and at the base of the *Galium* clade are not well supported.

Within the first group, clade 1 consists of members of sect. *Baccogalium.* Clades 2 and 3 consist of members of sect. *Lophogalium.* Within the second group, clade 4 consists of members of sect. *Bataprine*, which form a paraphyletic grade from which a small group comprising members of sect. *Platygalium* are derived. Clade 5 is composed of members of sect. *Lophogalium*. Clade 6 consists primarily of members of sect. *Relbunium*, in addition to one member of sect. *Lophogalium* (*G. gracilicaule*) and two unassigned species (*G. latoramosum, G. lilloi*). Clade 7 consists of annual taxa from an unnamed group. Clade 8 consists primarily of members of sect. *Lophogalium*, and one member of sect. *Baccogalium* (*G.*

aschenbornii). Clade 9 comprises members from sects. *Lophogalium* and *Platygalium* (Appendix B), in addition to two unassigned species (*G. correllii, G. hintoniorum*).

Ancestral state reconstructions—Parsimony reconstruction of breeding systems required 15 transformations and reconstructed an equivocal origin of the CGV clade as andromonoecious or hermaphroditic (Fig. 2.5). Likelihood reconstruction of ancestral breeding systems (-ln L = 68.979) reconstructed a hermaphroditic origin of the CGV clade with proportional likelihood of 0.790 (Fig. 2.5).

Andromonoecy has arisen at least twice from hermaphroditism in *Cruciata* and *Valantia* (Fig. 2.5). Dioecy has arisen at least three times from hermaphroditism: in the common ancestor of clades 1—3 and in clades 6 and 9 (Fig. 2.5). Polygamy has arisen at least twice from dioecy in clades 1 and 2 and at least six times from hermaphroditism in clades 5—6 and 8—9, with a reversal to hermaphroditism in clade 9 (Fig. 2.5).

The most parsimonious and highest likelihood (-ln L = 75.875) reconstruction of fruit hairs required 18 transformations and reconstructed an unequivocal origin of the CGV clade with no fruit hairs (Fig. 2.6). Fruits with long-straight hairs have arisen at least five times in clades 2—3, 5—6, and 8—9 (Fig. 2.6). Fruits with hooked hairs have arisen at least four times in clades 2 and 4 and at the base of the *Galium* clade (Fig. 2.6). Pubescent fruits have arisen at least twice in clades 1 and 6 (Fig. 2.6).

Parsimony and likelihood (-ln L = 22.911) reconstruction of fruit type required 5 transformations, and reconstructed an unequivocal origin of the CGV clade with nutlets (Fig. 2.6). Berries have arisen at least four times from nutlets in clades 1, 4, 6, and 8, with a probable reversal to nutlets in clade 4 (Fig. 2.6).

Topology testing—All five alternative topologies, indicating polygamous taxa as intermediate between hermaphroditic and dioecious taxa, were rejected by the approximately unbiased test (P = 0.000; Table 2.4). The Bayesian consensus tree was ranked number one, and the only topology that was not rejected (P = 1.000).

DISCUSSION

Phylogeny—Clade 1 (Fig. 2.4) corresponds to *Galium* sect. *Baccogalium*, the fleshy-fruited species distributed from Oregon to Baja California (Dempster and Stebbins, 1965, 1968). The group contains approximately 13 perennial species and up to 24 taxa including infraspecific taxa (Dempster, 1993). Members are mostly dioecious, with the exception of one polygamous species (*G. grande*). This group includes both diploid and polyploid taxa (Dempster and Stebbins, 1965, 1968).

Other fleshy-fruited species from southeastern USA and Mexico, once thought to be closely related to sect. *Baccogalium* (Dempster, 1978), occur elsewhere throughout the CGV clade: in clade 4 (*G. bermudense*, *G. uniflorum*) and clade 8 (*G. aschenbornii*; Figs. 2.4, 2.6). Monophyly has been confirmed for the fleshy-fruited group from Oregon to Baja California only.

Clade 2 (Fig. 2.4) corresponds to the group described as the *Galium angustifolium* complex (Ehrendorfer, 1956; Dempster and Stebbins, 1971). On the basis of our results, *G. hallii* also is a member, previously suspected of close affinities with this group but assigned to the *Galium multiflorum* complex (Ehrendorfer, 1956). *Galium stellatum* is sister to clade 2, but the inclusive clade is not strongly supported. This taxon was previously assigned to the *Galium multiflorum* complex (Ehrendorfer, 1956; Dempster and Ehrendorfer, 1965).

The *G. angustifolium* complex was previously described as strictly dioecious (Ehrendorfer, 1956; Dempster and Stebbins, 1971). However, our results show that a polygamous taxon (*G. catalinense*), an endangered species endemic to the southern Channel Islands (Dempster, 1993), also belongs to this group. This complex now comprises approximately five species and up to 13 taxa including infraspecific taxa (Dempster, 1993). This group is restricted to the Channel Islands and coastal ranges of southern California and Baja California, east to the Colorado and Mojave deserts (Dempster and Ehrendorfer, 1965; Dempster and Stebbins, 1971; Dempster, 1973). Members are herbaceous perennials to shrubs, bearing linear leaves with apically directed, short hairs along the margins (Ehrendorfer, 1956; Dempster and Ehrendorfer, 1965; Dempster and Stebbins, 1971). Most are diploid, with four reported polyploids (Ehrendorfer, 1961; Dempster and Ehrendorfer, 1965; Dempster and Stebbins, 1971; Dempster, 1993).

Clade 3 (Fig. 2.4) corresponds to the the *Galium multiflorum* complex, first described by Ehrendorfer (1956), then narrowly circumscribed by Dempster (1959),

and finally revised as the *Galium multiflorum* aggregate (Dempster and Ehrendorfer, 1965). Approximately 14 species, and possibly up to 37 taxa including infraspecific taxa, constitute the group. Members are herbaceous to suffrutescent perennials, bearing lanceolate to orbicular leaves lacking curved hairs along the margins (Ehrendorfer, 1956; Dempster and Ehrendorfer, 1965), and are strictly dioecious. On the basis of our results, the group's distribution is restricted to interior California, north to Washington, and east to central Colorado, primarily distributed in the Great Basin mountains and southern deserts (Ehrendorfer, 1956; Dempster, 1959; Ehrendorfer, 1961; Dempster and Ehrendorfer, 1965). This group includes both diploid and polyploid taxa (Ehrendorfer, 1961; Dempster and Ehrendorfer, 1965).

Clade 4 (Fig. 2.4) is composed of representatives from sects. *Bataprine* and *Platygalium.* Section *Bataprine* forms a paraphyletic grade from which representatives of sect. *Platygalium* are derived. This clade is distributed in eastern North America, from Texas and Florida north to Ontario and Quebec, and includes approximately six perennial, hermaphroditic species. Taxa are variable, with glabrous, fleshy fruits to hooked-hairy, dry fruits (Fig. 2.6). Ploidy levels are unknown for this group.

Clade 5 (Fig. 2.4) is composed of one species (*G. gilliesii*) from South America, previously included in sect. *Lophogalium* (Dempster, 1980). This species may turn out to be closely allied to clade 6 with additional sampling from South America.

Clade 6 (Fig. 2.4) is predominantly South American, containing sect. *Relbunium,* a member of sect. *Lophogalium* (*G. gracilicaule*), and two unassigned taxa from South America (*G. latoramosum, G. lilloi*). Our results show that sect. *Relbunium* is not monophyletic because *G. latoramosum* is nested within it. Dempster (1982) was skeptical of sect. *Relbunium*, as defined by Endlicher (1839) and Ehrendorfer (1955), as a monophyletic group. Dempster (1982) restricted the section to include only those species with all flowers solitary, sessile, and involucrate. She excluded species with involucrate inflorescences like *G. microphyllum* and *G. richardianum,* in which not all flowers are sessile and individually involucrate. Clade 6 contains a well-supported group that corresponds to Dempster's (1990) notion of sect. *Relbunium* sensu stricto (s.s.), as represented by the clade that contains *G. nigroramosum* and *G. corymbosum*.

Clade 6, as sampled here, contains mostly hermaphroditic species, one dioecious species (*G. latoramosum*), and two polygamous species (*G. megapotamicum, G. richardianum*). Most of the species are perennial, with several annuals. The distribution for this group is centered in the southern half of South America, with a few species extending along western South America, north to Mexico, and east to the Caribbean. Up to 49 species from South America may belong to this clade (Dempster 1980, 1982, 1990). Ploidy levels are unknown for the group, except for diploid *G. hypocarpium* (Cavalli-Molina et al., 1989).

Galium lilloi (clade 6) previously was considered primitive within *Galium* because of its two-leaved habit (Dempster, 1982). However, this two-leaved habit is

now inferred to be a reduction from four leaves at a node (Soza and Olmstead, 2010a). *Galium lilloi* is closely related to *G. gracilicaule*, which was previously included in sect. *Lophogalium* (Dempster, 1980). Both species share the features of solitary flowers in axils and creeping habit (Dempster 1980, 1982).

Clade 7 (Fig. 2.4) is composed of three annual species, once thought closely related to sect. *Relbunium* because of their sessile flowers, each subtended by two involucral bracts (Ehrendorfer, 1955). These three species are hermaphroditic, bearing dry fruits that are hooked-hairy and reflexed (Fig. 2.1F). This group is distributed predominantly in southern USA, just barely extending into northern Mexico (Dempster, 1978). Ploidy levels are unknown for this group.

Clade 8 (Fig. 2.4) corresponds to the group described by Dempster (1973) as "the polygamous species of…*Galium*…section *Lophogalium,* of Mexico and southwestern United States" (excluding *G. catalinense*). We have shown that *G. aschenbornii* and *G. carterae* also belong to this group, extending its distribution south to Central America. Approximately 12 species belong to clade 8, distributed in mountains from southwestern USA to Central America (Dempster, 1973, 1978). Members are perennial, apparently diploid, and all polygamous. Fruits are generally dry with long-straight hairs, except for *G. aschenbornii,* which has fleshy fruits (Dempster 1973, 1978).

Clade 9 (Fig. 2.4) is composed primarily of representatives from sects. *Lophogalium* and *Platygalium.* At least 17 perennial species belong to this group. Taxa are variable, exhibiting fruit with no hairs, hooked hairs, or long-straight hairs

(Fig. 2.6). Most members are hermaphroditic, with four polygamous taxa and one dioecious taxon (Dempster, 1978; Turner and Turner, 1983). The group is distributed predominantly in Mexico, extending north into southern USA and south to Central America (Dempster, 1978; Turner and Turner, 1983). Taxa are montane in distribution, and most species grow on calcareous substrates (Dempster, 1978; Turner and Turner, 1983). Ploidy levels are unknown for this group.

Our results from the combined cp + nr data sets show *Valantia* as sister to the remaining CGV clade (Fig. 2.4). These results conflict with the Rubieae phylogeny based entirely on cpDNA sequences presented by Soza and Olmstead (2010a), in which *Valantia* is sister to *Cruciata* with moderate support (74% bootstrap in parsimony, 0.56 pp in Bayesian analyses). This conflict may be caused by addition of the ETS data set. Our combined cp phylogeny (Fig. 2.2) shows a strongly supported CGV clade, but relations among *Cruciata, Galium,* and *Valantia* are not well supported. The ETS phylogeny (Fig. 2.3), however, shows a strongly supported clade of *Cruciata* and *Galium* that excludes *Valantia*. *Valantia* is a variable genus with both annual and perennial species, widespread and restricted species, and different base chromosome numbers. Additional sampling of *Valantia*, in addition to the widespread species *V. muralis*, may resolve this conflict.

Breeding system evolution—Dioecy is inferred to have arisen at least three times directly from hermaphroditism in this clade of *Galium* (Fig. 2.5). This direct pathway to dioecy from hermaphroditism is not one of the two main pathways commonly inferred, that is via gynodioecy (Lloyd, 1980; Hart, 1985; Ainsworth et al.,

1998; Weller and Sakai, 1999; Weiblen et al., 2000; Barrett, 2002) or monoecy (Lewis, 1942; Lloyd, 1980; Renner and Ricklefs, 1995; Ainsworth et al., 1998; Renner, 1998; Renner and Won, 2001; Barrett, 2002). In addition, no monoecious or gynodioecious species of *Galium* have been described within this group. However, a direct transformation from hermaphroditism to dioecy may be more likely within this clade of *Galium,* since rudimentary organs of the opposite sex remain in dioecious species.

Self-fertilization is known to occur in hermaphroditic species of *Galium* (sect. *Relbunium*; Cavalli-Molina et al., 1989; Brandão de Freitas et al., 1995), and evolution of dioecy may have resulted to avoid inbreeding. Dioecy in this clade of *Galium* has proved to be evolutionarily successful, as evidenced by the diversification of clades 1—3.

Dioecy often has been correlated with wind pollination, especially in temperate regions (Freeman et al., 1980; Givnish, 1980; Renner and Ricklefs, 1995; Vamosi et al., 2003). However, in *Galium*, the small, rotate, fragrant, white or yellow flowers are visited by a variety of lepidopterans, beetles, flies, ants, wasps, and short or longue-tongued bees (Batra, 1984). This association of dioecy with small flowers pollinated by unspecialized insects has been found in other cases of dioecy as well (Bawa and Opler, 1975; Bawa, 1980; Ibarra-Manríquez and Oyama, 1992; Sakai et al., 1995; Vamosi et al., 2003).

Our results indicate andromonoecy arises twice from hermaphroditism in *Cruciata* and *Valantia,* with a reversal to hermaphroditism in *Cruciata pedemontana*

(Fig. 2.5). However, *Cruciata* and *Valantia* may form a clade together, as prior studies have shown (Soza and Olmstead, 2010a). In this case, andromonoecy would have a single origin from hermaphroditism in the CGV clade, and the origin of the CGV clade would be unequivocally hermaphroditic.

In *Cruciata* and *Valantia,* the central flowers of cymes are hermaphroditic, and lateral flowers are male or absent (Ehrendorfer and Schönbeck-Temesy, 1982; Ehrendorfer et al., 2005). Andromonoecious members of *Cruciata* are outcrossing, producing large, yellow, fragrant, nectar-producing flowers (Ehrendorfer, 1965), whereas, *C. pedemontana* has become autogamous, with reduced hermaphroditic flowers and lacking male flowers (Ehrendorfer, 1965, 1971). All annual species of *Valantia* are autogamous and bear reduced male flowers, except for the single perennial species, which is allogamous (*V. aprica*; Ehrendorfer, 1965, 1971; Devesa and Ortega-Olivencia, 2003).

Polygamy is thought to have arisen at least eight times in *Galium* (Fig. 2.5). All known cases in the genus occur in this clade of *Galium* (Soza and Olmstead, 2010a). In all but one instance, polygamy has been a terminal condition. The one exception is the return to hermaphroditism in clade 9. Polygamy is inferred to have arisen at least six times from hermaphroditic ancestors and twice from dioecious ancestors. No evidence exists in the CGV phylogeny of polygamy as a pathway from hermaphroditism to dioecy. However, if we assume that dioecy evolved from hermaphroditism via polygamy and perform a weighted parsimony reconstruction of ancestral states, it is equally possible that dioecious *G. latoramosum* in clade 6 (Fig.

2.5) arose from a hermaphroditc or polygamous ancestor. This may be the only instance in which dioecy may have evolved from polygamy in the CGV clade.

In the two cases where polygamy has arisen from dioecy in *G. catalinense* and *G. grande* (clades 1 and 2, Fig. 2.5), this may be indicative of a breakdown of dioecy. Both polygamous taxa are endangered or sensitive species with small, isolated populations (Dempster, 1993), in which selection for the potential to self-fertilize may be particularly strong.

Unfortunately, not much is known about the ploidy level of taxa in clades 4—9, except for the diploid, polygamous species of clade 8 that Dempster (1973) referred to in her original hypothesis of polygamy. Dempster's hypothesis, based on the observation of dioecious, polyploid taxa, was founded on her knowledge of clades 1—3, in which polygamy had arisen as a breakdown of dioecy. However, dioecious, diploid taxa also occur in clades 1—3. The diploid, polygamous taxa Dempster referred to in clade 8 are not closely related to clades 1—3, as Dempster had previously thought. Dempster's original hypothesis of polygamy as ancestral to dioecy in *Galium* is now likely refuted.

Fruit evolution—We have shown that historical sections described for *Galium* in the CGV clade are not monophyletic. The main features used to define these sections have been fruit type and hairiness. Reconstruction of ancestral states of fruit type and hairiness (Fig. 2.6) also confirm that groups defined by these traits are not monophyletic and that these traits are not good indicators of shared evolutionary history.

Another recent study on members of *Galium* outside the CGV clade (Abdel Khalik et al., 2008) also has shown that sections based on external fruit morphology and seed characters are artificial. Abdel Khalik et al. (2008) showed that SEM studies of fruit and seed characters are useful for distinguishing between closely related taxa but are not indicative of historical groups.

Bremer and Eriksson (1992; Bremer, 1996) showed that fleshy fruits have arisen multiple times in Rubiaceae and were derived from dry fruits at least once in *Galium.* We infer that fleshy fruits have arisen at least four times in this clade of *Galium*. However, berries do define clade 1 (as section *Baccogalium* s.s.) and a subclade of clade 6 (as section *Relbunium* sensu lato), with fleshy fruits inferred in the common ancestors of both groups.

We found in examing the evolution of fruit types and breeding systems in the CGV clade (Fig. 2.7), that fleshy fruits do not appear to be correlated with dioecy, as has been observed in other studies (Bawa, 1980; Givnish, 1980; Flores and Schemske, 1984; Ibarra-Manríquez and Oyama, 1992; Renner and Ricklefs, 1995; Sakai et al., 1995; Webb et al., 1999; Vamosi et al., 2003; Vamosi and Vamosi, 2004). Most of these studies have been based on various floras. However, Vamosi et al. (2003) used a phylogenetic approach across angiosperms and found that dioecy is more likely to evolve in asterids that already bear fleshy fruits. In the CGV clade, fleshy fruits have arisen independently in hermaphroditic (clades 4 and 6), polygamous (clade 8), and dioecious (clade 1) species. In the one case in which

fleshy fruits are correlated with dioecy (clade 1), dioecy appears to have been established before the origin of fleshy fruits (Fig. 2.7).

Fruit hairs also are evolutionarily labile in this clade of *Galium* (Fig. 2.6). Glabrous fruits occur at the origin of the CGV clade in *Cruciata, Valantia,* and *Galium*. Hooked hairs and long-straight hairs, associated with dry fruits, have arisen multiple times in this clade of *Galium*.

These fruit characteristics may have been important to the success of this clade of *Galium*, with initial colonization of the New World by an ancestor with dry fruits and hooked hairs (Fig. 2.6). Subsequent evolution of long-straight hairs and fleshy fruits (Vamosi and Vamosi, 2004) may have aided further diversification in the New World.

In a study done in eastern North America (Matlack, 1994), fruits of understory species that were ingested by animals had higher migration rates than adhesive (i.e., hooked-hairs) fruits. Both these animal-dispersed fruit types had much higher migration rates than fruits dispersed by wind, followed by ant dispersal, and by fruits lacking known dispersal modes (Matlack, 1994).

In mammals of central Europe, such as wild boar and roe deer, hooked hairs and bristles on fruits have been shown to aid animal dispersal more than other fruit characteristics have (Heinken and Raudnitschka, 2002). Long-straight hairs also have been suggested to aid wind dispersal (Ehrendorfer, 1961).

Fleshy fruits, most likely eaten by animals functioning as dispersal agents, are correlated with glabrous or pubescent fruits, especially in the *Galium* portion of the

CGV clade (Fig. 2.6). No published observations of birds eating fleshy fruits of *Galium* have been made (Cavalli-Molina and Winge, 1988). However, lizards have been shown to eat the fleshy, orange fruits of *Galium hypocarpium* in South America and to transport intact, germinable seeds (Willson et al., 1996). *Galium* fruits also have been recorded in the diet of mantled ground squirrels in western North America (Martin et al., 1951).

Conclusions—The use of fruit morphology alone does not allow for unambiguous delimitation of sections of *Galium* within the CGV clade. Fruits with hooked hairs appear to be a plesiomorphic feature of this clade of *Galium*, as evidenced by the paraphyletic grade of members of sect. *Platygalium* at the base of the clade. Fleshy fruits are inferred to have arisen at least four times within this clade of *Galium*. Likewise, dry fruits with long-straight hairs are thought to have arisen at least five times within this clade of *Galium*. Of the sections historically described for *Galium* within the CGV clade, only the following two sections are monophyletic: sect. *Baccogalium*, if *G. aschenbornii* is excluded, and sect. *Relbunium*, if *G. latoramosum* is included, or if the group is constrained to Dempster's more limited circumscription.

Our results indicate both andromonoecy and dioecy have arisen directly from hermaphroditism in the CGV clade, which is in contrast to commonly reported pathways of monoecy and gynodioecy in angiosperms and the hypothesized pathway of polygamy in *Galium*. In addition, both dioecy and polygamy have arisen multiple times in the CGV clade, with polygamy representing a terminal condition in the majority of cases and not a pathway to dioecy. Multiple origins of dioecy and

polygamy from hermaphroditism within this clade may be due to the presence of type I unisexual flowers, in which sex is determined late in development.

Geographic distribution appears to be a better indicator of shared evolutionary history than fruit type or breeding system for the nine main branches occurring in this clade of *Galium*.

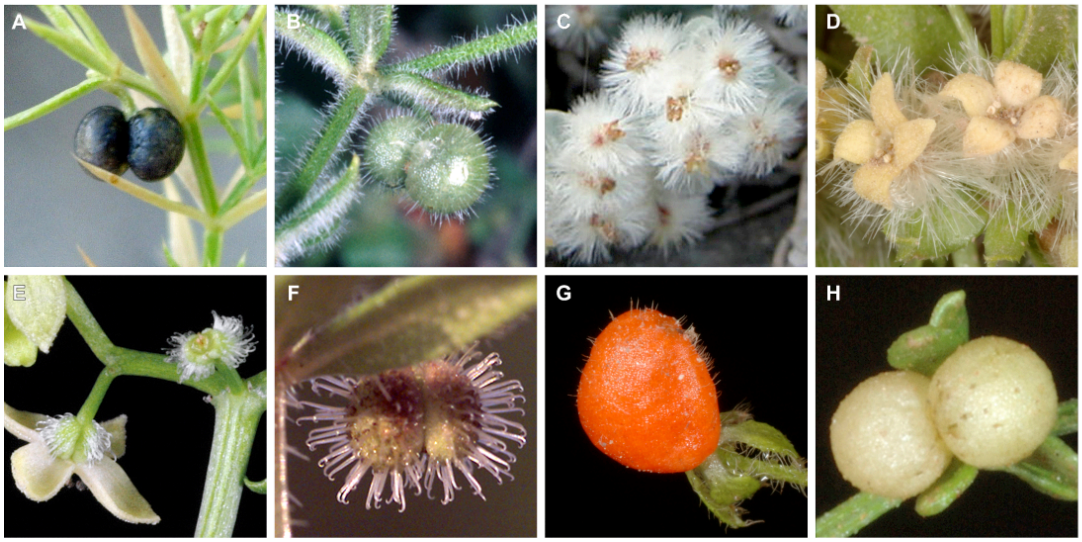

Figure 2.1. Fruit types and hairs of New World *Galium* in the *Cruciata-Galium-Valantia* clade. (A) Fleshy, glabrous fruit of *G. andrewsii*. (B) Fleshy, pubescent fruit of *G. grande*. (C) Dry fruits with long-straight hairs of *G. hypotrichium* subsp. *tomentellum*. (D) Dry fruits with long-straight hairs of *G. gilliesii* subsp. *gilliesii* (photo by J. T. Columbus). (E) Dry fruit and ovary with hooked hairs of *G. oreganum* (photo by G. D. Carr). (F) Dry fruit with hooked hairs of *G. virgatum* (photo by H. Wilson). (G) Fleshy, pubescent fruit of *G. hypocarpium* (photo by J. T. Columbus). (H) Fleshy, glabrous fruit of *G. bigeminum* (photo by J. T. Columbus).

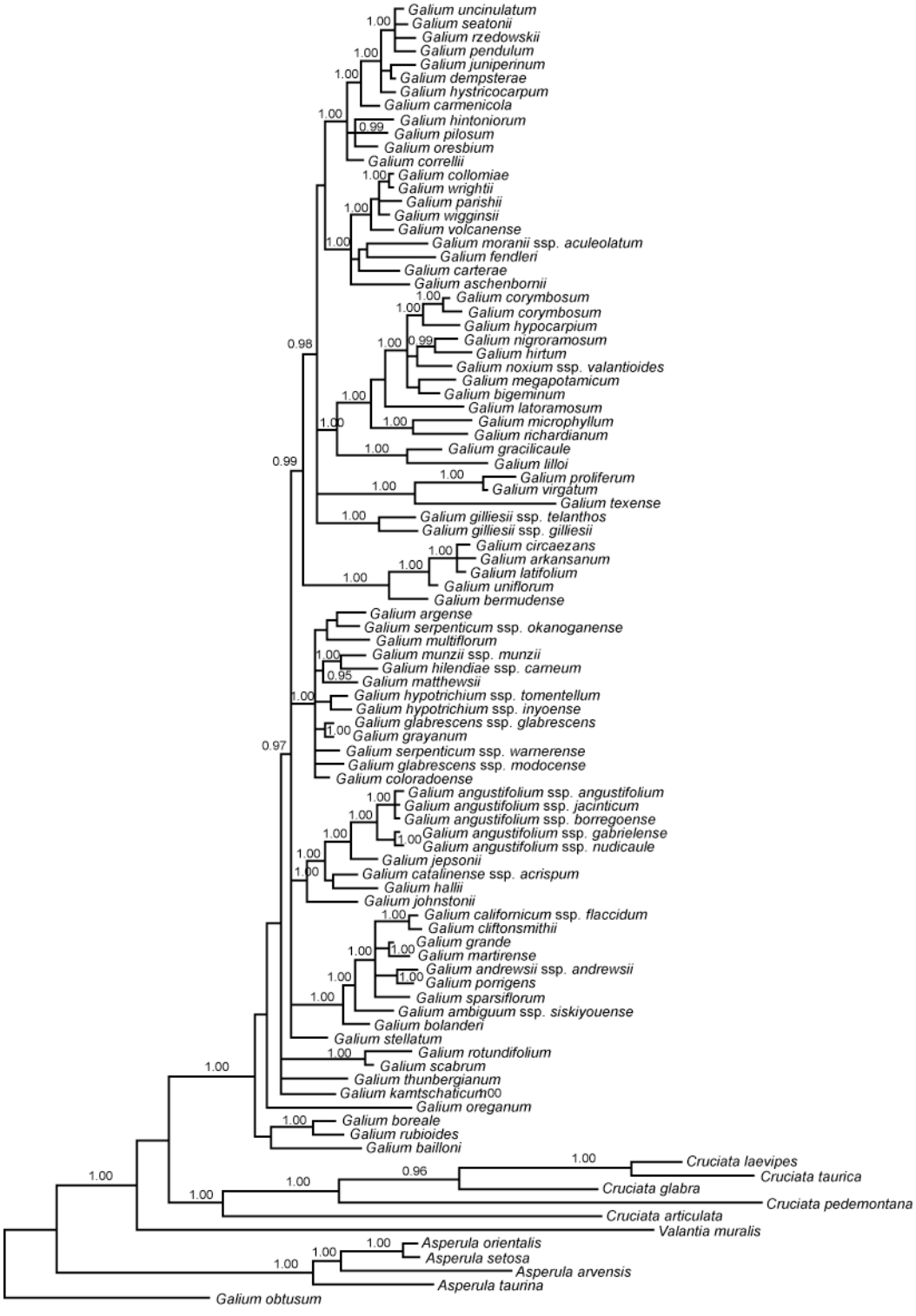

Figure 2.2. Phylogeny of *Cruciata-Galium-Valantia* clade, estimated from the 50% majority rule Bayesian consensus tree based on three combined cp regions (*rpoB-trnC, trnC-psbM, trnL-trnF-ndhJ*). Posterior probabilities ≥ 0.95 displayed above branches.

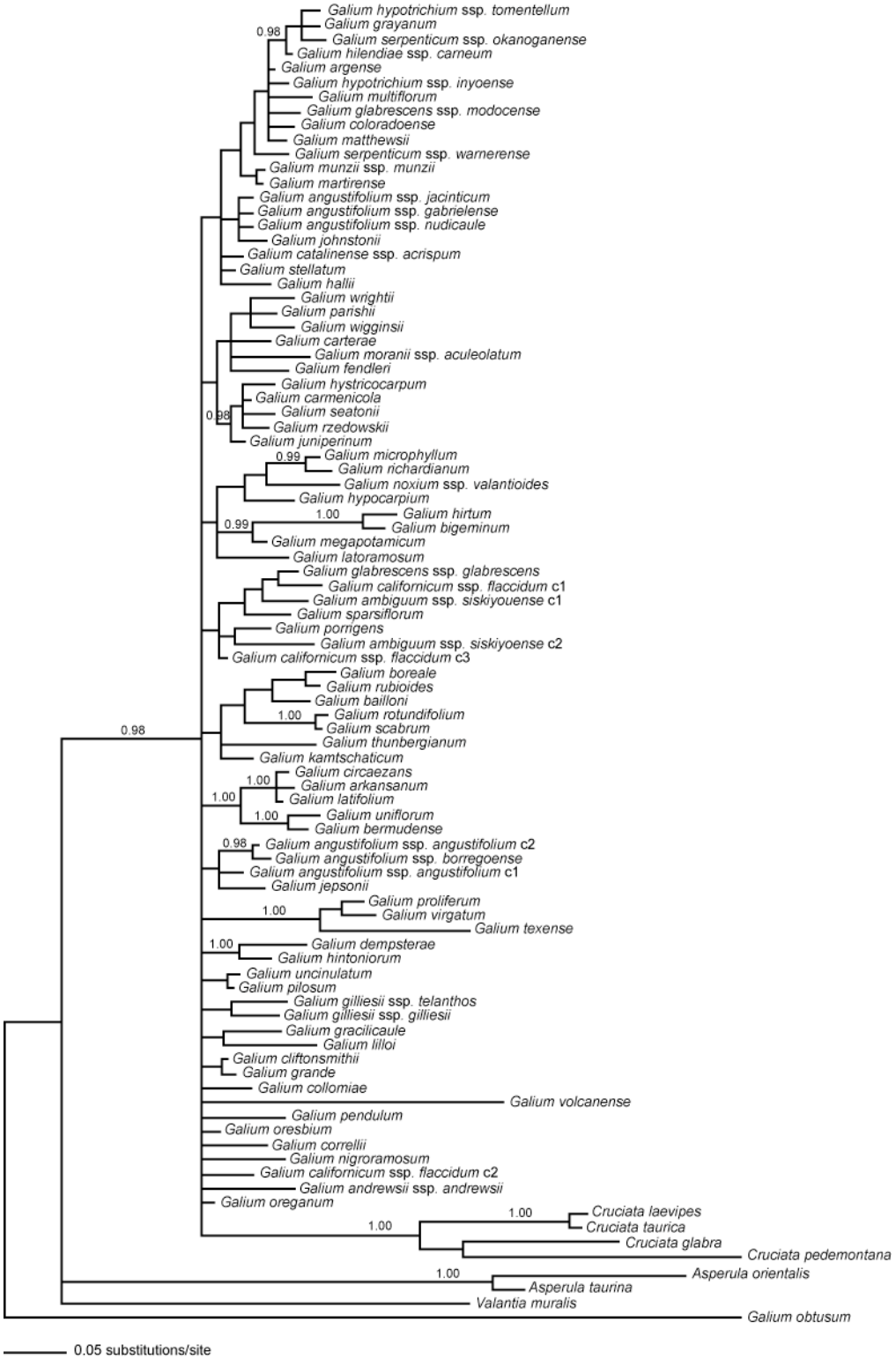

Figure 2.3. Phylogeny of *Cruciata-Galium-Valantia* clade, estimated from the 50% majority rule Bayesian consensus tree based on the nr external transcribed spacer (ETS). Posterior probabilities ≥ 0.95 displayed above branches.

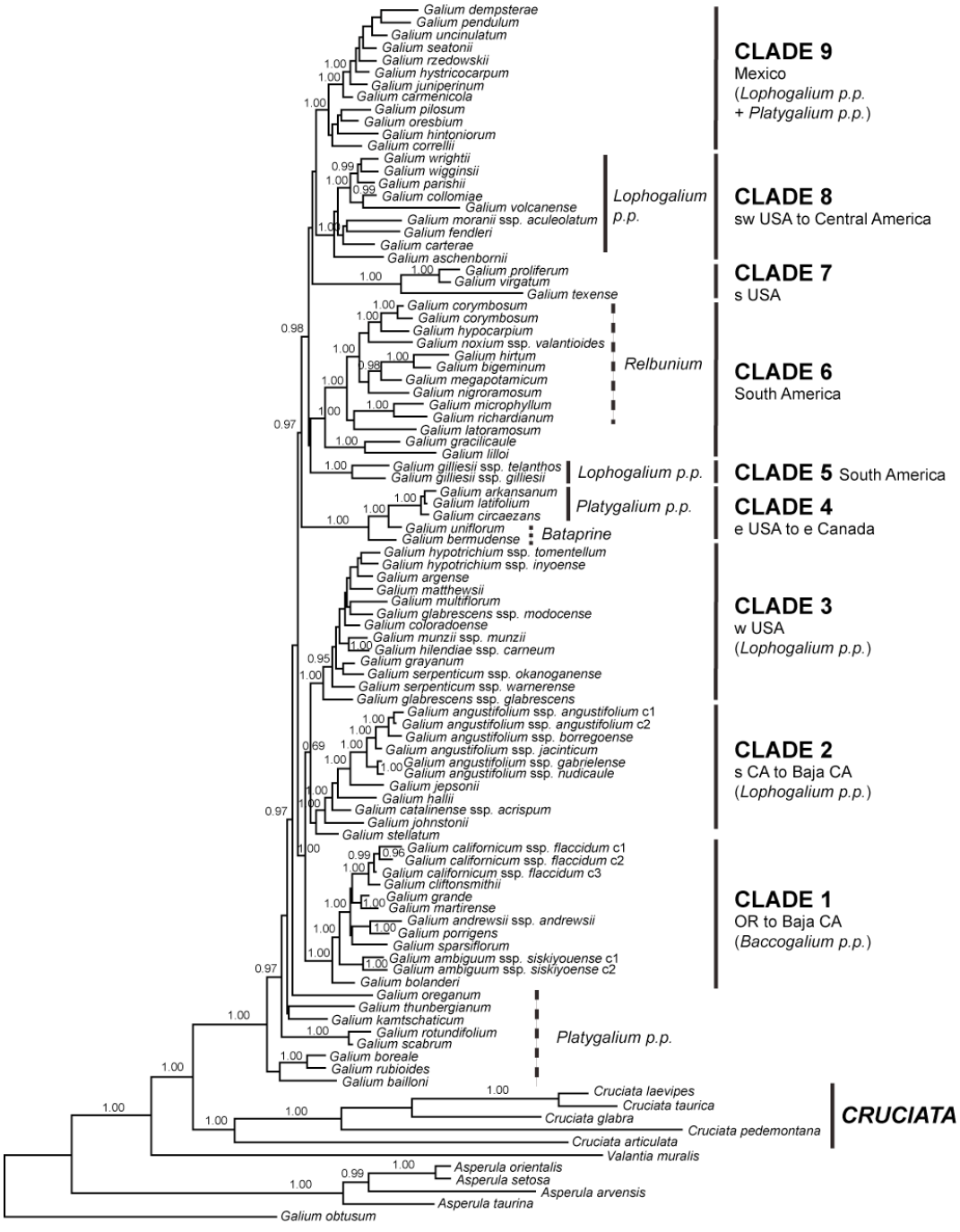

Figure 2.4. Phylogeny of *Cruciata-Galium-Valantia* clade, estimated from the Bayesian consensus tree based on the combined data set of three chloroplast regions (*rpoB-trnC, trnC-psbM, trnL-trnF-ndhJ*) and one nuclear ribosomal region (external transcribed spacer). Posterior probabilities ≥ 0.95 displayed above branches, including lower posterior probabilities at ancestral node of clades 2 and 3. Sectional affiliations shown for clades (solid lines) or grades (dashed lines) of two or more taxa. Primary geographic distribution indicated for clades 1—9. *Figure abbreviations*: CA, California; e, eastern; OR, Oregon; p.p., pro parte; s, southern; sw, southwestern; USA, United States of America; w, western.

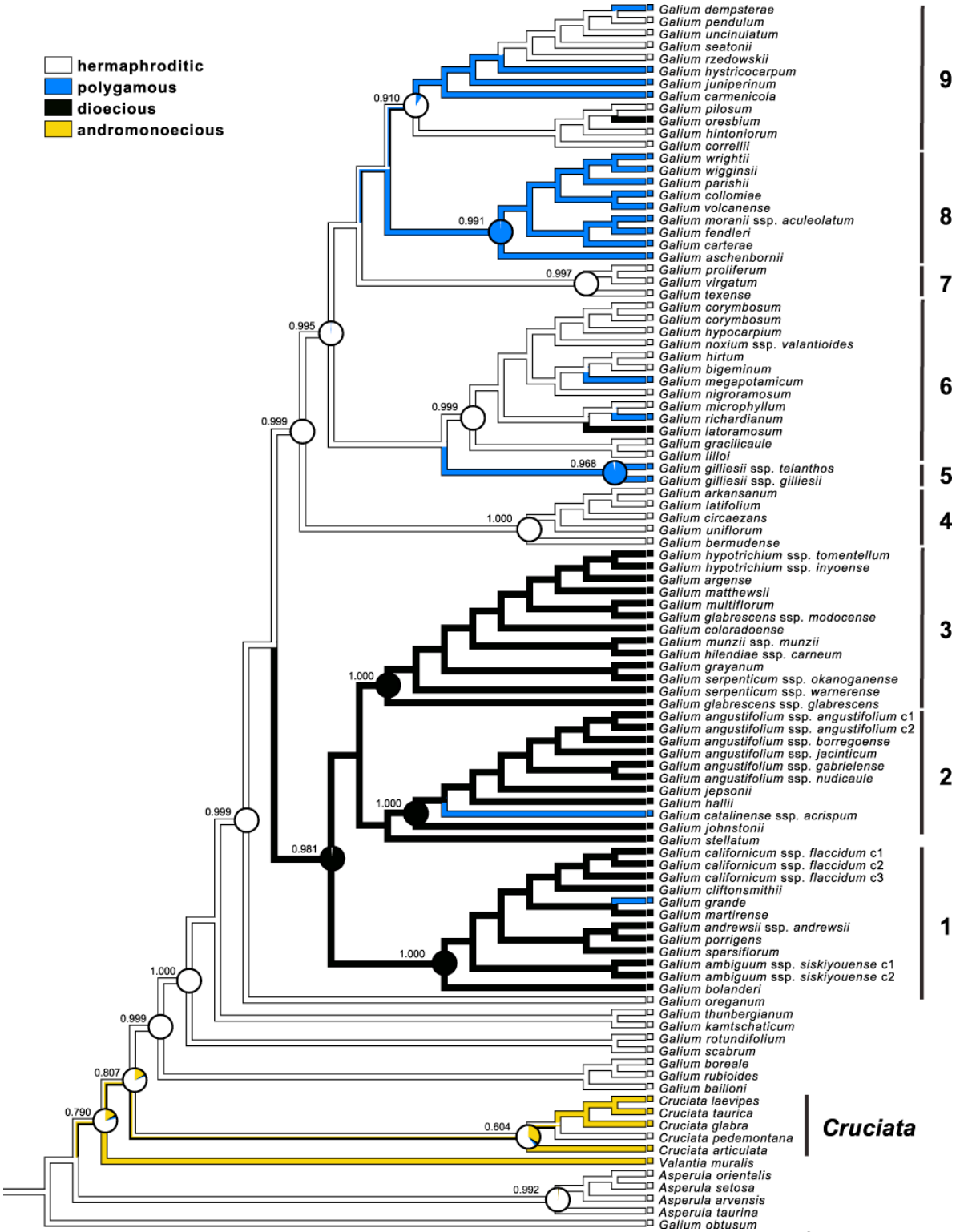

Figure 2.5. Bayesian consensus tree with parsimony and likelihood reconstructions of ancestral states of breeding systems. Proportional likelihoods of most likely state shown at strongly supported (i.e., Bayesian posterior probabilities ≥ 0.95) ancestral nodes along backbone of *Cruciata-Galium-Valantia* clade for nine major *Galium* clades, *Cruciata* clade, and outgroup.

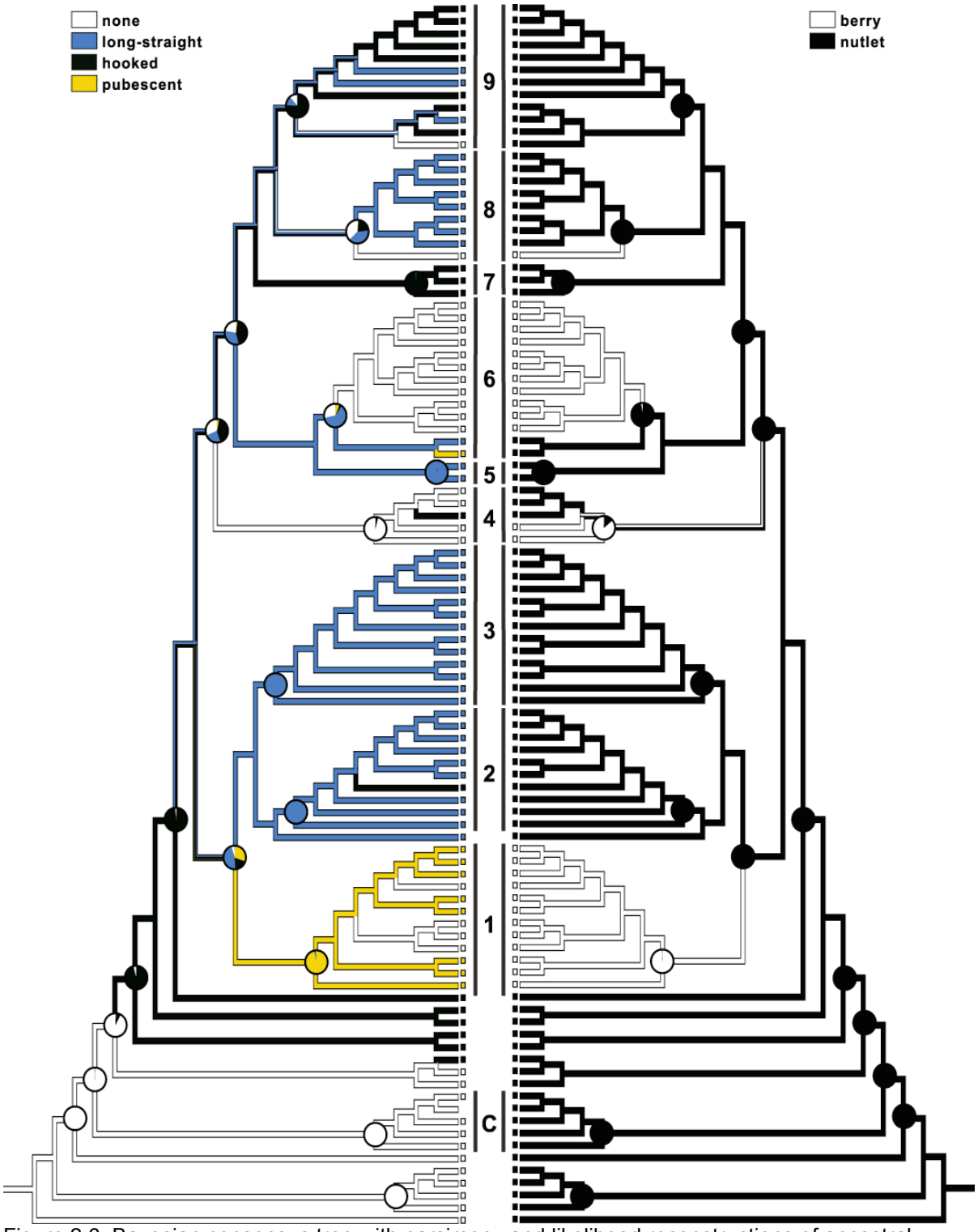

Figure 2.6. Bayesian consensus tree with parsimony and likelihood reconstructions of ancestral states of fruit hairs (left) and fruit types (right). Pie diagrams with relative likelihoods shown at strongly supported (i.e., Bayesian posterior probabilities ≥ 0.95) ancestral nodes along backbone of *Cruciata-Galium-Valantia* clade for nine major *Galium* clades, *Cruciata* clade, and outgroup.

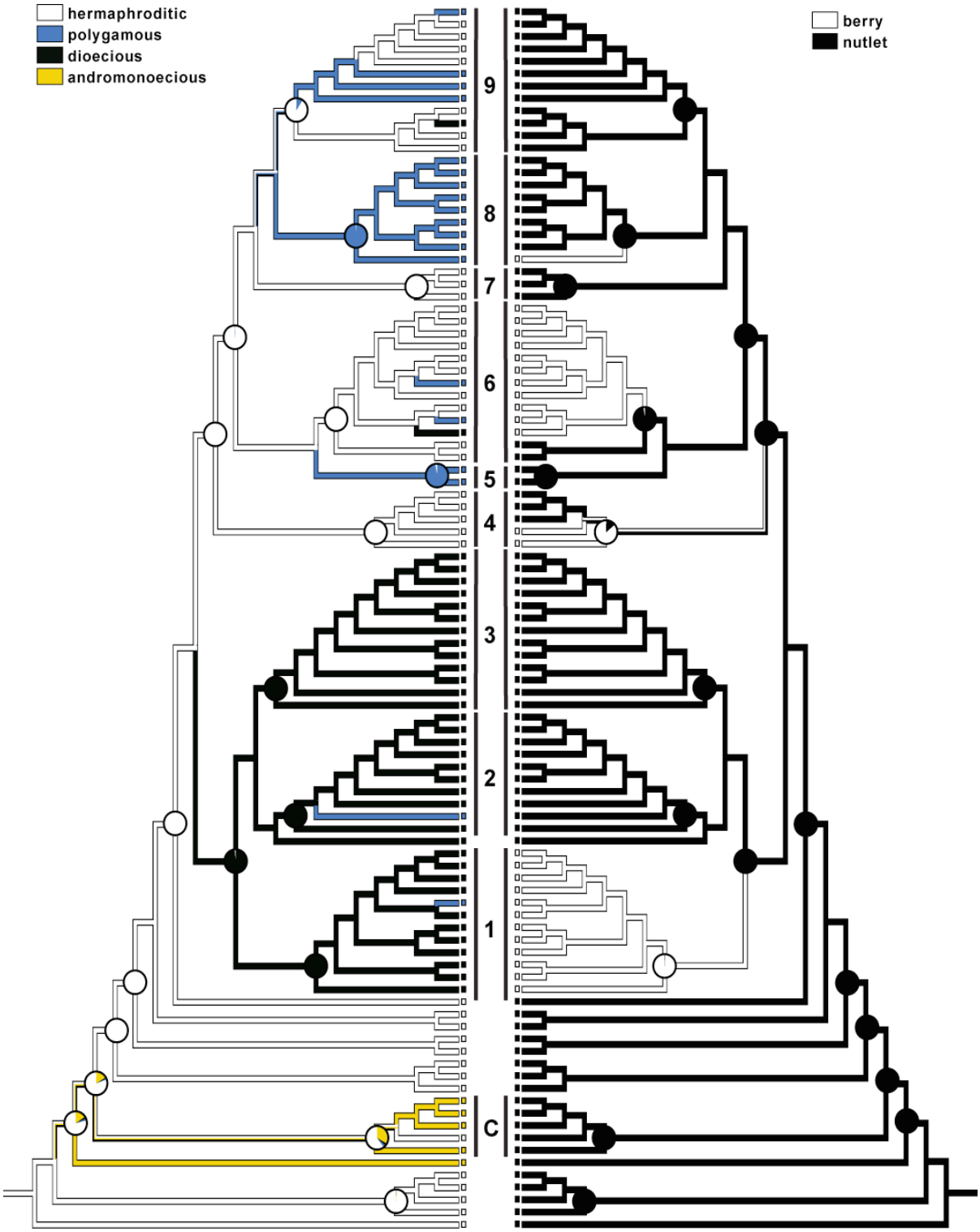

Figure 2.7. Bayesian consensus tree with parsimony and likelihood reconstructions of ancestral states of breeding systems (left) and fruit types (right). Pie diagrams with relative likelihoods shown at strongly supported (i.e., Bayesian posterior probabilities ≥ 0.95) ancestral nodes along backbone of *Cruciata-Galium-Valantia* clade for nine major *Galium* clades, *Cruciata* clade, and outgroup.

Table 2.1. Sections sampled among genera of the *Cruciata-Galium-Valantia* clade.

Genus	Section	No. Taxa Sampled
Cruciata Mill.		5
Galium L.	*"Baccogalium"*	10
	Bataprine Nwd.	2
	Lophogalium K. Schum.	39
	Platygalium W. Koch	16
	Relbunium Endl.	9
	Unassigned taxa	7
Valantia L.		1

Note: Classification follows Ehrendorfer et al. (2005) and F. Ehrendorfer (University of Vienna, personal communication).

Table 2.2. Internal primers designed for sequencing chloroplast and nuclear ribosomal regions.

Region	Primer	Sequence (5'--3')
rpoB-trnC	rpoBb	CGGATATTAATAKMTACATACG
	rpoBbR	CGTATGTAKMTATTAATATCCG
	rpoBd	GTTGGGGTTTACATATACT
	rpoBdR	AGTATATGTAAACCCCAAC
trnC-psbM	psbMa	GACATCRTGGTTGTCKAACGAG
	psbMb	GGTAAGAACCYRTTGATTGAAATAG
	psbMc	CGAATRCATAACCCTTTTCRA
trnL-trnF	d	(Taberlet, 1991)
	e	(Taberlet, 1991)
trnF-ndhJ	ndhJa	GATTTCTTYRTTTCKCTTA
	ndhJb	AATCTCTAATTGTAYTATCTT
	ndhJbR	AAGATARTACAATTAGAGATT
	trnFF	CTCGTGTCACCAGTTCAAATC
External transcribed spacer	18S-E	(Baldwin and Markos, 1998)

Table 2.3. Molecular results of chloroplast and nuclear ribosomal regions sequenced in this study.

Data summary	*rpoB-trnC*	*trnC-psbM*	*trnL-trnF-ndhJ*	ETS	Combined
Unaligned length (bp)	1053—1188	1248—1609	1602—1819	398--415	4301—5031
Aligned length (bp)	1484	2232	2266	438	6420
Excluded regions (bp)	16	0	0	0	16
No. base pairs analyzed	1468	2232	2266	438	6404
Uncorrected pairwise distances	0—0.075	0—0.112	0—0.044	0--0.297	0—0.065
No. gaps scored	23	34	43	4	104
No. accessions completed	88	92	95	88	84
No. accessions partially sequenced	7	3	0	0	11

Table 2.4. Five candidate trees of evolution of polygamy in the *Cruciata-Galium-Valantia* clade and *P* values from approximately unbiased test.

Tree	Rank	*P* value
1	2	0.000
2	5	0.000
3	6	0.000
4	4	0.000
5	3	0.000
6	1	1.000

Notes: See Appendix C for tree form. *P* values rounded off to 3 decimal places.

CHAPTER III

EXAMINATION OF NUCLEAR *RPB2* AND CHLOROPLAST DNA IN RECONSTRUCTING RELATIONSHIPS AMONG RARE TAXA IN *GALIUM* SECT. *BACCOGALIUM* (RUBIACEAE)

SUMMARY

Approximately 76 *Galium* taxa (Rubiaceae) occur in California, 30 of which are designated rare by the California Native Plant Society (CNPS). We examined *Galium* sect. *Baccogalium*, a clade primarily distributed in California that contains 10 CNPS-listed taxa - 4 species and 6 subspecies. We sought to use the *D* and *I* copies of nuclear *RPB2* in combination with chloroplast DNA (*rpoB-trnC, trnC-psbM, trnL-ndhJ*) to reconstruct evolutionary relationships among species to inform management of rare taxa. Bayesian analyses showed the two copies of *RPB2* in *Galium* do not correspond to the *D* and *I* copies, but rather to a duplication of the *D* copy (*D1*, *D2*). The *RPB2-D1* locus appears to have undergone a period of rapid sequence divergence, but since their divergence, both *RPB2-D1* and *RPB2-D2* exhibit typical selective constraints and appear to be functional with very low estimates of *dN*/*dS*. Within *Galium* sect. *Baccogalium,* relationships among taxa are not well resolved with either cp or *RPB2* data. However, according to cp data, subspecies from three species complexes do not form respective monophyletic groups, which may have implications for management of rare infraspecific taxa. This suggests that many subspecies may be distinct lineages from other conspecific

subspecies, may be hybridizing with other species, or that taxa within *Galium* sect. *Baccogalium* may be too closely related to resolve relationships.

INTRODUCTION

Thirty *Galium* taxa (Rubiaceae) are currently listed in the California Native Plant Society (CNPS) Inventory of Rare and Endangered Plants (CNPS 2010), out of ~76 taxa that occur in California (Dempster, 1993). The inventory categorizes sensitive taxa into five lists: (1A) plants presumed extinct in California, (1B) plants rare, threatened, or endangered in California and elsewhere, (2) plants rare, threatened, or endangered in California, but more common elsewhere, (3) plants about which we need more information – a review list, and (4) plants of limited distribution – a watch list (CNPS 2010). Of the 30 *Galium* taxa in the inventory, 18 belong to List 1B, two belong to List 2, one belongs to List 3, and nine belong to List 4 (CNPS 2010).

We have been trying to understand the evolutionary relationships within the polyphyletic genus *Galium* to understand the evolution and classification of the group and to aid management of rare taxa within the group. Former studies have identified three major clades of *Galium,* distributed throughout tribe Rubieae, that collectively do not form a monophyletic group (Manen et al., 1994; Natali et al., 1995, 1996; Soza and Olmstead, 2010a). These studies utilized the chloroplast (cp) *atpB-rbcL*, *rpoB-trnC*, *trnC-psbM*, and *trnL-ndhJ* intergenic regions to uncover relationships among these groups (Manen et al., 1994; Natali et al., 1995, 1996;

Soza and Olmstead, 2010a). Soza and Olmstead (2010b) examined one of the three major clades of *Galium,* primarily distributed in the New World, in more detail to uncover relationships within this group. From that study, we identified a small clade of *Galium* sect. *Baccogalium* that included several listed species in California and proposed to test the use of nuclear regions in species-level phylogenetic reconstruction in the group in order to assess their status as evolutionary units for conservation purposes.

Galium sect. *Baccogalium* is one of two sections within this New World clade of *Galium* that is strongly supported as a monophyletic group (Soza and Olmstead, 2010b). The section has never been typified or validly published, but has been characterized as dioecious and bearing fleshy fruits lacking specialized hairs, and is distributed from Oregon to Baja California (Dempster and Stebbins, 1965, 1968). The group contains approximately 13 perennial species, and up to 24 taxa including infraspecific taxa (Dempster, 1993). Members are mostly dioecious, with the exception of one polygamous species (*G. grande*). This group includes both diploid and polyploid taxa (Dempster and Stebbins, 1965, 1968). In addition, ten taxa from this group are listed in the CNPS Inventory as sensitive taxa: four species and six subspecies (Table 3.1).

Four different classes of RNA polymerases (I—IV) transcribe all the major RNAs in the nucleus (Denton et al., 1998; Oxelman and Bremer, 2000; Luo and Hall, 2007). Each enzyme is composed of two large and eight to ten smaller subunits (Luo

and Hall, 2007). Most subunits are encoded by a single-copy gene (Oxelman and Bremer, 2000).

In *Arabidopsis thaliana*, the second largest subunit of RNA polymerase II (*RPB2*) is a single-copy gene with 3,564 base pairs of coding sequence and 24 introns (Denton et al., 1998; Oxelman and Bremer, 2000). Denton et al. (1998) assessed the use of *RPB2* for phylogenetic studies across green plants. In all green plants examined, location of the 24 introns is conserved, but intron length and sequence is highly variable, suggesting applicability to resolving species-level relationships (Denton et al. 1998). As seen among species of *Litsea* (Lauraceae), *RPB2* sequences were more successful in resolving phylogenetic relationships than cp DNA and nuclear ribosomal internal transcribed spacer regions (Fijridiyanto and Murakami, 2009).

Two functional copies of *RPB2* have been found throughout asterids (Oxelman and Bremer, 2000; Oxelman et al., 2004; Luo et al., 2007). This duplication appears to have occurred early in core eudicots (Oxelman et al., 2004; Luo et al., 2007). However, one of the copies has been lost in several groups, including Rosids (Luo et al., 2007), as evidenced by *Arabidopsis thaliana*. The "*D*" copy of *RPB2* is the major functional *RPB2* gene in most tissues and the only copy expressed in vegetative tissues; whereas, the "*I*" copy is expressed in reproductive organs, primarily in pollen (Luo et al., 2007). Oxelman et al. (2004) demonstrated that the two copies were distinguishable from one another across eudicots and inferred separate phylogenies for the two copies.

The goal of the present study was to (1) use two nuclear loci, in addition to cp DNA data, to resolve species-level relationships within *Galium* sect. *Baccogalium*, and (2) apply knowledge of species relationships to inform management of rare plant taxa within the section. To achieve these goals, we increased sampling within *Galium* sect. *Baccogalium*, and sought to incorporate the two functional copies of *RPB2* with previously used cp regions for this group. Data were used from three cp regions (*rpoB-trnC, trnC-psbM, trnL-ndhJ*) and a region of *RPB2* that corresponds to exons 11—17, which is under less functional constraint and spans six introns that are variable for species-level relationships (Denton et al., 1998; Cramer 2001).

MATERIALS AND METHODS

Sampling methods—We sampled 25 accessions, representing 25 taxa, from *Galium* sect. *Baccogalium* and outgroups (Appendix D). These accessions represent all 13 species in *Galium* sect. *Baccogalium* and 79% of all infraspecific taxa. For outgroups, we sampled seven known diploid species from major clades of New World *Galium* (Soza and Olmstead, 2010b): *G. angustifolium* subsp. *angustifolium*, *G. aschenbornii*, *G. bailloni*, *G. coloradoense*, *G. correllii*, *G. hypocarpium*, and *G rotundifolium*. For *RPB2* analyses, we also included sequences of both *RPB2-D* and *RPB2-I* copies from eight asterids (Appendix D) and one outgroup sequence (*Platanus orientalis*).

Molecular methods—DNA samples were obtained from field-collected, silica gel-dried tissue, herbarium specimens, or other Rubiaceae researchers (Appendix

D). We extracted DNA using the 2% CTAB procedure (Doyle and Doyle, 1987) and purified as outlined in Soza and Olmstead (2010a, 2010b).

We amplified the cp *rpoB-trnC, trnC-psbM,* and *trnL-trnF-ndhJ* regions as outlined in Soza and Olmstead (2010a, 2010b). Exons 11—17 of *RPB2* were amplified using primers P6F2 (5'-TGGGGMATGATGTGTCCWGC-3') and P7R2 (5'-CCCATDGCTTGYTTDCCCAT-3'), modified from Denton et al. (1998) by adding degeneracies to incorporate both *RPB2* copies across asterids.

For *RPB2* amplification, polymerase chain reactions (PCR) were conducted in a MJ Research PTC-100 Peltier thermal cycler (Biorad, Hercules, California, USA) in 25 μL volumes: 2.5 μL 10x 30 mM $MgCl_2$ reaction buffer, 2.5 μL 10x Taq diluent, 2.5 μL dNTPs (10 mM), 1.25 μL each primer (5 μM), 0.125 μL Taq, 1 μL template, and 13.875 μL H_20. PCR conditions were an initial denaturation of 94°C for 4 min, followed by 35 cycles of 94°C denaturation for 15 s, 48°C annealing for 5 s with an increase of 1°C per 5 s to 65°C, 72°C extension for 2 min, and a final extension at 72°C for 10 min. PCR products were purified by a 20% polyethylene glycol precipitation (Sambrook et al., 1989) before cloning.

TOPO® cloning reactions and One Shot® (Invitrogen, Carlsbad, California, USA) chemical transformation were performed following the manufacturer's instructions in quarter reactions. Twenty to 64 colonies, depending on ploidy level, were picked from each PCR product, and screened and amplified by PCR with "M13*F" (5'-GTAAAACGACGGCCAGTGAAT-3') and "M13*R" (5'-CAGGAAACAGCTATGACCATG-3'; primers modified by K. Karol, New York

Botanical Garden) in 20 μL volumes: 2.0 μL 10x 30 mM $MgCl_2$ reaction buffer, 2.0 μL 10x Taq diluent, 2.0 μL dNTPs (10 mM), 1.2 μL each primer (5 μM), 0.1 μL Taq, and 11.5 μL H_20. PCR conditions were an initial denaturation of 94°C for 2 min, followed by 30 cycles of 94°C denaturation for 15 s, 55°C annealing for 15 s, 72°C extension for 2 min, and a final extension at 72°C for 10 min. Cloned PCR products were purified as outlined above, and 16—27 positive clones per accession were sequenced as described below.

Sequencing was performed with the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Buckinghamshire, UK). Both strands of each cp region were sequenced with the same amplification primers and several internal primers, as outlined in Soza and Olmstead (2010a, 2010b). For sequencing of cloned *RPB2* products, we used the T3 and T7 primers (Invitrogen, Carlsbad, California, USA), as well as internal primers listed in Table 3.2. Sequencing reactions were done in 5 μL volumes: 2 μL five-fold diluted dRhodamine sequencing reagent premix, 0.25 μL primer (5 μM), 1 μL template, and 1.75 μL H_20. Sequencing conditions were an initial denaturation of 94°C for 2 min, followed by 35 cycles of 92°C denaturation for 10 s, 55°C annealing for 5 s, and 60°C extension for 2.5 min. Sequencing products were purified with a sodium acetate/EDTA and ethanol precipitation and then analyzed on an ABI Prism 377 DNA Sequencer (Applied Biosystems, Carlsbad, California, USA).

Phylogenetic methods—Sequences were initially aligned in ClustalW (Chenna et al., 2003) and then manually adjusted in MacClade 4.08 (Maddison and

Maddison, 2000). Unambiguously aligned gaps that were phylogenetically informative for ingroup were coded as presence/absence characters for cp regions (Graham et al., 2000; Simmons and Ochoterena, 2000). Regions in which the alignment was ambiguous were excluded from the analyses. All *RPB2* sequences were aligned in one alignment; coding sequences were easily alignable, but introns from different *D* copies within *Galium* were aligned separately within the alignment (Steane et al., 1999).

All cp regions were combined and analyzed separately from the *RPB2* dataset with Bayesian analyses. The majority rule consensus tree based on the full *RPB2* dataset, including both introns and exons, was used to identify monophyletic groups of clonal sequences from the same accession.

For taxa with multiple clonal *RPB2* sequences, one sequence was selected from each strongly supported (i.e., ≥ 0.95 posterior probability) monophyletic group of sequences representing a given taxon. All clonal sequences, not forming monophyletic groups with other clones from the same accession, were included in subsequent analyses. A second analysis was performed just on the exon sequences of this *RPB2* subset with Bayesian analyses. Exon sequences were also translated into amino acid sequences and manually checked for any frameshift mutations or stop codons that would indicate a pseudogene. A third Bayesian analysis was performed on this *RPB2* subset, including both introns and exons.

We conducted Bayesian MCMC (Yang and Rannala, 1997) phylogenetic analyses to estimate the phylogeny of *Galium* sect. *Baccogalium*. For Bayesian

analyses, models of evolution for the combined cp dataset and *RPB2* datasets were determined separately by Modeltest 3.7 (Posada and Crandall, 1998). The models selected under the Akaike information criterion (AIC; Akaike, 1974) were K81uf + I + Γ (combined cp), TVM + I + Γ (*RPB2* introns + exons), and GTR+ I + Γ (*RPB2* exons only). In addition, the binary model was used for gap data, with ascertainment coding bias set to variable, in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Bayesian analyses were conducted with the use of the CIPRES Portal 2.0 (Miller et al., 2010), with data partitioned under the selected models, and default priors of no prior knowledge for the parameters of these models.

The prior probability density for the six nucleotide substitution rates and four stationary nucleotide frequencies was a flat Dirichlet, with all values set to 1.0. The prior for the proportion of invariable sites and gamma shape parameter for among-site rate variation was a uniform distribution, from 0 to 1 and 0 to 200 for α, respectively. The default prior probability for topology was uniform, with all possible trees equally probable a priori. The default prior probability distribution on branch lengths was unconstrained and exponential with a parameter of 10. Parameters for nucleotide frequencies (statefreq), substitution rates (revmat), and gamma shape (shape) were unlinked across both data partitions. All partitions were allowed to evolve under different rates, and site-specific rates were allowed to vary under a flat Dirichlet prior across partitions.

Bayesian analyses were conducted with three independent Markov Chain Monte Carlo analyses of 1 million generations for the combined cp dataset and 40 million generations for the *RPB2* datasets, with a sampling frequency of every 1000th generation. Metropolis coupling (Yang and Rannala, 1997) for each analysis was conducted with the default of four chains started from different random trees, three heated and one cold chain, temperature of 0.2, and one swap of states tried between chains every generation. Convergence was determined when the average standard deviation of split frequencies remained less than 0.01.

For the combined cp dataset, the first 43% of trees were discarded before convergence. For the *RPB2* datasets, the first 25% of trees were discarded. For each analysis, the remaining trees from each run were pooled to construct a 50% majority rule consensus tree to obtain posterior probabilities (pp).

Detection of recombination and selection methods—The *RPB2* subset alignment of 128 exon sequences from *Galium* only was analyzed by Hypermut 2.0 to examine nucleotide substitutions in a sequence population relative to the reference *RPB2-D* sequence of *Gardenia* sp., the closest available sequence from a related species of Rubiaceae (http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html; Rose and Korber, 2000).

The same alignment was screened for evidence of recombination using Single Breakpoint Recombination analysis (SBP; Kosakovsky Pond et al., 2006a) online at Datamonkey (http://www.datamonkey.org/; Kosakovsky Pond et al., 2006b;

Delport et al., 2010), a web-server of the HyPhy package (Kosakovsky Pond et al., 2005), to screen for PCR recombinants. The Datamonkey automatic model selection tool was used to select the best model under the AIC prior to SBP analysis (Kosakovsky Pond and Frost, 2005a). Due to computational time, SBP was conducted under the recommended default criterion of small sample AIC (AIC_c) with the selected model, no site-to-site rate variation, and two rate classes.

To obtain estimates of overall nonsynonymous (*dN*) to synonymous (*dS*) substitution rates and to detect sites in *RPB2* coding sequences under positive or negative selection, we used three different codon-based maximum likelihood methods implemented online through Datamonkey: single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), and random effects likelihood (REL; Kosakovsky Pond and Frost, 2005b, 2005c). We obtained estimations of average *dN*/*dS* ratios for each copy and *dN* and *dS* substitutions rates at every codon. Sequences from the two different copies of *RPB2* in *Galium* were analyzed separately in two different alignments with the *RPB2-D* sequence of *Gardenia* sp. For each alignment, the Datamonkey automatic model selection tool was used to select the best model under the AIC prior to analyses (Kosakovsky Pond and Frost, 2005a). A user tree was imported from the second Bayesian analyses above on *RPB2* exon sequences for all analyses. For SLAC analyses, the global *dN*/*dS* values were estimated from the data, ambiguous characters were averaged, and the significance level was $P = 0.1$. For FEL analyses, the significance level was $P = 0.1$. For REL analyses, the significance level for the Bayes factor was 50. All three

analyses were compared using the Integrative Selection Analysis tool in Datamonkey.

To determine if any branches at the base of a duplicated copy's clade may be undergoing positive selection, we conducted codeml analyses in PAML 4.4 (Yang, 2007) with the entire *RPB2* subset alignment of 128 exon sequences from *Galium,* including the *RPB2-D* sequences of *Antirrhinum majus* and *Gardenia* sp., to obtain estimates of *dN*/*dS* for a particular branch of the tree. We used an unrooted phylogeny with branch lengths, estimated by GARLI 1.0 (Zwickl, 2006) under the default settings, to test nested models in codeml under the branch-site test of positive selection with model A (Yang, 2007).

RESULTS

Sampling and molecular results—Summarized in Table 3.3 are the unaligned and aligned lengths, excluded regions, total base pairs analyzed, uncorrected pairwise distances, gaps scored, and number of completely or partially sequenced accessions for each cp and *RPB2* region. Two different sized copies of *RPB2* were amplified using our PCR protocols (*RPB2-D1, RPB2-D2*). The cp DNA and both *RPB2-D1* and *RPB2-D2* sequences exhibit low sequence divergence among *Galium* samples, as evidenced by uncorrected pairwise distances that ranged from 0--0.017, 0—0.048, and 0--0.033, respectively (Table 3.3).

We found no evidence of the *RPB2-I* copy in PCR products from *Galium* taxa examined in this study. Two copies of *RPB2-D* were found in all except four of the

taxa sampled. We were only able to amplify one copy of *RPB2-D* for *G. bailloni, G. correllii, G. hardhamae,* and *G. rotundifolium*. We did not amplify any copies of *RPB2* for five taxa (*G. californicum* subsp. *miguelense, G. clementis, G. muricatum, G. nuttallii* subsp. *insulare, G. sparsiflorum*). Lack of amplification of copies in these nine taxa may be primarily due to low quality DNA from herbarium specimens. Clonal sequences per accession per copy ranged from 0—9 (Figs. 3.2—3.3).

Phylogenetic results—According to the majority rule consensus tree from combined cp DNA, *Galium* sect. *Baccogalium* is strongly supported as a monophyletic group (pp = 1.00; Fig. 3.1), with the exclusion of *G. muricatum.* Within *Galium* sect. *Baccogalium,* relationships among taxa are not well resolved. However, at the base of the clade, *G. bolanderi* and *G. hardhamae* are unresolved with respect to the remaining members, which form a clade (pp = 1.00; Fig. 3.1). Above this node, *G. ambiguum* subsp. *siskiyouense* is sister to the remaining members in *G.* sect. *Baccogalium* (pp = 1.00; Fig.3.1).

Three species complexes do not form monophyletic groups (Fig. 3.1): (1) *Galium californicum* subsp. *flaccidum* is strongly supported as more closely related to *G. cliftonsmithii* (pp = 1.00) than to other subspecies of *G. californicum. Galium californicum* subsp. *primum* is strongly supported as more closely related to *G. porrigens* (pp = 1.00) than to other subspecies of *G. californicum. Galium californicum* subsp. *miguelense* is more closely related to *G. sparsiflorum* (pp = 0.92) than to other subspecies of *G. californicum.* (2) *Galium nuttallii* subsp. *nuttallii* is strongly supported as more closely related to *G. andrewsii, G. californicum* subsp.

primum, and *G. porrigens* (pp = 1.00) than to other subspecies of *G. nuttallii. Galium nuttallii* subsp. *insulare* is strongly supported as more closely related to *G. grande* and *G. martirense* (pp = 0.97) than to other subspecies of *G. nuttallii* (Fig. 3.1). (3) *Galium andrewsii* is strongly supported as more closely related to *G. californicum* subsp*. primum, G. nuttallii* subsp*. nuttallii,* and *G. porrigens* (pp = 1.00) than to other subspecies of *G. andrewsii* (Fig. 3.1).

According to the majority rule consensus trees from exon only sequences and intron + exon sequences of *RPB2*, the two copies of *RPB2* amplified in *Galium* taxa represent a duplication within the *D* copy (*D1* and *D2*), rather than the *D* and *I* copies (Figs. 3.2—3.3). The two copies within *Galium* are strongly supported as a monophyletic group (pp = 1.00) and as sister to the *Gardenia* sp. *RPB2-D* sequence (pp=1.00; Figs. 3.2—3.3). The *RPB2-D1* sequences form a strongly supported clade (pp = 1.00; Figs. 3.2—3.3); but the relationships among the *RPB2-D2* sequences are unresolved (Fig. 3.2), or do not form a clade (Fig. 3.3). In addition, the *RPB2-D1* copy has accumulated many more substitutions per site from its divergence from the common ancestor with *RPB2-D2* than the *RPB2-D2* copy, as evidenced by the long branch subtending the *RPB2-D1* clade (Figs. 3.2—3.3).

Relationships among *Galium* sequences inferred from each *RPB2* copy are not well resolved nor strongly supported; and clonal sequences from individual taxa do not form monophyletic groups (Figs. 3.2—3.3). *Galium* sect. *Baccogalium* is strongly supported as a monophyletic group by *RPB2-D1* exon sequences with the inclusion of *G. coloradoense* and the exclusion of a clone of *G. andrewsii* (*D1b*)

nested within *G. angustifolium* subsp. *angustifolium* (Fig. 3.2). However, including both introns and exons in analyses, *Galium* sect. *Baccogalium* is only weakly supported as a monophyletic group by the *RPB2-D1* copy (Fig. 3.3).

Detection of recombination and selection results—Based on Hypermut results, the number of sites in which a *Galium RPB2-D1* sequence differs from the *Gardenia RPB2-D* sequence ranges from 145 to 183 of 708 sites (20.5—25.8%). The number of sites in which a *Galium RPB2-D2* sequence differs from the *Gardenia RPB2-D* sequence ranges from 109 to 127 of 708 sites (15.4—17.9%).

Based on analyses with Datamonkey, five sequences were identified with internal stop codons and two duplicate sequences were found (Table 3.4). Under the model selected for *RPB2-D* (010234), no evidence of recombination was detected in the alignment of *Galium* exon sequences using SBP analysis.

Under the model selected for *RPB2-D1* (001123), SLAC and REL analyses estimate the average *dN*/*dS* as 0.10 and 0.22, respectively. All three analyses (SLAC, FEL, REL) do not identify any codons under positive or diversifying selection (Table 3.5). Under SLAC and FEL analyses, 21 and 60 (of 236) codons are under negative or purifying selection, respectively (Table 3.5). Of these results, 21 codons were identified by both SLAC and FEL analyses as under negative or purifying selection (Table 3.5). No specific, negatively selected sites were identified with REL analyses.

Under the model selected for *RPB2-D2* (000121), SLAC and REL analyses estimate the average *dN*/*dS* as 0.08 and 0.12, respectively. All three analyses

(SLAC, FEL, REL) do not identify any codons under positive or diversifying selection (Table 3.6). Under SLAC and FEL analyses, 12 and 57 (of 236) codons are under negative or purifying selection, respectively (Table 3.6). Of these results, 12 codons were identified by both SLAC and FEL analyses as under negative or purifying selection (Table 3.6). No specific, negatively selected sites were identified with REL analyses.

Based on codeml analyses in PAML, the branch at the base of the *RPB2-D1* clade may have experienced positive selection at some point along the branch. The long branch that subtends the *RPB2-D1* clade (Fig. 3.2) has an estimated *dN*/*dS* of 4.06 ($p = 0.076$). This corresponds to a relatively high *dN*/*dS* in comparison to the within copy *dN*/*dS* ratios above.

DISCUSSION

RPB2 copies among eudicots—Absence of the *RPB2-I* copy is not unusual in core eudicots. Oxelman et al. (2004) observed that across eudicots, the *I* copy has been lost several times; and in groups where the *I* copy is not found, the function of this supplementary copy is presumed to be lost. Luo et al. (2007) found evidence that the *I* copy has been lost at least eight times, and the *D* copy has been lost at least four times, in the evolution of angiosperms. Rosids and Caryophyllales lack the *I* copy (Luo et al., 2007), as well as several genera in the asterids (*Borago, Eucommia, Garrya*; Oxelman et al., 2004).

In addition, duplication of *RPB2-D* is not unusual among core eudicots either, with several instances of *RPB2-D* duplications occurring among terminal lineages (Oxelman et al., 2004; Luo et al., 2007). Several cases of duplication of the *D* copy in the absence of an *I* copy are known from the literature (*Borago; Hibiscus* s.l.; *Armeria, Pereskia, Rivina*; Oxelman et al., 2004; Pfeil et al., 2004; Luo et al., 2007). The correlation between the lack of an *I* copy and the presence of a *D* duplicate may suggest that a *D* duplicate is being co-opted for the *I* function.

Phylogeny—*Galium* sect. *Baccogalium* is strongly supported as a monophyletic group based on cp DNA with the exclusion of *G. muricatum* from northern California coastal ranges (Fig. 3.1); however, evidence from *RPB2* data is unclear (Figs. 3.2—3.3). Apart from three small terminal clades and two nodes near the base of the tree, relationships within *G.* sect. *Baccogalium* are not well resolved with cp DNA (Fig. 3.1) and the *RPB2* data afford virtually no resolution among species within *G.* sect. *Baccogalium* (Figs. 3.2—3.3). Dempster and Stebbins (1968) noted that this section is unusual relative to other groups of *Galium,* because it has not been divided into isolated geographic races, and is continuously distributed over its entire range from southern Oregon to Baja California. The virtually complete lack of differentiation among taxa and extremely low sequence divergence in *G.* sect. *Baccogalium*, suggests a recent diversification, and either substantial gene flow, or incomplete lineage sorting among taxa. Despite the slower substitution rate of cp DNA, the four-fold faster coalescence of organellar genome evolution (Moore, 1995)

and restriction to a maternal gene tree, may help explain the greater resolution possible with cp DNA in this group.

Data from cp regions in this study do provide some insights into the maternal gene tree of sect. *Baccogalium.* In particular, species complexes comprising multiple subspecies do not represent maternal lineages (i.e., *G. andrewsii, G. californicum, G. nuttallii*; Fig. 3.1). This cp DNA pattern may represent an accurate picture of the phylogenetic relationships within the clade; or, terminal clades that include one subspecies from a species complex among other species may represent chloroplast transfer following hybridization within sect. *Baccogalium* (i.e., *G. californicum* subsp. *primum* + *G. porrigens, G. californicum* subsp. *flaccidum* + *G. cliftonsmithii*; Fig. 3.1).

Based on cp DNA, *G. californicum* subsp*. flaccidum* is most closely related to *G. cliftonsmithii* (Fig. 3.1), a relationship suggested by Dempster and Stebbins (1965); these two taxa also occur in sympatry (Dempster and Stebbins, 1968).

Galium californicum subsp*. primum* is a CNPS List 1B plant, but concern has been raised whether this subspecies is actually different from its morphologically similar, sympatric species, *G. porrigens.* Hybrid swarms between the two taxa occur in the San Jacinto Mountains in California; and *G. californicum* subsp*. primum* appears to have been completely submerged by introgression (Dempster and Stebbins, 1968). According to cp DNA, these two taxa are closely related (Fig. 3.1).

Galium porrigens is the most widespread species within the section and is also recorded as hybridizing with *G. andrewsii* (Dempster and Stebbins, 1968). This may explain why *G. andrewsii* forms a strongly supported clade with *G. porrigens, G.*

californicum subsp. *primum,* and *G. nuttallii* (Fig. 3.1). All four taxa occur in Riverside County, California (Dempster and Stebbins, 1968).

Based on the cp DNA results, *G. nuttallii* subsp. *insulare* forms a strongly supported clade with *G. grande* and *G. martirense* (Fig. 3.1). *Galium grande*, a CNPS List 1B plant, and *G. martirense* are morphologically similar, despite their geographic disjunction. *Galium grande* has a very restricted distribution in the San Gabriel Mountains and *G. martirense* occurs in Baja California (Dempster and Stebbins, 1965, 1968). *Galium martirense*, on the other hand, does grow sympatrically with *G. nuttallii* in some areas, but does not appear morphologically similar (Dempster and Stebbins, 1965).

According to Dempster and Stebbins (1965), most of the polyploids in the group are likely allopolyploids resulting from hybridization followed by genome doubling; and evidence exists for introgression within the group based on chromosome numbers, morphological and anatomical characters, and geographic and ecological distributions (Dempster and Stebbins, 1968). Many of the subspecies in the group were named at this rank because they are polyploids that resemble a diploid species, but have a distinct geographic distribution (Dempster and Stebbins, 1968). The high number of clonal sequences per accession per *RPB2* copy is probably indicative of polyploidization within the group, with some taxa having as many as 18 and 20 sets of chromosomes (i.e. *G. cliftonsmithii* and *G. grande*, respectively, x = 11; Dempster and Stebbins, 1965, 1968). Approximately half of the taxa sampled in this study are diploid; the other half are polyploid.

Relationships among taxa based on cp DNA suggest that many subspecies may be distinct lineages from other conspecific subspecies, may be hybridizing with other species, or that taxa within *Galium* sect. *Baccogalium* may be too closely related to resolve relationships. However, due to the presumed allopolyploid history of many taxa in this group, hybridization is more likely to account for the relationships observed with the maternal phylogeny.

Detection of recombination and selection— Because we identified two copies of *RPB2-D* in *Galium* taxa during this study, we wanted to determine whether both copies of *RPB2-D* were under functional constraints, or whether one copy may be evolving adaptively and undergoing subfunctionalization or neofunctionalization. We examined each copy for evidence of negative selection (indicating functional constraints) or positive selection (indicating subfunctionalization or neofunctionalization). Estimates of overall nonsynonymous (*dN*) to synonymous (*dS*) substitution rates provide evidence of positive selection when $dN/dS > 1$, negative selection when $dN/dS < 1$, and neutral selection when $dN/dS = 1$. We also employed methods implemented in Datamonkey to study site-by-site selection to determine if any sites in an alignment may be undergoing selection despite the overall *dN*/*dS* ratio (Kosakovsky Pond and Frost, 2005c).

Five clonal sequences contain internal stop codons (Table 3.4). Three of these sequences (*Galium andrewsii* subsp. *intermedium D1b, Galium grande D1f, Galium porrigens D2a*) are nested within clusters of other clonal sequences from the same accession and the remaining two sequences are either not closely related to

other clonal sequences from the same accession (*Galium nuttallii* subsp. *nuttallii D1d*), or represent the only clonal sequence amplified for an accession (*G. hardhamae D1*). These may represent either experimental error (e.g., PCR misincorporation) or pseudogenization in a redundant gene family (especially in polyploid taxa).

No evidence of recombination was detected in the alignment of *Galium* exon sequences using SBP analysis. This suggests that PCR recombinants do not contribute to the number of clonal sequences observed per accession per copy.

RPB2-D1 and *RPB2-D2* copies have an average *dN*/*dS* of 0.10--0.22 and 0.08—0.12, respectively, suggesting strong purifying selection. Both copies appear to be under functional constraints, without evidence of sub- or neo-functionalization. For *RPB2-D1,* 21 codons were identified by both SLAC and FEL analyses as under negative or purifying selection (Table 3.5). For *RPB2-D2,* 12 codons were identified by both SLAC and FEL analyses as under negative or purifying selection (Table 3.6). Both copies share six codons (2, 6, 198, 232, 233, and 235) under negative selection from both SLAC and FEL analyses.

The *RPB2-D1* copy within *Galium* has diverged more from the common ancestor with *Gardenia* than the *RPB2-D2* copy*,* as observed by Bayesian, Hypermut, and SLAC results (Figs. 3.2—3.3). The majority of these substitutions has been nonsynonymous along this branch (*dN*/*dS* = 4.06). This indicates that positive selection occurred at some point along this branch, followed by a return to normal

selective constraints as evidenced by the overall low *dN*/*dS* (0.10—0.22) for this copy.

Exons 11-17 correspond to regions External 1 and External 2 of *RPB2*, consisting of α helices, β strands, and loops located on the outer surface of RNA Polymerase II (Cramer et al., 2001). This region of *RPB2* does not contain any interaction sites for other RNA polymerase II subunits (Cramer et al., 2001), and thus may be less conserved. Examining the entire *RPB2* region will provide further insights into the duplication of the *D* copy within *Galium.*

Conclusions—*RPB2-D* has undergone a duplication within Rubiaceae in the lineage leading to *Galium*, since the divergence of that lineage from *Gardenia*, which has a single copy of this locus. The *D1* locus seems to have undergone a period of rapid sequence divergence, but since their divergence, both copies have undergone negative selection and appear to be under functional constraints. Further research that samples genera outside of *Galium* will determine where the *RPB2* duplication occurred, after the divergence of *Gardenia*. *RPB2* data perform poorly in resolving species-level relationships within *Galium*; however, cp data provide some insight into the maternal gene tree of *Galium* sect. *Baccogalium*. Other data besides cp DNA are required to determine the evolutionary relationships among *Galium* species and subspecies to inform management of rare taxa within this genus. Chloroplast data give us only one side of the story. Relationships within *Galium* may be further confounded by hybridization.

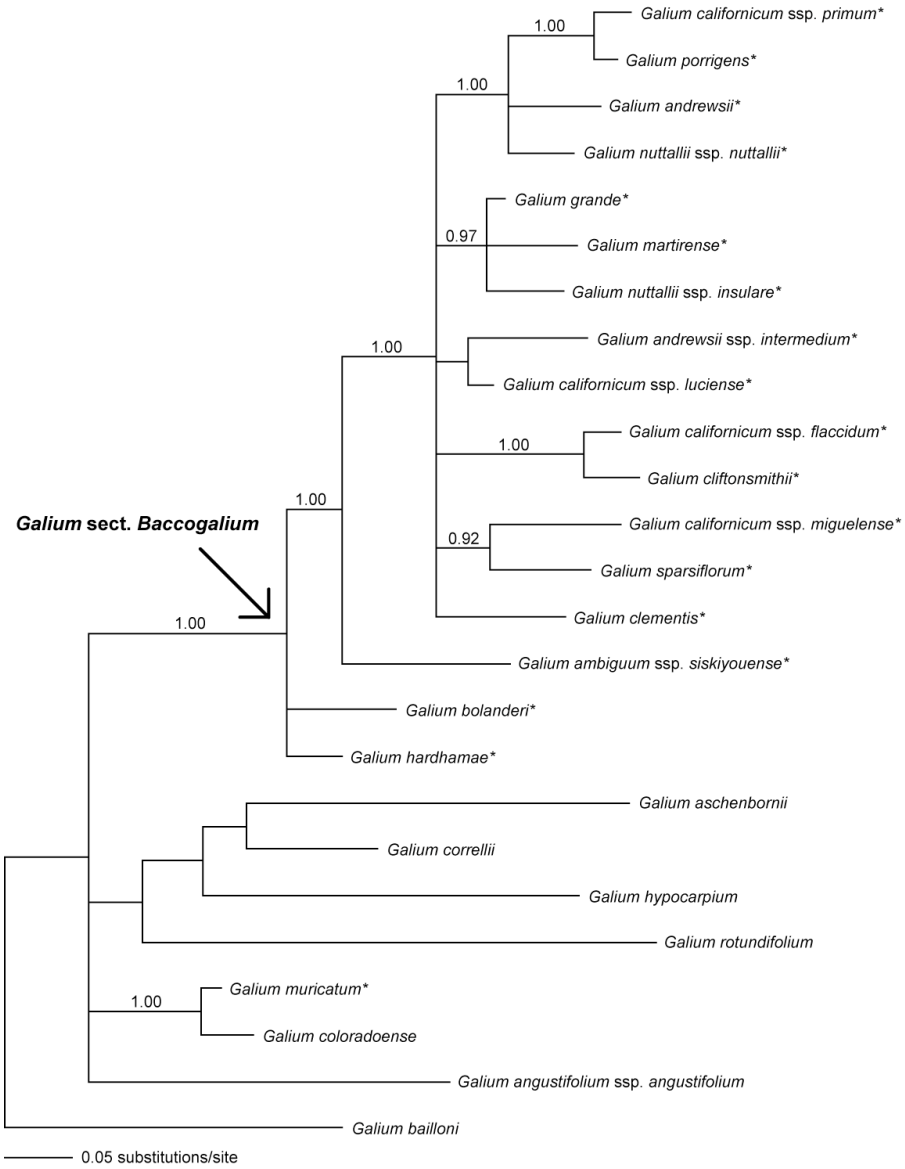

Figure 3.1. Phylogeny of *Galium* sect. *Baccogalium* and outgroups, estimated from the Bayesian 50% majority rule consensus tree based on the combined dataset of three cp regions (*rpoB-trnC, trnC-psbM, trnL-trnF-ndhJ*). Posterior probabilities (pp) ≥ 0.90 displayed above branches. Asterisks denote members of *G.* sect. *Baccogalium.*

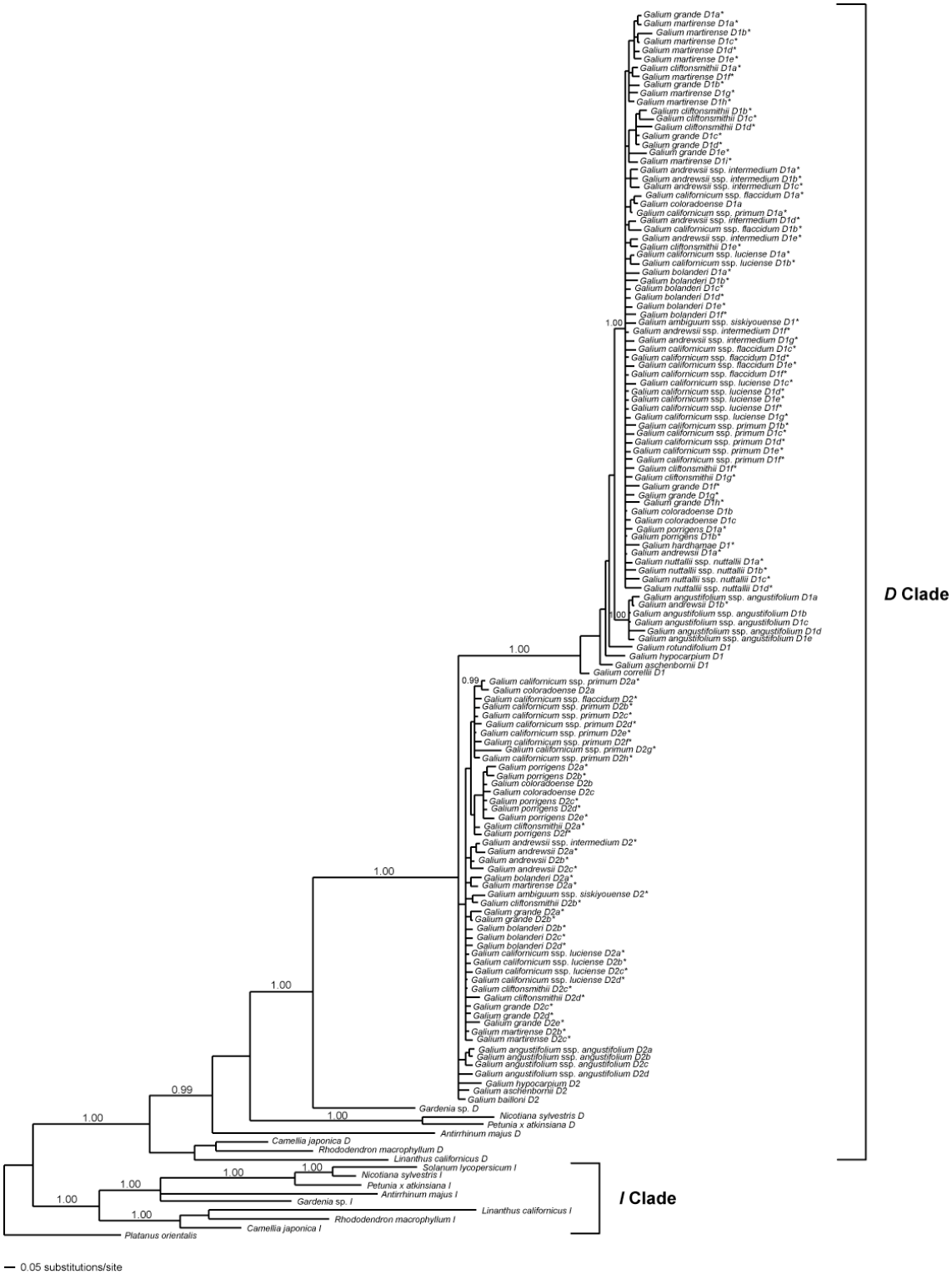

Figure 3.2. Phylogeny of *Galium* sect. *Baccogalium* and outgroups, estimated from the Bayesian 50% majority rule consensus tree based on nuclear *RPB2* exon sequences. *D* and *I* clades represent the two well-documented *RPB2* copies in eudicots. Posterior probabilities (pp) ≥ 0.95 displayed above branches. Asterisks denote members of *G*. sect. *Baccogalium.*

Figure 3.3. Phylogeny of *Galium* sect. *Baccogalium* and outgroups, estimated from the Bayesian 50% majority rule consensus tree based on nuclear *RPB2* sequences, including both exons and introns. Only the *RPB2-D* clade is represented here, and all outgroups outside of Rubiaceae have been removed from the tree. Posterior probabilities (pp) ≥ 0.95 displayed above branches. Asterisks denote members of *G.* sect. *Baccogalium.*

Table 3.1. *Galium* sect. *Baccogalium* taxa listed in the California Native Plant Society Inventory of Rare and Endangered Plants, 8th edition.

Taxon	CNPS List	State List	Federal List
Galium andrewsii subsp. *gatense*	4.2	None	None
Galium californicum subsp. *luciense*	1B.3	None	None
Galium californicum subsp. *miguelense*	4.2	None	None
Galium californicum subsp. *primum*	1B.2	None	None
Galium californicum subsp. *sierrae*	1B.2	Rare	Endangered
Galium clementis	1B.3	None	None
Galium cliftonsmithii	4.3	None	None
Galium grande	1B.2	None	None
Galium hardhamae	1B.3	None	None
Galium nuttallii subsp. *insulare*	4.3	None	None

Table 3.2. Internal primers designed for sequencing nuclear *RPB2* loci.

Region	Primer	Sequence (5'--3')
RPB2-D1	450R	CCAGCCACCTTCTTCCTCCTGT
	599F	ACMGATTATGGGCGRTGCAGYCG
	802R	GATCAMGGTGTATTCCTACCCARC
RPB2-D2	408F	TGCGGCGTTTGWKAAGAAGGGT
	597R	GAGTAAGGAWCTGAATGTAAAGC
	289F	GGATTCATAGAATACATTGACACTG
	493R	ATYTCACAGTGCGTATAAGTATCYG

Table 3.3. Molecular results of chloroplast and nuclear regions sequenced from members of *Galium* in this study.

	rpoB-trnC	***trnC-psbM***	***trnL-ndhJ***	***RPB2-D1***	***RPB2-D2***
Unaligned length (bp)	1096—1124	1362—1591	1704—1786	1151--1332	1435--1501
Aligned length (bp)	1164	1741	1927	1562	1708
Excluded regions (bp)	0	0	0	0	0
# base pairs analyzed	1164	1741	1927	1562	1708
Uncorrected pairwise distances	0—0.017	0—0.012	0—0.013	0--0.048	0.001--0.033
# gaps scored	1	1	4	NA	NA
# accessions completed	24	25	25	20	16
# accessions partially sequenced	1	0	0	0	0

Table 3.4. *RPB2* sequences removed from the alignment prior to SBP, SLAC, FEL, and REL analyses.

Sequence name	Reason removed
Galium andrewsii subsp. *intermedium D1b*	Stop codon
Galium californicum subsp. *luciense D2a*	Duplicate sequence
Galium grande D1d	Duplicate sequence
Galium grande D1f	Stop codon
Galium hardhamae D1	Stop codon
Galium nuttallii subsp. *nuttallii D1d*	Stop codon
Galium porrigens D2a	Stop codon

Table 3.5. Selected sites from *RPB2-D1* sequences identified by at least the single likelihood ancestor counting (SLAC) or fixed effects likelihood (FEL) method implemented at Datamonkey web-server. Codon sites in bold were selected by both analyses.

	SLAC		FEL	
Codon	**Normalized *dN-dS***	***p*-value**	**Normalized *dN-dS***	***p*-value**
2	**-4.561**	**0.000**	**-14.923**	**0.000**
6	**-5.399**	**0.000**	**-27.076**	**0.000**
10			-0.598	0.100
14			-1.130	0.023
15			-1.332	0.042
16	**-3.906**	**0.000**	**-10.929**	**0.000**
19			-0.861	0.066
20			-0.724	0.071
28			-0.598	0.094
30	**-0.689**	**0.037**	**-1.479**	**0.011**
32			-0.598	0.099
36			-0.794	0.073
40			-0.761	0.054
52			-0.598	0.099
58			-0.786	0.085
59			-0.767	0.040
60	**-5.514**	**0.000**	**-23.574**	**0.000**
62	**-0.876**	**0.018**	**-1.468**	**0.006**
65			-1.332	0.041
67	**-8.042**	**0.000**	**-36.163**	**0.000**
71			-1.031	0.049
82			-0.650	0.084
83			-0.716	0.075
90			-1.302	0.044
96	**-0.689**	**0.037**	**-1.175**	**0.012**
105			-0.755	0.074
108	**-1.540**	**0.002**	**-2.964**	**0.000**
112			-1.304	0.044
115			-0.806	0.045
117	**-1.535**	**0.005**	**-3.233**	**0.002**
119			-0.598	0.100
120			-0.887	0.048
122	**-3.357**	**0.000**	**-8.871**	**0.000**
124			-0.803	0.073
138			-0.636	0.087
162	**-0.815**	**0.035**	**-1.227**	**0.016**
166	**-0.780**	**0.045**	**-1.732**	**0.011**

Table 3.5 continued

170			-0.986	0.025
173			-0.785	0.073
177	**-0.921**	**0.038**	**-2.748**	**0.012**
179	**-1.222**	**0.007**	**-5.040**	**0.000**
182			-1.148	0.077
184			-0.573	0.093
190	**-0.931**	**0.012**	**-2.008**	**0.002**
192			-0.986	0.036
198			-0.599	0.092
203			-0.914	0.032
205			-0.979	0.092
206			-1.031	0.049
208			-0.986	0.029
214			-1.031	0.049
218			-1.040	0.053
220	**-0.815**	**0.035**	**-2.140**	**0.006**
225			-1.040	0.051
227			-1.313	0.053
229			-0.984	0.097
230	**-0.689**	**0.037**	**-1.622**	**0.006**
232	**-4.939**	**0.000**	**-14.030**	**0.000**
233	**-7.903**	**0.000**	**-32.613**	**0.000**
235	**-7.467**	**0.000**	**-40.965**	**0.000**

Table 3.6. Selected sites from *RPB2-D2* sequences identified by at least the single likelihood ancestor counting (SLAC) or fixed effects likelihood (FEL) method implemented at Datamonkey web-server. Codon sites in bold were selected by both analyses.

	SLAC		FEL	
Codon	**Normalized *dN-dS***	***p*-value**	**Normalized *dN-dS***	***p*-value**
2	**-54.799**	**0.000**	**-42.066**	**0.000**
6	**-46.702**	**0.000**	**-53.789**	**0.000**
10			-2.025	0.089
14			-2.985	0.029
16			-2.893	0.033
17			-2.272	0.063
28			-4.107	0.015
30			-2.289	0.039
32			-2.025	0.087
40			-2.056	0.077
52			-2.025	0.090
54			-4.238	0.017
56			-2.801	0.064
67			-5.797	0.031
80			-1.868	0.082
84			-1.894	0.098
87			-1.770	0.086
91			-2.370	0.044
92			-1.573	0.099
93			-1.878	0.093
98			-2.025	0.094
103			-4.202	0.017
108	**-6.012**	**0.090**	**-3.348**	**0.023**
109			-3.183	0.048
119			-2.025	0.089
124			-2.336	0.075
129			-3.879	0.023
132			-2.580	0.068
134			-3.455	0.049
135	**-9.429**	**0.019**	**-11.515**	**0.001**
148			-3.440	0.021
159			-2.640	0.069
162			-1.696	0.095
165	**-8.556**	**0.044**	**-6.396**	**0.008**
170			-3.243	0.022
179			-5.797	0.032
182			-2.781	0.029

Table 3.6 continued				
183			-2.797	0.030
184			-1.787	0.089
192	**-17.545**	**0.001**	**-13.145**	**0.000**
194	**-7.519**	**0.037**	**-6.678**	**0.003**
198			-1.696	0.097
200	**-8.604**	**0.044**	**-5.064**	**0.011**
203			-2.750	0.032
205			-3.018	0.088
206	**-6.297**	**0.070**	**-6.111**	**0.008**
211			-1.892	0.096
214			-3.183	0.048
217			-3.277	0.024
218			-3.455	0.049
220			-3.455	0.048
225			-3.455	0.048
229			-1.867	0.097
230			-3.419	0.022
232	**-48.860**	**0.000**	**-44.445**	**0.000**
233	**-49.765**	**0.000**	**-71.412**	**0.000**
235	**-42.610**	**0.000**	**-51.077**	**0.000**

REFERENCES

Abdel Khalik, K., M. Abd El-Ghani, and A. El Kordy. 2008. Fruit and seed morphology in *Galium* L. (Rubiaceae) and its importance for taxonomic identification. *Acta Botanica Croatica* 67: 1—20.

Aikake, H, 1974. A new look at the statistical model identification. *IEEE Transactions on Automatic Control* 19: 716—723.

Ainsworth, C. 2000. Boys and girls come out to play: The molecular biology of dioecious plants. *Annals of Botany* 86**:** 211--221.

Ainsworth, C., J. Parker, and V. Buchanan-Wollaston. 1998. Sex determination in plants. *Current Topics in Developmental Biology* 38: 167--223.

Andersson, L., and J. H. E. Rova. 1999. The rps16 intron and the phylogeny of the Rubioideae (Rubiaceae). *Plant Systematics and Evolution* 214: 161—186.

Backlund, M. 2005. *Phylogenetic studies in the Gentianales: Approaches at different taxonomic levels*. Dissertation, Uppsala University, Uppsala.

Backlund, M., B. Bremer, and M. Thullin. 2007. Paraphyly of Paederieae, recognition of Putorieae and expansion of *Plocama* (Rubiaceae-Rubioideae). *Taxon* 56: 315—328.

Baillon, H. E. 1880. *Historie des plantes*, vol. 7. Hachette, Paris.

Baldwin, B. G., and S. Markos. 1998. Phylogenetic utility of the external transcribed spacer (ETS) of 18S-26S rDNA: Congruence of ETS and ITS trees of *Calycadenia* (Compositae). *Molecular Phylogenetics and Evolution* 10: 449—463.

Barrett, S. C. H. 2002. The evolution of plant sexual diversity. *Nature Reviews Genetics* 3: 274--294.

Batra, S. W. T. 1984. Phytophages and pollinators of *Galium* (Rubiaceae) in Eurasia and North America. *Environmental Entomology* 13: 1113--1124.

Bawa, K. S. 1980. Evolution of dioecy in flowering plants. *Annual Review of Ecology and Systematics* 11: 15--39.

Bawa, K. S., and P. A. Opler. 1975. Dioecism in tropical forest trees. *Evolution; International Journal of Organic Evolution* 29: 167—179.

Behling, H. 1997. Late Quaternary vegetation, climate and fire history from the tropical mountain region of Morro de Itapeva, SE Brazil. *Palaeogeography, Palaeoclimatology, Palaeoecology* 129: 407—422.

Bisby, F. A, Y. R. Roskov, T. M. Orrell, D. Nicolson, L. E. Paglinawan, N. Bailly, P. M. Kirk, et al. (eds.). 2009. Species 2000 & ITIS Catalogue of Life: 2009 Annual Checklist. Website http://www.catalogueoflife.org/annual-checklist/2009/ [accessed 30 October 2009].

Brandão de Freitas, L., S. Cavalli-Molina, and H. Winge. 1995. Sources of genetic variability in a neotropical cleistogamous species, *Relbunium hypocarpium,* Rubiaceae. *Revista Brasileira de Genetica* 18: 289—296.

Bremekamp, C. E. B. 1966. Remarks on the position, the delimitation and the subdivision of the Rubiaceae. *Acta Botanica Neerlandica* 15: 1—33.

Bremer, B. 1996. Phylogenetic studies within Rubiaceae and relationships to other families based on molecular data. *Opera Botanica Belgica* 7: 33—50.

Bremer, B., and T. Eriksson. 1992. Evolution of fruit characters and dispersal modes in the tropical family Rubiaceae. *Biological Journal of the Linnean Society. Linnean Society of London* 47: 79—95.

Bremer, B., and T. Eriksson. 2009. Time tree of Rubiaceae: phylogeny and dating the family, subfamilies, and tribes. *International Journal of Plant Sciences* 170: 766--793.

Bremer, B., and J.-F. Manen. 2000. Phylogeny and classification of the subfamily Rubioideae (Rubiaceae). *Plant Systematics and Evolution* 225: 43--72.

California Native Plant Society (CNPS). 2010. Inventory of Rare and Endangered Plants (online edition, v8-01a). CNPS, Sacramento, California, USA. Website http://www.rareplants.cnps.org/ [accessed 08 Dec 2010].

Catalán, P., E. A. Kellogg, and R. G. Olmstead. 1997. Phylogeny of Poaceae subfamily Pooideae based on chloroplast *ndh*F gene sequences. *Molecular Phylogenetics and Evolution* 8: 150—166.

Cavalli-Molina, S., V. E. P. Motta, M. Schiengold, and H. Winge. 1989. Identical isoenzyme patterns in sib plants of *Relbunium hypocarpium* (Rubiaceae). *Revista Brasileira de Genetica* 12: 361—368.

Cavalli-Molina, S., and H. Winge. 1988. Phenetic relationships among populations of the autogamous plant *Relbunium hypocarpium* (Rubiaceae). *Revista Brasileira de Genetica* 11: 401—418.

Charlesworth, D., and D. S. Guttman. 1999. The evolution of dioecy and plant sex chromosome systems. Pp. 25—49 in*:* C. C. Ainsworth (ed.), *Sex determination in plants*. Bios Scientific, Oxford, U.K.

Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, and J. D. Thompson. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research* 31: 3497—3500.

Cox, C. B., and P. D. Moore. 2000. *Biogeography: An ecological and evolutionary approach.* Blackwell Science, London, England, UK.

Cramer, P., D. A. Bushnell, and R. D. Kornberg. 2001. Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution. *Science* 292: 1863—1876.

Cronquist, A. 1968. *The evolution and classification of flowering plants*. Houghton Mifflin Company, Boston, Massachusetts, USA.

DeBry, R. W., and R.G. Olmstead. 2000. A simulation study of reduced tree-search effort in bootstrap resampling analysis. *Systematic Biology* 49: 171—179.

De Candolle, A. P. 1830. *Prodomus systematis naturalis, regni vegetabilis*, vol. 4. Treuttel et Würtz, Paris.

De Jussieu, A. L. 1789. *Genera plantarum secundum ordines naturales disposita.* Herrisant, Paris.

Delph, L. F., and D. E. Wolf. 2005. Evolutionary consequences of gender plasticity in genetically dimorphic breeding systems. *New Phytologist* 166: 119--128.

Delport, W., A. F. Y. Poon, S. D. W. Frost, and S. L. Kosakovsky Pond. 2010. Datamonkey 2010: A suite of phylogenetic analysis tools for evolutionary biology. *Bioinformatics* 26: 2455—2457.

Dempster, L. T. 1959. A re-evaluation of *Galium multiflorum* and related taxa. *Brittonia* 11: 105—122.

Dempster, L. T. 1973. The polygamous species of the genus *Galium* (Rubiaceae), section *Lophogalium*, of Mexico and southwestern United States. *University of California Publications in Botany* 64: 1--36.

Dempster, L. T. 1978. The genus *Galium* (Rubiaceae) in Mexico and Central America. *University of California Publications in Botany* 73: 1--33.

Dempster, L. T. 1980. The genus *Galium* section *Lophogalium* (Rubiaceae) in South America. *Allertonia* 2: 247—279.

Dempster, L. T. 1981. The genus *Galium* (Rubiaceae) in South America. II. *Allertonia* 2: 393—426.

Dempster, L. T. 1982. The genus *Galium* (Rubiaceae) in South America. III. *Allertonia* 3: 211—258.

Dempster, L. T. 1990. The genus *Galium* (Rubiaceae) in South America. IV. *Allertonia* 5: 283—345.

Dempster, L. T. 1993. Rubiaceae. Pp. 976—986 in: J. C. Hickman (ed.), *The Jepson manual, higher plants of California*. University of California Press, Berkeley, California, U.S.A.

Dempster, L. T., and F. Ehrendorfer. 1965. Evolution of the *Galium multiflorum* complex in western North America. II. Critical taxonomic revision. *Brittonia* 17: 289—334.

Dempster, L. T., and G. L. Stebbins. 1965. The fleshy-fruited *Galium* species of California (Rubiaceae). I. Cytological findings and some taxonomic conclusions. *Madroño* 18: 105—113.

Dempster, L. T., and G. L. Stebbins. 1968. A cytotaxonomic revision of the fleshy-fruited *Galium* species of the Californias and southern Oregon (Rubiaceae). *University of California Publications in Botany* 46: 1--52.

Dempster, L. T., and G. L. Stebbins. 1971. The *Galium angustifolium* complex (Rubiaceae) of California and Baja California. *Madroño* 21: 70—95.

Denton, A. L., B. L. McConaughy, and B. D. Hall. 1998. Usefulness of RNA polymerase II coding sequences for estimation of green plant phylogeny. *Molecular Biology and Evolution* 16: 1082—1085.

Devesa, J. A., and A. Ortega-Olivencia. 2003. A new species of *Valantia* (Rubiaceae) from Spain. *Botanical Journal of the Linnean Society* 143: 331—335.

Doyle, J. J., and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11—15.

Ehrendorfer, F. 1955. Revision of the genus *Relbunium* (Endl.) Benth. et Hook. (Rubiaceae-Galieae). *Botanische Jahrbücher* 76: 516—553.

Ehrendorfer, F. 1956. Survey of the *Galium multiflorum* complex in western North America. *Contributions from the Dudley Herbarium* 5: 1—21.

Ehrendorfer, F. 1958. Critical notes on Turkish Rubiaceae. *Notes from the Royal Botanic Garden, Edinburgh* 22: 323—401.

Ehrendorfer, F. 1961. Evolution of the *Galium multiflorum* complex in western North America. I. Diploids and polyploids in this dioecious group. *Madroño* 16: 109—140.

Ehrendorfer, F. 1965. Dispersal mechanisms, genetic systems, and colonizing abilities in some flowering plant families. Pp. 331—352 in: H. G. Baker and G. L. Stebbins (eds.), *The genetics of colonizing species*. Academic Press, New York, New York, U.S.A.

Ehrendorfer, F. 1971. Evolution and eco-geographical differentiation in some South-West Asiatic Rubiaceae. Pp. 195—215 in: P. H. Davis, P. C. Harper, and I. C. Hedge (eds.), *Plant life of South-West Asia*. Botanical Society of Edinburgh, Edinburgh, U.K.

Ehrendorfer, F., F. Krendl, and C. Puff. 1976. *Galium* L. Pp. 14—36 in: T. G. Tutin, V. H. Heywood, N. A. Burges, D. M. Moore, D. H. Valentine, S. M. Walters, and D. A. Webb (eds.), *Flora Europaea*, vol. 4. Cambridge University Press, New York, New York, U.S.A.

Ehrendorfer, F., and E. Schönbeck-Temesy. 1982. *Galium* L. Pp. 767—849 in: P. H. Davis (ed.), *Flora of Turkey and the East Aegean Islands*, vol. 7. Edinburgh University Press, Edinburgh, Scotland, U.K.

Ehrendorfer, F., E. Schönbeck-Temesy, C. Puff, and W. Rechinger. 2005. Rubiaceae. Part 176 of: K. H. Rechinger, *Flora Iranica*. Verlag des Naturhistorischen Museums Wien, Vienna, Austria.

Endlicher, S. 1839. *Genera plantarum secundum ordines naturales disposita*. Friedrich Beck, Vienna, Austria.

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

Fijridiyanto, I. A., and N. Murakami. 2009. Phylogeny of *Litsea* and related genera (Laureae-Lauraceae) based on analysis of *rpb2* gene sequences. *Journal of Plant Research* 122: 283—298.

Fitch, W. M. 1971. Toward defining the course of evolution: Minimum change for a specific tree topology. *Systematic Zoology* 20: 406—416.

Flores, S., and D. W. Schemske. 1984. Dioecy and monoecy in the flora of Puerto Rico and the Virgin Islands: Ecological correlates. *Biotropica* 16: 132—139.

Freeman, D. C., K. T. Harper. and W. K. Ostler. 1980. Ecology of plant dioecy in the intermountain region of western North America and California. *Oecologia* 44: 410—417.

Fukuda, Y. 1988. Phyllotaxis in two species of *Rubia*, *R. akane* and *R. sikkimensis*. *Botanical Magazine (Tokyo)* 101: 25—38.

Givnish, T. J. 1980. Ecological constraints on the evolution of breeding systems in seed plants: Dioecy and dispersal in gymnosperms. *Evolution; International Journal of Organic Evolution* 34: 959—972.

Govaerts, R. (ed.) 2006. World checklist of selected plant families. In: F. A. Bisby, Y. R. Roskov, M. A. Ruggiero, T. M. Orrell, L. E. Paglinawan, P. W. Brewer, N. Bailly, and J. van Hertum, (eds.), *Species 2000 & ITIS catalogue of life: 2007 annual checklist*. Digital resource at www.catalogueoflife.org/annual-checklist/2007/. Species 2000, Reading, U. K.

Graham, A. 2009. Fossil record of the Rubiaceae. *Annals of the Missouri Botanical Garden* 96: 90—108.

Graham, S. W., P. A. Reeves, A. C. E. Burns, and R. G. Olmstead. 2000. Microstructural changes in noncoding chloroplast DNA: Interpretation, evolution, and utility of indels and inversions in basal angiosperm phylogenetic inference. *International Journal of Plant Sciences* 161 (6 Supplement): S83--S96.

Hart, J. A. 1985. Evolution of dioecism in *Lepechinia* Willd. sect. *Parviflorae* (Lamiaceae). *Systematic Botany* 10: 147—154.

Heinken, T., and D. Raudnitschka. 2002. Do wild ungulates contribute to the dispersal of vascular plants in central European forests by epizoochory? A case study in NE Germany. *Forstwissenschaftliches Centralblatt* 121: 179—194.

Hooker, J. D. 1873. Rubiaceae. Pp. 7--151 in: G. Bentham and J. D. Hooker, *Genera plantarum*, vol. 2. Lovell Reeve & Co., Williams & Norgate, London, England, UK.

Huelsenbeck, J. P., and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754--755.

Huysmans, S., S. Dessein, E. Smets, and E. Robbrecht. 2003. Pollen morphology of NW European representatives confirms monophyly of Rubieae (Rubiaceae). *Review of Paleobotany and Palynology* 127: 219—240.

Ibarra-Manriquez, G., and K. Oyama. 1992. Ecological correlates of reproductive traits of Mexican rain forest trees. *American Journal of Botany* 79: 383—394.

Jansen, S., S. Dessein, F. Piesschaert, E. Robbrecht, and E. Smets. 2000. Aluminum accumulation in leaves of Rubiaceae: systematic and phylogenetic implications. *Annals of Botany (Oxford)* 85: 91—101.

Jeune, B. 1980. Croissance des feuilles et stipules du *Galium palustre* L. subsp. *elongatum* (Presl) Lange et valeur phylogénique de ces données de morphogénèse. *Adansonia* 19: 451—465.

Kårehed, J., I. Groeninckx, S. Dessein, T. J. Motley, and B. Bremer. 2008. The phylogenetic utility of chloroplast and nuclear DNA markers and the phylogeny of the Rubiaceae tribe Spermacoceae. *Molecular Phylogenetics and Evolution* 49: 843—866.

Kosakovsky Pond, S. L., and S. D. W. Frost. 2005a. A simple hierarchical approach to modeling distributions of substitution rates. *Molecular Biology and Evolution* 22: 223—234.

Kosakovsky Pond, S. L., and S. D. W. Frost. 2005b. Datamonkey: Rapid detection of selective pressure on individual sites of codon alignments. *Bioinformatics* 21: 2531—2533.

Kosakovsky Pond, S. L., and S. D. W. Frost. 2005c. Not so different after all: A comparison of methods for detecting amino acid sites under selection. *Molecular Biology and Evolution* 22: 1208—1222.

Kosakovsky Pond, S. L., S. D. W. Frost, and S. V. Muse. 2005. HyPhy: Hypothesis testing using phylogenies. *Bioinformatics* 21: 676—679.

Kosakovsky Pond, S. L., D. Posada, M. B. Gravenor, C. H. Woelk, and S. D. W. Frost. 2006a. Automated phylogenetic detection of recombination using a genetic algorithm. *Molecular Biology and Evolution* 23: 1891—1901.

Kosakovsky Pond, S. L., D. Posada, M. B. Gravenor, C. H. Woelk, and S. D. W. Frost. 2006b. GARD: Genetic algorithm for recombination detection. *Bioinformatics* 22: 3096—3098.

Kumar, S., R. K. Mishra, A. Kumar, S. Srivastava, and S. Chaudhary. 2009. Regulation of stipule development by *COCHLEATA* and *STIPULE-REDUCED* genes in pea *Pisum sativum*. *Planta* 230: 449—458.

Latorre, C., J. L. Betancourt, K. A. Rylander, J. Quade, and O. Matthei. 2003. A vegetation history from the arid prepuna of northern Chile (22—23 ºS) over the last 13,500 years. *Palaeogeography, Palaeoclimatology, Palaeoecology* 194: 223—246.

Lewis, D. 1942. The evolution of sex in flowering plants. *Biological Review of the Cambridge Philosophical Society* 17: 46--67.

Llyod, D. G. 1980. The distributions of gender in four angiosperm species illustrating two evolutionary pathways to dioecy. *Evolution; International Journal of Organic Evolution* 34: 123--134.

Lozano-García, M. S., B. Ortega-Guerrero, and S. Sosa-Nájera. 2002. Mid- to late-Wisconsin pollen record of San Felipe Basin, Baja California. *Quaternary Research* 58: 84—92.

Luo, J., and B. D. Hall. 2007. A multistep process gave rise to RNA polymerase IV of land plants. *Journal of Molecular Evolution* 64: 101—112.

Luo, J., N. Yoshikawa, M. C. Hodson, and B. D. Hall. 2007. Duplication and paralog sorting of *RPB2* and *RPB1* genes in core eudicots. *Molecular Phylogenetics and Evolution* 44: 850—862.

Maddison, D. R., and W. P. Maddison. 2000. *MacClade 4: Analysis of phylogeny and character evolution, version 4.0*. Sinauer Associates, Sunderland, Massachusetts, U.S.A.

Maddison, W. P., and D. R. Maddison. 2009. *Mesquite: A modular system for evolutionary analysis, version 2.6, 2.72.* Website http://mesquiteproject.org [accessed 10 Jan 2010].

Manen, J.-F., and A. Natali. 1995. Comparison of the evolution of ribulose-1, 5-biphosphate carboxylase (*rbcL*) and *atpB-rbcL* noncoding spacer sequences in a recent plant group, the tribe Rubieae (Rubiaceae). *Journal of Molecular Evolution* 41: 920—927.

Manen, J.-F., A. Natali, and F. Ehrendorfer. 1994. Phylogeny of Rubiaceae-Rubieae inferred from the sequence of a cpDNA intergene region. *Plant Systematics and Evolution* 190: 195--211.

Martin, A. C., H. S. Zim, and A. L. Nelson. 1951. *American wildlife and plants*. McGraw-Hill, New York, New York, U.S.A.

Matlack, G. R. 1994. Plant species migration in a mixed-history forest landscape in eastern North America. *Ecology* 75: 1491—1502.

Miller, M. A., M. T. Holder, R. Vos, P. E. Midford, T. Liebowitz, L. Chan, P. Hoover, and T. Warnow. 2010. The CIPRES Portals. CIPRES. Website http://www.phylo.org/sub_sections/portal [accessed 06 Jan 2010].

Mindell, D. P. 1991. Aligning DNA sequences: Homology and phylogenetic weighting. Pp. 73—89 ın: M. M. Miyamoto and J. Cracraft (eds.), *Phylogenetic analysis of DNA sequences*. Oxford University Press, New York, New York, U.S.A.

Mitchell, C. H., and P. K. Diggle. 2005. The evolution of unisexual flowers: Morphological and functional convergence results from diverse developmental transitions. *American Journal of Botany* 92: 1068--1076.

Moore, W. S. 1995. Inferring phylogenies from mtDNA variation: Mitochondrial-gene trees versus nuclear-gene trees. *Evolution* 49: 718—726.

Mort, M. E., J. K. Archibald, C. P. Randle, N. D. Levsen, T. R. O'Leary, K. Topalov, C. M. Wiegand, and D. J. Crawford. 2007. Inferring phylogeny at low taxonomic

levels: Utility of rapidly evolving cpDNA and nuclear ITS loci. *American Journal of Botany* 94: 173—183.

Natali, A., J.-F. Manen, and F. Ehrendorfer. 1995. Phylogeny of the Rubiaceae-Rubioideae, in particular the tribe Rubieae: Evidence from a non-coding chloroplast DNA sequence. *Annals of the Missouri Botanical Garden* 82: 428--439.

Natali, A., J.-F. Manen, M. Kiehn, and F. Ehrendorfer. 1996. Tribal, generic and specific relationships in the Rubioideae-Rubieae (Rubiaceae) based on sequence data of a cpDNA intergene region. *Opera Botanica Belgica* 7: 193—203.

Nie, Z.-L., J. Wen, H. Sun, and B. Bartholomew. 2005. Monophyly of *Kelloggia* Torrey ex Benth. (Rubiaceae) and evolution of its intercontinental disjunction between western North America and eastern Asia. *American Journal of Botany* 92: 642—652.

Niewland, J. A. 1910. A new genus of Rubiaceae. *American Midland Naturalist* 1: 263—264.

Niklas, K. J. 1998. Light harvesting “fitness landscapes” for vertical shoots with different phyllotactic patterns. Pp. 759—773 in: V. Jean and D. Barabé (eds.), *Symmetry in plants*. World Scientific Publishing Co., River Edge, New Jersey, USA.

Nylander, J. A. A., J. C. Wilgenbusch, D. L. Warren, and D. L. Swofford. 2008. AWTY (are we there yet?): A system for graphical exploration of MCMC convergence in Bayesian phylogenetics. *Bioinformatics* 24: 581--583.

Oxelman, B., and B. Bremer. 2000. Discovery of paralogous nuclear gene sequences coding for the second-largest subunit of RNA polymerase II (*RPB2*) and their phylogenetic utility in Gentianales of the asterids. *Molecular Biology and Evolution* 17: 1131—1145.

Oxelman, B., N. Yoshikawa, B. L. McConaughy, J. Luo, A. L. Denton, and B. D. Hall. 2004. *RPB2* gene phylogeny in flowering plants, with particular emphasis on asterids. *Molecular Phylogenetics and Evolution* 32: 462—479.

Pfeil, B. E., C. L. Brubaker, L. A. Craven, and M. D. Crisp. 2004. Paralogy and orthology in the Malvaceae *rpb2* gene family: Investigation of gene duplication in *Hibiscus. Molecular Biology and Evolution* 21: 1428—1437.

Pobedimova, E. G. 2000. *Galium* L. pp. 345—459 in: B. K. Schischkin (ed.), *Flora of the U.S.S.R*, vol. 23. Bishen Singh Mahendra Pal Singh, Dehra Dun. India, and Koeltz Scientific Books, Koenigstein, Germany.

Porto, M. L., J. E. A. Mariath, M. L. Detoni, S. S. Cavalli, H. Winge, and F. Ehrendorfer. 1977. New species of *Relbunium* (Rubiaceae) from Brazil, with notes on flavonoid and peroxidase patterns. *Plant Systematics and Evolution* 128: 177—193.

Posada, D., and K. A. Crandall. 1998. Modeltest: Testing the model of DNA substitution. *Bioinformatics* 14: 817--818.

Pötter, U., and K. Klopfer. 1987. Untersuchungen zur Blatt- und Blütenentwicklung bei *Galium aparine* L. (Rubiaceae). *Flora* 179: 305—314.

Renner, S. S. 1998. Phylogenetic affinities of Monimiaceae based on cpDNA gene and spacer sequences. *Perspectives in Plant Ecology, Evolution, and Systematics* 1: 61—77.

Renner, S. S., and R. E. Ricklefs. 1995. Dioecy and its correlates in the flowering plants. *American Journal of Botany* 82: 596--606.

Renner, S. S., and H. Won. 2001. Repeated evolution of dioecy from monoecy in Siparunaceae (Laurales). *Systematic Biology* 50: 700—712.

Richard, M. A. 1829. *Mémoire sur la famille des Rubiacées.* J. Tastu, Paris.

Richards, A. J. 1997. *Plant breeding systems*, 2nd ed. Chapman and Hall, London, England, U.K.

Robbrecht, E. 1988. Tropical woody Rubiaceae. *Opera Botanica Belgica* 1: 1—171.

Robbrecht, E., and J.-F. Manen. 2006. The major evolutionary lineages of the coffee family (Rubiaceae, angiosperms). Combined analysis (nDNA and cpDNA) to infer the position of *Coptosapelta* and *Luculia*, and supertree construction based on *rbcL*, *rps16*, *trnL-trnF* and *atpB-rbcL* data. A new classification in two subfamilies, Cinchonoideae and Rubioideae. *Systematics and Geography of Plants* 76: 85—146.

Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572—1574.

Rose, P. P., and B. T. Korber. 2000. Detecting hypermutations in viral sequences with an emphasis on G → A hypermutation. *Bioinformatics* 16: 400—401.

Ross, M. D. 1982. Five evolutionary pathways to subdioecy. *American Naturalist* 119: 297--318.

Rutishauser, R. 1999. Polymerous leaf whorls in vascular plants: Developmental morphology and fuzziness of organ identities. *International Journal of Plant Sciences* 160 (6 Suppl.): S81—S103.

Rydin, C., K. Kainulainen, S. G. Razafimandimbison, J. E. E. Smedmark, and B. Bremer. 2009a. Deep divergences in the coffee family and the systematic position of *Acranthera. Plant Systematics and Evolution* 278: 101—123.

Rydin, C., S. G. Razafimandimbison, A. Khodabandeh, and B. Bremer. 2009b. Evolutionary relationships in the Spermacoceae alliance (Rubiaceae) using information from six molecular loci: Insights into systematic affinities of *Neohymenopogon* and *Mouretia*. *Taxon* 58: 793—810.

Sakai, A. K., W. L. Wagner, D. M. Ferguson, and D. R. Herbst. 1995. Biogeographical and ecological correlates of dioecy in the Hawaiian flora. *Ecology* 76: 2530—2543.

Sambrook, J., E. F. Fritsch, and T. Maniatus. 1989. *Molecular cloning: A laboratoy manual*, 2nd ed, vol. 1--3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, U.S.A.

Schischkin, B. K., (ed.). 2000. *Flora of the U.S.S.R.,* vol. 23. Bishen Singh Mahendra Pal Singh, Delhra Dunn, India, and Koeltz Scientific Books, Koenigstein.

Schoute, J. C. 1938. On whorled phyllotaxis IV. Early binding whorls. *Recueil des Travaux Botaniques Néerlandais* 35: 415—558.

Schumann, K. 1897. Rubiaceae. Pp. 1--156 in: A. Engler and K. Prantl. (eds.), *Die natürlichen Pflanzenfamilien*, Teil IV, Abt. 4. Engelmann, Leipzig, Germany.

Shaw, J., E. B. Lickey, J. T. Beck, S. B. Farmer, W. Liu, J. Miller, K. C. Siripun, C. T. Winder, E. E. Schilling, and R. L. Small. 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92: 142—166.

Shaw, J., E. B. Lickey, E. E. Schilling, and R. L. Small. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. *American Journal of Botany* 94: 275—288.

Shimodaira, H. 2002. An approximately unbiased test of phylogenetic tree selection. *Systematic Biology* 51: 492—508.

Shimodaira, H., and M. Hasegawa. 2001. CONSEL: For assessing the confidence of phylogenetic tree selection. *Bioinformatics* 17: 1246—1247.

Simmons, M. P. 2004. Independence of alignment and tree search. *Molecular Phylgenetics and Evolution* 31: 874—879.

Simmons, M. P., and H. Ochoterena. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* 49: 369--381.

Soza, V. L., and R. G. Olmstead. 2010a. Molecular systematics of tribe Rubieae (Rubiaceae): Evolution of major clades, development of leaf-like whorls, and biogeography. *Taxon* 59: 755--771.

Soza, V. L., and R. G. Olmstead. 2010b. Evolution of breeding systems and fruits in New World *Galium* and relatives (Rubiaceae). *American Journal of Botany* 97: 1630--1646.

Steane, D. A., R. W. Scotland, D. J. Mabberley, and R. G. Olmstead. 1999. Molecular systematics of *Clerodendrum* (Lamiaceae): ITS sequences and total evidence. *American Journal of Botany* 86: 98—107.

Swofford, D. L. 2002. *PAUP*: Phylogenetic analysis using parsimony (*and other methods)*, *version 4.0*. Sinauer Associates, Sunderland, Massachusetts, U.S.A.

Taberlet, P., L. Gielly, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105—1109.

Takeda, H. 1916. Some points in the morphology of the stipules in the Stellatae, with special reference to *Galium. Annals of Botany (Oxford)* 30: 197—214.

Tao, C. and Z. Hua. In preparation. Rubiaceae. In: Z. Y. Wu, P. H. Raven, D. Y. Hong (eds.), *Flora of China*, vol. 19. Science Press, Beijing, China, and Missouri Botanical Garden Press, St. Louis, Missouri, U.S.A. Website http://flora.huh.harvard.edu/china/mss/volume19/Rubiaceae-MO_original.htm [accessed 17 February 2009].

Thompson, R. S. 1990. Late Quaternary vegetation and climate in the Great Basin. Pp. 200—239 in: J. L. Betancourt, T. R. Van Devender, and P. S. Martin (eds.), *Packrat Middens: The last 40,000 years of biotic changes*. University of Arizona Press, Tucson, Arizona, USA.

Turner, B. L., and G. Turner. 1983. A new gypsophilic species of *Galium* (Rubiaceae) from north-central Mexico. *Madroño* 30: 31—33.

Vamosi, J. C., S. P. Otto, and S. C. H. Barrett. 2003. Phylogenetic analysis of the ecological correlates of dioecy in angiosperms. *Journal of Evolutionary Biology* 16: 1006—1018.

Vamosi, J. C., and S. M. Vamosi. 2004. The role of diversification in causing the correlates of dioecy. *Evolution; International Journal of Organic Evolution* 58: 723—731.

Webb, C. J., D. G. Lloyd, and L. F. Delph. 1999. Gender dimorphism in indigenous New Zealand plants. *New Zealand Journal of Botany* 37: 119—130.

Weiblen, G. D., R. K. Oyama, and M. J. Donoghue. 2000. Phylogenetic analysis of dioecy in monocotyledons. *American Naturalist* 155: 46--58.

Weller, S. G., and A. K. Sakai. 1999. Using phylogenetic approaches for the analysis of plant breeding system evolution. *Annual Review of Ecology and Systematics* 30: 167--199.

White, J. M., and T. A. Ager. 1994. Palynology, paleoclimatology and correlation of middle Miocene beds from Porcupine River (locality 90-1), Alaska. *Quaternary Research* 22/23: 43—77.

Willson, M. F., C. Sabag, J. Figueroa, J. J. Armesto, and M. Caviedes. 1996. Seed dispersal by lizards in Chilean rainforest. *Revista Chilena de Historia Natural* 69: 339—342.

Wright, S. D., C. G. Yong, S. R. Wichman, J. W. Dawson, and R. C. Gardner. 2001. Stepping stones to Hawaii: A trans-equatorial dispersal pathway for *Metrosideros* (Myrtaceae) inferred from nrDNA (ITS+ETS). *Journal of Biogeography* 28: 769—774.

Yamazaki, T. 1993. Rubiaceae. Pp. 206—240 in: K. Iwatsuki, T. Yamazaki, D. E. Boufford, H. Ohba (eds.), *Flora of Japan*, vol. 3a. Kodansha, Tokyo, Japan.

Yang, Z. 2007. PAML 4: Phylogenetic analysis by maximum likelihood. *Molecular Biology and Evolution* 24: 1586—1591.

Yang, Z. and B. Rannala. 1997. Bayesian phylogenetic inference using DNA sequences: A Markov Chain Monte Carlo method. *Molecular Biology and Evolution* 14: 717—724.

Zwickl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation, University of Texas, Austin, Texas, U.S.A. Website http://garli.nescent.org [accessed 06 Jan 2010].

Appendix A. Voucher information, GenBank accessions, and character states for taxa sampled in Chapter I.

Taxon, country, political subdivision, collector, number (herbarium), *rpoB-trnC, trnC-psbM, trnL-trnF-ndhJ,* distribution state (OW = Old World, NA = North America, or SA = South America), number of whorl organs (2, 4, 4--6, or 6+)

Asperula albovii Manden., Georgia,----, Jazhugze P. s.n. (MO), GU357269, GU357396, GU357139, OW, 6+; *Asperula arvensis* L., Greece, Crete, Kyriakopoulos & Turland sub Turland 1178 (MO), GU357244, GU357372, GU357114, OW, 6+; *Asperula chlorantha* Boiss. & Heldr.,Greece, Epirus, F. Ehrendorfer 930413-4401 (WU), GU357259, GU357386, GU357129, OW, 6+; *Asperula cynanchica* L., Switzerland, Geneva Botanical Garden, JBG 861771/0 (G), GU357256, GU357383, GU357126, OW, 4; *Asperula glomerata* (M. Bieb.) Griseb., Georgia, Garcani, J.F. Gaskin 607 (MO), GU357268, GU357395, GU357138, OW, 6+; *Asperula glomerata* (M. Bieb.) Griseb. subsp. *turcomanica* (Pobed.) Ehrend. & Schönb.-Tem., Turkmenistan, Balkan, D. Kurbanov 700 (MO), GU357267, GU357394, GU357137, OW, 6+; *Asperula gussonei* Boiss., Switzerland, Geneva Botanical Garden, JBG 783214/0 (G), GU357257, GU357384, GU357127, OW, 4; *Asperula hirta* Ramond, Switzerland, Geneva Botanical Garden, JBG 814140/0 M (G), GU357261, GU357388, GU357131, OW, 6+; *Asperula laevigata* L., Italy, Tuscany, A. Natali & M.-A. Thiébaud s.n. (G), GU357253, GU357381, GU357123, OW, 4; *Asperula molluginoides* Rchb., Sweden, Bergius Botanic Garden, Andreasen 343 (SBT), GU357270, GU357397, GU357140, OW, 6+; *Asperula orientalis* Boiss. & Hohen., Georgia, Meskheti, D. Mtskhvetadze 6 (MO), GU357245, GU357373, GU357115, OW, 6+; *Asperula purpurea* (L.) Ehrend., Italy, Alpi Apuane, A. Natali & J.F. Manen 011 (G), GU357260, GU357387, GU357130, OW, 6+; *Asperula setosa* Jaub. & Spach, Turkmenistan, Balkan, D. Kurbanov 68 (MO), GU357246, GU357374, GU357116, OW, 6+; *Asperula* sp. Georgia, Mtiuleti, M. Merello, J. Stone & M. Chiboshvili 2448 (MO), GU357258, GU357385, GU357128, OW, 4; *Asperula taurina* L., Sweden, Bergius Botanic Garden, L.G. Reinhammar & K. Kustas 729 (WTU), GU357242, GU357370, GU357112, OW, 4; *Asperula taurina* L., Georgia, Kartli, M. Merello, J. Stone, J. Gaskin, & M. Khutsishvili 2264 (MO), GU357243, GU357371, GU357113, OW, 4; *Asperula tinctoria* L., U.S.A., University of Washington Medicinal Herb Garden, V. Soza 1773 (WTU), GU357254, GU357382, GU357124, OW, 4--6; *Asperula tinctoria* L., Sweden, Bergius Botanic Garden, L.G. Reinhammar & K. Kustas 727 (WTU), GU357255,----, GU357125, OW, 4--6; *Callipeltis cucullaris* (L.) DC., Turkmenistan, Ahal, D. Kurbanov 2211 (MO), GU357272, GU357399, GU357142, OW, 4; *Crucianella angustifolia* L., France, Corsica, D. Jeanmonod & A. Natali J5044 (G), GU357265, GU357392, GU357135, OW, 4; *Crucianella chlorostachys* Fisch. & Mey., Turkmenistan, Balkan, D. Kurbanov 669 (MO), GU357266, GU357393, GU357136, OW, 4; *Crucianella filifolia* Regel & Schmalh., Turkmenistan, Tashauz, D. Kurbanov 568 (MO), GU357263, GU357390, GU357133, OW, 4; *Crucianella sintenisii* Bornm., Turkmenistan, Western Kopet Dag, D. Kurbanov 1605 (MO), GU357264, GU357391, GU357134, OW, 6+; *Cruciata*

glabra (L.) Ehrend., Italy, Tuscany, A. Natali & M.-A. Thiébaud N57761 (G), GU357239, GU357367, GU357109, OW, 4; *Cruciata laevipes* Opiz, France, Corsica, D. Jeanmonod, A. Natali, & R. Palese J4198 (G), GU357237, GU357365, GU357107, OW, 4; *Cruciata pedemontana* (All.) Ehrend., U.S.A., Washington, S. Rodman, P.F. Zika, S. Bagshaw, & D. Blum 508 (WTU), GU357240, GU357368, GU357110, OW, 4; *Cruciata taurica* (Pall.) Ehrend., Crimea, Ukraine, M. Popov & D. Dobrochaeva s.n. (MO), GU357238, GU357366, GU357108, OW, 4; *Didymaea alsinoides* (Cham. & Schltdl.) Standl., Mexico, Chiapas, T.J. Pacheco 6 (MEXU), GU357313, GU357441, GU357184, NA, 6+; *Didymaea floribunda* Rzed., Mexico, Mexico, Distrito Federal, T.J. Pacheco 16 (MEXU), GU357314, GU357442, GU357185, NA, 6+; *Galianthe brasiliensis* (Spreng.) E.L. Cabral & Bacigalupo, Argentina, Misiones, V. Soza, J.T. Columbus, & G. Ocampo 1806 (WTU), GU357317, GU357446, GU357189,----, 6+; *Galium aetnicum* Bivona., Italy, Tuscany, A. Natali & M.-A. Thiébaud N57944 (G), GU357281, GU357408, GU357151, OW, 6+; *Galium album* Mill., France, Corsica, D. Jeanmonod, A. Natali, & R. Palese s.n. (G), GU357276, GU357403, GU357146, OW, 6+; *Galium ambiguum* W. Wight subsp. *siskiyouense* (Ferris) Dempster & Stebbins, U.S.A., Oregon, V. Soza 1760 (WTU), GU357216, GU357344, GU357086, NA, 4; *Galium andrewsii* A. Gray subsp. *andrewsii,* U.S.A., California, V. Soza 1729 (WTU), GU357213, GU357341, GU357083, NA, 4; *Galium angustifolium* Nutt. ex Torr. & A. Gray subsp*. angustifolium* U.S.A., California, S. Boyd 11551b (RSA), GU357218, GU357346, GU357088, NA, 4; *Galium aparine* L., U.S.A., California, N. Fraga 1282 (RSA), GU357303, GU357431, GU357174, OW, 6+; *Galium argense* Dempster & Ehrend., U.S.A., California, V. Soza 1742a (WTU), GU357221, GU357349, GU357091, NA, 4; *Galium bailloni* Brandza, Romania, Arges, F. Ehrendorfer 890821-3001 (WU), GU357233, GU357361, GU357103, OW, 4; *Galium bifolium* S. Watson, U.S.A., California, V. Soza 1748 (WTU), GU357247, GU357375, GU357117, NA, 4; *Galium bigeminum* Griseb., Argentina, Misiones, V. Soza, J.T. Columbus, & G. Ocampo 1809 (WTU), GU357204, GU357332, GU357074, SA, 4; *Galium bolanderi* A. Gray, U.S.A., Oregon, V. Soza 1758a (WTU), GU357217, GU357345, GU357087, NA, 4; *Galium boreale* L., U.S.A., California, V. Soza 1755 (WTU), GU357235, GU357363, GU357105, OW, 4; *Galium* cf*. cespitosum* Lam., Spain, Huesca, Villar & Perez s.n. (JACA), GU357292, GU357420, GU357163, OW, 6+; *Galium circaezans* Michx., U.S.A., Texas, J. Quayle, Varnum, & Douglass 0656 (TEX), GU357228, GU357356, GU357098, NA, 4; *Galium collomiae* J.T. Howell, U.S.A., Arizona, V. Soza & Y. Yaowu 1785 (WTU), GU357190, GU357318, GU357060, NA, 4; *Galium coloradoense* W. Wight, U.S.A., New Mexico, V. Soza & Y. Yaowu 1784 (WTU), GU357226, GU357354, GU357096, NA, 4; *Galium cometerhizon* Lapeyr., Spain, Huesca, Villar & Gomez s.n. (JACA), GU357290, GU357418, GU357161, OW, 6+; *Galium corrudifolium* Vill., Italy, Tuscany, A. Natali & M.-A. Thiébaud N56941 (G), GU357282, GU357409, GU357152, OW, 6+; *Galium corsicum* Spreng., France, Corsica, D. Jeanmonod & A. Natali J4931 (G), GU357298, GU357426, GU357169, OW, 6+; *Galium divaricatum* Lam., France, Corsica, D. Jeanmonod, A. Natali, & C. Zelweger J3394 (G), GU357295, GU357423,

GU357166, OW, 6+; *Galium elongatum* Presl, France, Corsica, D. Jeanmonod & A. Natali J4966 (G), GU357252, GU357380, GU357122, OW, 4--6; *Galium estebani* Sennen, Spain, Huesca, Montserrat & al. s.n. (JACA), GU357287, GU357415, GU357158, OW, 6+; *Galium fendleri* A. Gray, U.S.A., Arizona, V. Soza & Y. Yaowu 1778 (WTU), GU357195, GU357323, GU357065, NA, 4; *Galium friedrichii* N. Torres, L. Sáenz, Mus & Rosselló, Spain, Valencia Jardín Botánico, R.G. Olmstead 2004-4 (WTU), GU357285, GU357412, GU357155, OW, 6+; *Galium fruticescens* Cav., Spain, Zaragoza, Year s.n. (DAHU), GU357273, GU357400, GU357143, OW, 6+; *Galium gilliesii* Hook. & Arn. subsp*. gilliesii,* Argentina, Mendoza, V. Soza, J.T. Columbus, & G. Ocampo 1848 (WTU), GU357201, GU357329, GU357071, SA, 4; *Galium glabrescens* (Ehrend.) Dempster & Ehrend. subsp*. modocense* Dempster & Ehrend., U.S.A., California, V. Soza 1749b (WTU), GU357225, GU357353, GU357095, NA, 4; *Galium gracilicaule* Ehrend. & Bacigalupo, Argentina, Jujuy, V. Soza, J.T. Columbus, & G. Ocampo 1829 (WTU), GU357208, GU357336, GU357078, SA, 4; *Galium grayanum* Ehrend., U.S.A., California, Ertter & Schoolcraft 8641 (WTU), GU357227, GU357355, GU357097, NA, 4; *Galium hallii* Munz & I.M. Johnst., U.S.A., California, V. Soza 1724a (WTU), GU357220, GU357348, GU357090, NA, 4; *Galium hilendiae* Dempster & Ehrend. subsp. *carneum* (Hilend & J.T. Howell) Dempster & Ehrend., U.S.A., California, V. Soza 1740b (WTU), GU357224, GU357352, GU357094, NA, 4; *Galium hintoniorum* B.L. Turner, Mexico, Tamaulipas, G. Nesom, M. Mayfield, & J. Hinton 7460 (TEX), GU357199, GU357327, GU357069, NA, 4; *Galium hirtum* Lam., Argentina, Corrientes, V. Soza, J.T. Columbus, & G. Ocampo 1801 (WTU), GU357202, GU357330, GU357072, SA, 4; *Galium hypocarpium* Endl. ex Griseb., Argentina, Corrientes, V. Soza, J.T. Columbus, & G. Ocampo 1804 (WTU), GU357205, GU357333, GU357075, SA, 4; *Galium hypotrichium* A. Gray subsp*. inyoense* Dempster & Ehrend., U.S.A., California, V. Soza 1743b (WTU), GU357222, GU357350, GU357092, NA, 4; *Galium hystricocarpum* Greenm., Mexico, Sonora, T.R. Van Devender 2000-820 (TEX), GU357212, GU357340, GU357082, NA, 4; *Galium intricatum* Reut., Greece, Ionian Islands, F. Ehrendorfer 930409-2501 (WU), GU357299, GU357427, GU357170, OW, 6+; *Galium jepsonii* Hilend & J.T. Howell, U.S.A., California, V. Soza 1723a (WTU), GU357219, GU357347, GU357089, NA, 4; *Galium juniperinum* Standl., Mexico, Nuevo Leon, T.F. Patterson, K. & J. Clary 7474 (TEX), GU357210, GU357338, GU357080, NA, 4; *Galium kamtschaticum* Steller ex Schult., Russia, Kuril Archipelago, S. Gage SG4583 (WTU), GU357232, GU357360, GU357102, OW or NA, 4; *Galium latoramosum* Clos, Argentina, Cordoba, V. Soza, J.T. Columbus, & G. Ocampo 1844 (WTU), GU357206, GU357334, GU357076, SA, 4; *Galium lilloi* Hicken, Argentina, Jujuy, V. Soza, J.T. Columbus, & G. Ocampo 1820 (WTU), GU357209, GU357337, GU357079, SA, 2; *Galium lucidum* All., Spain, Huesca, Montserrat & al. s.n. (JACA), GU357283, GU357410, GU357153, OW, 6+; *Galium martirense* Dempster & Stebbins, Mexico, Baja California, V. Soza, S. Avila Moreno, & Y. Yaowu 1788 (WTU), GU357215, GU357343, GU357085, NA, 4; *Galium megapotamicum* Spreng., Argentina, Corrientes, V. Soza, J.T. Columbus, & G. Ocampo 1800 (WTU), GU357203,

GU357331, GU357073, SA, 4; *Galium mexicanum* Kunth subsp. *asperrimum* (A. Gray) Dempster, U.S.A., Arizona, V. Soza & Y. Yaowu 1774 (WTU), GU357277, GU357404, GU357147, NA, 6+; *Galium mollugo* L., Spain, Huesca, Montserrat s.n. (JACA), GU357275, GU357402, GU357145, OW, 6+; *Galium moranii* Dempster subsp. *aculeolatum* (Dempster) Dempster, Mexico, Baja California, V. Soza, S. Avila Moreno, & Y. Yaowu 1787 (WTU), GU357194, GU357322, GU357064, NA, 4; *Galium multiflorum* Kellogg, U.S.A., California, S. Boyd 11558 (RSA), GU357223, GU357351, GU357093, NA, 4; *Galium murale* All., Spain, Lleida, Pedrol s.n. (DAHU), GU357297, GU357425, GU357168, OW, 4--6; *Galium odoratum* Scop., Switzerland, Geneva, A. Natali & J.F. Manen 016 (G), GU357304, GU357432, GU357175, OW, 6+; *Galium oreganum* Britton, U.S.A., Oregon, V. Soza 1761 (WTU), GU357234, GU357362, GU357104, NA, 4; *Galium ossirwaense* Krause, Africa, Kenya, Luke 8877 (SBT), GU357284, GU357411, GU357154, OW, 6+; *Galium palustre* L., Spain, Huesca, Catalan & Muller s.n. (UZ), GU357249, GU357377, GU357119, OW, 4--6; *Galium parishii* Hilend & J.T. Howell, U.S.A., California, V. Soza 1736 (WTU), GU357192, GU357320, GU357062, NA, 4; *Galium parisiense* L., U.S.A., Oregon, V. Soza 1756 (WTU), GU357296, GU357424, GU357167, OW, 6+; *Galium perralderii* Coss., Africa, Algeria, F. Ehrendorfer 930626-1001 (WU), GU357300, GU357428, GU357171, OW, 6+; *Galium pilosum* Aiton, U.S.A., Arizona, V. Soza & Y. Yaowu 1779 (WTU), GU357200, GU357328, GU357070, NA, 4; *Galium porrigens* Dempster, U.S.A., California, N. Fraga 1281b (RSA), GU357214, GU357342, GU357084, NA, 4; *Galium productum* Lowe, Portugal, Madeira, Catalan & Sequeira s.n. (MS), GU357274, GU357401, GU357144, OW, 6+; *Galium proliferum* A. Gray, U.S.A., Texas, B.L. Turner 24-155 (TEX), GU357196, GU357324, GU357066, NA, 4; *Galium pumilum* Murray, Spain, Huesca, Catalan & Muller s.n. (UZ), GU357288, GU357416, GU357159, OW, 6+; *Galium pyrenaicum* Gouan, France, Pyrénées-Orientales, Montserrat & Villar s.n. (JACA), GU357294, GU357422, GU357165, OW, 6+; *Galium richardianum* Endl. ex Walp., Argentina, Corrientes, V. Soza, J.T. Columbus, & G. Ocampo 1799 (WTU), GU357207, GU357335, GU357077, SA, 4; *Galium rivale* Griseb., Poland, Subcarpathian Voivodeship, I. Kucowa 265 (WTU), GU357286, GU357413, GU357156, OW, 6+; *Galium rotundifolium* L., Spain, Huesca, Montserrat & al. s.n. (JACA), GU357230, GU357358, GU357100, OW, 4; *Galium rubioides* L., Switzerland, Geneva Botanical Garden, A. Natali & J.F. Manen 013 (G), GU357236, GU357364, GU357106, OW, 4; *Galium saxatile* L., Sweden, Västergötland, J. Rova 2511 (WTU), GU357291, GU357419, GU357162, OW, 6+; *Galium scabrum* L., Portugal, Madeira, Catalan & Sequeira s.n. (MS), GU357231, GU357359, GU357101, OW, 4; *Galium songaricum* Schrenk, China, Xinjiang, B. Bartholomew, I. Al-Shehbaz, A. Abbas, & A. Tumur 8506 (MO), GU357248, GU357376, GU357118, OW, 4; *Galium* sp., Russia, Sakhalin Island, B. Legler 1328 (WTU), GU357278, GU357405, GU357148, OW, 6+; *Galium stellatum* Kellogg, U.S.A., Arizona, Messinger 274 (WTU), GU357229, GU357357, GU357099, NA, 4; *Galium suecicum* (Sterner) Ehrend., Sweden, Västergötland, J. Rova & O. Janson 2510 (WTU), GU357289, GU357417, GU357160, OW, 6+; *Galium sylvaticum* L., France,

Pyrénées-Atlantiques, Montserrat & Villar s.n. (JACA), GU357280, GU357407, GU357150, OW, 6+; *Galium texense* A. Gray, U.S.A., Texas, W. R. Carr 22860 (TEX), GU357198, GU357326, GU357068, NA, 4; *Galium tinctorium* L., U.S.A., Texas, D.J. Rosen 4043 (TEX), GU357251, GU357379, GU357121, NA, 4--6; *Galium tomentosum* Thunb., South Africa,----, B. Bremer & al. 4370 (SBT), GU357306, GU357434, GU357177, OW, 6+; *Galium tricornutum* Dandy, Switzerland, Geneva Botanical Garden, A. Natali & J.F. Manen 014 (G), GU357302, GU357430, GU357173, OW, 6+; *Galium trifidum* L., U.S.A., Washington, Weinmann 01-19 (WTU), GU357250, GU357378, GU357120, OW or NA, 4--6; *Galium triflorum* Michx., U.S.A., California, Soza 1753 (WTU), GU357305, GU357433, GU357176, OW or NA, 6+; *Galium uliginosum* L., Sweden, Jämtland, J. Rova 2508 (WTU),----, GU357414, GU357157, OW, 6+; *Galium uncinulatum* DC., U.S.A., Texas, V. Soza & Y. Yaowu 1780 (WTU), GU357211, GU357339, GU357081, NA, 4; *Galium valdepilosum* Heinr. Braun, Poland, Gmina Miechów, Palkowa & Piekos 268 (WTU), GU357293, GU357421, GU357164, OW, 6+; *Galium verrucosum* Sm., France, Corsica, D. Jeanmonod, R. Palese, & D. Roguet J3980 (G), GU357301, GU357429, GU357172, OW, 6+; *Galium verum* L., U.S.A., University of Washington Medicinal Herb Garden, V. Soza 1768 (WTU), GU357279, GU357406, GU357149, OW, 6+; *Galium virgatum* Nutt. ex Torr. & A. Gray, U.S.A., Texas, W.R. Carr 22289 (TEX), GU357197, GU357325, GU357067, NA, 4; *Galium volcanense* Dempster, Mexico, Baja California, V. Soza, R. Arce, S. Avila Moreno, & Y. Yaowu 1786 (WTU), GU357193, GU357321, GU357063, NA, 4; *Galium wrightii* A. Gray, U.S.A., Arizona, Luckow & al. 2762 (WTU), GU357191, GU357319, GU357061, NA, 4; *Kelloggia galioides* Torr., U.S.A., California, G. Helmkamp s.n. (RSA), GU357315, GU357443, GU357186, OW, 6+; *Phuopsis stylosa* Benth. & Hook.f., Switzerland, Geneva Botanical Garden, JBG 916798 (G), GU357271, GU357398, GU357141, OW, 6+; *Rubia cordifolia* L., China, Yunnan, Li Heng, D. Zhiling, L. Rong, J. Zhutang, J. Yunheng, P. Fritsch, L. Zhou, & K. Armstrong 19917 (MO), GU357310, GU357438, GU357181, OW, 4; *Rubia florida* Boiss., Turkmenistan, Western Kopet Dag, D. Kurbanov 948 (MO), GU357309, GU357437, GU357180, OW, 2; *Rubia horrida* (Thunb.) Puff, South Africa,----, B. Bremer & al. 4266 (SBT), GU357312, GU357440, GU357183, OW, 6+; *Rubia oncotricha* Hand.-Mazz., China, Qinghai, T.N. Ho, B. Bartholomew, & M. Gilbert 90 (MO), GU357311, GU357439, GU357182, OW, 4; *Rubia tinctorum* L., U.S.A., University of Washington Medicinal Herb Garden, V. Soza 1771 (WTU), GU357307, GU357435, GU357178, OW, 4--6; *Rubia* sp., Georgia,----, J.F. Gaskin 258 (MO), GU357308, GU357436, GU357179, OW, 4--6; *Sherardia arvensis* L., U.S.A., California, S. Boyd 11633 (WTU), GU357262, GU357389, GU357132, OW, 6+; *Spermacoce brachystemonoides* (Cham. & Schltdl.) Kuntze, Argentina, Corrientes, R.G. Olmstead 2004-119 (WTU), GU357316, GU357445, GU357188,----, 6+; *Staelia thymoides* Cham. & Schltdl., Argentina, Misiones, R.G. Olmstead 2004-132 (WTU),----, GU357444, GU357187,----, 6+; *Valantia muralis* L., France, Corsica, D. Jeanmonod & A. Natali s.n. (G), GU357241, GU357369, GU357111, OW, 4.

Appendix B. Voucher information, section, character states, and GenBank accessions for taxa sampled in Chapter II.

Taxon, country, political subdivision, *collector & number* (herbarium), *section*, breeding system (A = andromonoecious, D = dioecious, H = hermaphroditic, P = polygamous), fruit hairs (N = none, L = long-straight, H = hooked, P = pubescent), fruit type (B = berry, N = nutlet), *rpoB-trnC, trnc-psbM, trnL-trnF-ndhJ,* ETS.

Asperula arvensis L., Greece, Crete, *Kyriakopoulos & Turland sub Turland 1178* (MO),----, H, N, N, GU357244, GU357372, GU357114,----; ***A. orientalis*** Boiss. & Hohen., Georgia, Meskheti, *D. Mtskhvetadze 6* (MO),----, H, N, N, GU357245, GU357373, GU357115, HM061072; ***A. setosa*** Jaub. & Spach, Turkmenistan, Balkan, *D. Kurbanov 68* (MO),----, H, N, N, GU357246, GU357374, GU357116,----; ***A. taurina*** L., Sweden, Bergius Botanic Garden, *L. G. Reinhammar & K. Kustas 729* (WTU),----, H, N, N, GU357242, GU357370, GU357112, HM061073; ***Cruciata articulata*** (L.) Ehrend., Israel, Jerusalem, *D. Atsmon s.n.* (WTU),----, A, N, N, HM055768, HM055807, HM055846,----; ***C. glabra*** (L.) Ehrend., Italy, Tuscany, *A. Natali & M.-A. Thiébaud N57761* (G),----, A, N, N, GU357239, GU357367, GU357109, HM061067; ***C. laevipes*** Opiz, France, Corsica, *D. Jeanmonod, A. Natali, & R. Palese J4198* (G),----, A, N, N, GU357237, GU357365, GU357107, HM061066; ***C. pedemontana*** (All.) Ehrend., U.S.A., Washington, *S. Rodman, P.F. Zika, S. Bagshaw, & D. Blum 508* (WTU),----, H, N, N, GU357240, GU357368, GU357110, HM061069; ***C. taurica*** (Pall.) Ehrend., Ukraine, Crimea, *M. Popov & D. Dobrochaeva s.n.* (MO),----, A,----, N, GU357238, GU357366, GU357108, HM061068; ***Galium ambiguum*** W. Wight **subsp. *siskiyouense*** (Ferris) Dempster & Stebbins, U.S.A., Oregon, *V. Soza 1760* (WTU), *Baccogalium,* D, P, B, GU357216, GU357344, GU357086, HM061055--HM061056; ***G. andrewsii*** A. Gray **subsp. *andrewsii,*** U.S.A., California, *V. Soza 1729* (WTU), *Baccogalium,* D, N, B, GU357213, GU357341, GU357083, HM061052; ***G. angustifolium*** Nutt. ex Torr. & A. Gray **subsp*. angustifolium*** U.S.A., California, *S. Boyd 11551b* (RSA), *Lophogalium,* D, L, N, GU357218, GU357346, GU357088, HM061036--HM061037; ***G. angustifolium*** Nutt. ex Torr. & A. Gray **subsp*. borregoense*** Dempster & Stebbins, U.S.A., California, *V. Soza 1730a* (WTU), *Lophogalium,* D, L, N, HM055769, HM055808, HM055847, HM061039; ***G. angustifolium*** Nutt. ex Torr. & A. Gray **subsp*. gabrielense*** (Munz & I.M. Johnst.) Dempster & Stebbins, U.S.A., California, *V. Soza 1734b* (WTU), *Lophogalium,* D, L, N, HM055770, HM055809, HM055848, HM061040; ***G. angustifolium*** Nutt. ex Torr. & A. Gray **subsp*. jacinticum*** Dempster & Stebbins, U.S.A., California, *V. Soza & S. Boyd 1727* (WTU), *Lophogalium,* D, L, N, HM055771, HM055810, HM055849, HM061038; ***G. angustifolium*** Nutt. ex Torr. & A. Gray **subsp*. nudicaule*** Dempster & Stebbins, U.S.A., California, *V. Soza 1735a* (WTU), *Lophogalium,* D, L, N, HM055772, HM055811, HM055850, HM061041; ***G. argense*** Dempster & Ehrend., U.S.A., California, *V. Soza 1742a* (WTU), *Lophogalium,* D, L, N, GU357221, GU357349, GU357091, HM061024; ***G. arkansanum*** A. Gray, U.S.A., Arkansas, *R.D. Thomas 7714* (WTU), *Platygalium,* H, N, N, HM055773, HM055812, HM055851, HM060993; ***G. aschenbornii*** S. Schauer, Mexico, Puebla, *J. Pacheco 21* (MEXU), *Baccogalium,*

P, N, B, HM055774, HM055813, HM055852,----; ***G. bailloni*** Brandza, Romania, Arges, *F. Ehrendorfer 890821-3001* (WU), *Platygalium,* H, N, N, GU357233, GU357361, GU357103, HM061064; ***G. bermudense*** L., U.S.A., South Carolina, *R.G. Olmstead 2005-1* (WTU), *Bataprine,* H, N or P, B, HM055787, HM055826, HM055865, HM060996; ***G. bigeminum*** Griseb., Argentina, Misiones, *V. Soza, J.T. Columbus, & G. Ocampo 1809* (WTU), *Relbunium,* H, N, B, GU357204, GU357332, GU357074, HM061015; **G. bolanderi** A. Gray, U.S.A., Oregon, *V. Soza 1758a* (WTU), *Baccogalium,* D, N or P, B, GU357217, GU357345, GU357087,----; ***G. boreale*** L., U.S.A., California, *V. Soza 1755* (WTU), *Platygalium,* H, H, N, GU357235, GU357363, GU357105, HM061062; ***G. californicum*** Hook. & Arn. **subsp. *flaccidum*** (Greene) Dempster & Stebbins, U.S.A., California, *V. Soza 1731* (WTU), *Baccogalium,* D, P, B, HM055775, HM055814, HM055853, HM061046--HM061048; ***G. carmenicola*** Dempster, Mexico, Coahuila, *J.M. Poole & W.A. Watson 2525* (TEX), *Lophogalium,* P, H, N, HM055776, HM055815, HM055854, HM061001; ***G. carterae*** Dempster, Mexico, Baja California, *A. Carter & R. Moran 5552* (MO), *Lophogalium,* P, L, N, HM055777, HM055816, HM055855, HM060987; ***G. catalinense*** A. Gray **subsp. *acrispum*** Dempster, U.S.A., California, *M. Elvin 158* (RSA), *Lophogalium,* P, L, N, HM055778, HM055817, HM055856, HM061044; ***G. circaezans*** Michx., U.S.A., Texas, *J. Quayle, Varnum, & Douglass 0656* (TEX), *Platygalium,* H, H, N, GU357228, GU357356, GU357098, HM060992; ***G. cliftonsmithii*** (Dempster) Dempster & Stebbins, U.S.A., California, *V. Soza 1720* (WTU), *Baccogalium,* D, N, B, HM055779, HM055818, HM055857, HM061049; ***G. collomiae*** J.T. Howell, U.S.A., Arizona, *V. Soza & Y. Yaowu 1785* (WTU), *Lophogalium,* P, L, N, GU357190, GU357318, GU357060, HM060982; ***G. coloradoense*** W. Wight, U.S.A., New Mexico, *V. Soza & Y. Yaowu 1784* (WTU), *Lophogalium,* D, L, N, GU357226, GU357354, GU357096, HM061032; ***G. correllii*** Dempster, U.S.A., Texas, *B.L. Turner 23-131* (TEX),----, H, N, N, HM055780, HM055819, HM055858, HM061008; ***G. corymbosum*** Ruiz & Pav., Bolivia, Cochabamba, *S.G. Beck & R. Seidel 14618* (WTU), *Relbunium,* H, N, B, HM055781, HM055820, HM055859,----; ***G. corymbosum*** Ruiz & Pav., Bolivia, La Paz, *N.F. Refulio-Rodriguez, T. Columbus, & J. Quisbert Quispe 198* (WTU), *Relbunium,* H, N, B, HM055782, HM055821, HM055860,----; ***G. dempsterae*** B.L. Turner, Mexico, Nuevo Leon, *G.B. Hinton et al. 25526* (TEX), *Lophogalium,* P, H, N, HM055783, HM055822, HM055861, HM060998; ***G. fendleri*** A. Gray, U.S.A., Arizona, *V. Soza & Y. Yaowu 1778* (WTU), *Lophogalium,* P, L, N, GU357195, GU357323, GU357065, HM061022; ***G. gilliesii*** Hook. & Arn. **subsp*. gilliesii,*** Argentina, Mendoza, *V. Soza, J.T. Columbus, & G. Ocampo 1848* (WTU), *Lophogalium,* P, L, N, GU357201, GU357329, GU357071, HM061010; ***G. gilliesii*** Hook. & Arn. **subsp*. telanthos*** (Philippi) Dempster*,* Argentina, Mendoza, *R.G. Olmstead 2004-202* (WTU), *Lophogalium,* P, L, N, HM055784, HM055823, HM055862, HM061009; ***G. glabrescens*** (Ehrend.) Dempster & Ehrend. **subsp*. glabrescens***, U.S.A., California, *A. Eckhert s.n.* (WTU), *Lophogalium,* D, L, N, HM055785, HM055824, HM055863, HM061026; ***G. glabrescens*** (Ehrend.) Dempster & Ehrend. **subsp*. modocense*** Dempster & Ehrend., U.S.A., California, *V. Soza 1749b* (WTU),

Lophogalium, D, L, N, GU357225, GU357353, GU357095, HM061031; ***G. gracilicaule*** Ehrend. & Bacigalupo, Argentina, Jujuy, *V. Soza, J.T. Columbus, & G. Ocampo 1829* (WTU), *Lophogalium,* H, L, N, GU357208, GU357336, GU357078, HM061020; ***G. grande*** McClatchie, U.S.A., California, *V. Soza 1733b* (WTU), *Baccogalium,* P, P, B, HM055786, HM055825, HM055864, HM061050; ***G. grayanum*** Ehrend., U.S.A., California, *Ertter & Schoolcraft 8641* (WTU), *Lophogalium,* D, L, N, GU357227, GU357355, GU357097, HM061027; ***G. hallii*** Munz & I.M. Johnst., U.S.A., California, *V. Soza 1724a* (WTU), *Lophogalium,* D, L, N, GU357220, GU357348, GU357090, HM061057; ***G. hilendiae*** Dempster & Ehrend. **subsp. *carneum*** (Hilend & J.T. Howell) Dempster & Ehrend., U.S.A., California, *V. Soza 1740b* (WTU), *Lophogalium,* D, L, N, GU357224, GU357352, GU357094, HM061034; ***G. hintoniorum*** B.L. Turner, Mexico, Tamaulipas, *G. Nesom, M. Mayfield, & J. Hinton 7460* (TEX),----, H, H, N, GU357199, GU357327, GU357069, HM061005; ***G. hirtum*** Lam., Argentina, Corrientes, *V. Soza, J.T. Columbus, & G. Ocampo 1801* (WTU), *Relbunium,* H, N, B, GU357202, GU357330, GU357072, HM061013; ***G. hypocarpium*** Endl. ex Griseb., Argentina, Corrientes, *V. Soza, J.T. Columbus, & G. Ocampo 1804* (WTU), *Relbunium,* H, N or P, B, GU357205, GU357333, GU357075, HM061016; ***G. hypotrichium*** A. Gray **subsp*. inyoense*** Dempster & Ehrend., U.S.A., California, *V. Soza 1743b* (WTU), *Lophogalium,* D, L, N, GU357222, GU357350, GU357092, HM061025; ***G. hypotrichium*** A. Gray **subsp*. tomentellum*** Ehrend., U.S.A., California, *V. Soza 1741a* (WTU), *Lophogalium,* D, L, N, HM055788, HM055827, HM055866, HM061023; ***G. hystricocarpum*** Greenm., Mexico, Sonora, *T.R. Van Devender 2000-820* (TEX), *Lophogalium,* P, L, N, GU357212, GU357340, GU357082, HM061000; ***G. jepsonii*** Hilend & J.T. Howell, U.S.A., California, *V. Soza 1723a* (WTU), *Lophogalium,* D, H, N, GU357219, GU357347, GU357089, HM061042; ***G. johnstonii*** Dempster & Stebbins, U.S.A., California, *V. Soza 1722b* (WTU), *Lophogalium,* D, L, N, HM055789, HM055828, HM055867, HM061043; ***G. juniperinum*** Standl., Mexico, Nuevo Leon, *T.F. Patterson, K. & J. Clary 7474* (TEX), *Lophogalium,* P, L, N, GU357210, GU357338, GU357080, HM060997; ***G. kamtschaticum*** Steller ex Schult., Russia, Kuril Archipelago, *S. Gage SG4583* (WTU), *Platygalium,* H, H, N, GU357232, GU357360, GU357102, HM061065; ***G. latifolium*** Michx., U.S.A., Virginia, *P.M. Mazzeo 1698* (WTU), *Platygalium,* H, N, N, HM055790, HM055829, HM055868, HM060994; ***G. latoramosum*** Clos, Argentina, Cordoba, *V. Soza, J.T. Columbus, & G. Ocampo 1844* (WTU),----, D, N, B, GU357206, GU357334, GU357076, HM061017; ***G. lilloi*** Hicken, Argentina, Jujuy, *V. Soza, J.T. Columbus, & G. Ocampo 1820* (WTU),----, H, P, N, GU357209, GU357337, GU357079, HM061021; ***G. martirense*** Dempster & Stebbins, Mexico, Baja California, *V. Soza, S. Avila Moreno, & Y. Yaowu 1788* (WTU), *Baccogalium,* D, P, B, GU357215, GU357343, GU357085, HM061051; ***G. matthewsii*** A. Gray, U.S.A., California, *V. Soza 1744a* (WTU), *Lophogalium,* D, L, N, HM055791, HM055830, HM055869, HM061035; ***G. megapotamicum*** Spreng., Argentina, Corrientes, *V. Soza, J.T. Columbus, & G. Ocampo 1800* (WTU), *Relbunium,* P, N, B, GU357203, GU357331, GU357073, HM061014; ***G. microphyllum*** A. Gray, U.S.A.,

Arizona, *V. Soza & Y. Yaowu 1776* (WTU), *Relbunium,* H, N, B, HM055792, HM055831, HM055870, HM061018; ***G. moranii*** Dempster **subsp*. aculeolatum*** (Dempster) Dempster, Mexico, Baja California, *V. Soza, S. Avila Moreno, & Y. Yaowu 1787* (WTU), *Lophogalium,* P, L, N, GU357194, GU357322, GU357064, HM060988; ***G. multiflorum*** Kellogg, U.S.A., California, *S. Boyd 11558* (RSA), *Lophogalium,* D, L, N, GU357223, GU357351, GU357093, HM061030; ***G. munzii*** Hilend & J.T. Howell **subsp. *munzii***, U.S.A., California, *V. Soza 1737* (WTU), *Lophogalium,* D, L, N, HM055793, HM055832, HM055871, HM061033; ***G. nigroramosum*** (Ehrend.) Dempster, Argentina, Tucuman, *V. Soza, J.T. Columbus, & G. Ocampo 1835* (WTU), *Relbunium,* H, N, B, HM055794, HM055833, HM055872, HM061012; ***G. noxium*** (A. St.-Hil.) Dempster **subsp. *valantioides*** (Cham. & Schlechtendal) Dempster, Argentina, Entre Rios, *V. Soza, J.T. Columbus, & G. Ocampo 1798a* (WTU), *Relbunium,* H, N, B, HM055795, HM055834, HM055873, HM061011; ***G. obtusum*** Bigelow, U.S.A., Texas, *D.J. Rosen 2759* (TEX), *Aparinoides,* H, N, N, HM055796, HM055835, HM055874, HM061071; ***G. oreganum*** Britton, U.S.A., Oregon, *V. Soza 1761* (WTU), *Platygalium,* H, H, N, GU357234, GU357362, GU357104, HM061058; ***G. oresbium*** Greenm., Mexico, Coahuila, *Henrickson 20543* (TEX), *Lophogalium,* D, L, N, HM055797, HM055836, HM055875, HM061007; ***G. parishii*** Hilend & J.T. Howell, U.S.A., California, *V. Soza 1736* (WTU), *Lophogalium,* P, L, N, GU357192, GU357320, GU357062, HM060984; ***G. pendulum*** Greenm., Mexico, Hidalgo, *P. Tenorio L. & C. Romero de T. 547* (MO), *Platygalium,* H, H, N, HM055798, HM055837, HM055876, HM061004; ***G. pilosum*** Aiton, U.S.A., Arizona, *V. Soza & Y. Yaowu 1779* (WTU), *Platygalium,* H, H, N, GU357200, GU357328, GU357070, HM061006; ***G. porrigens*** Dempster, U.S.A., California, *N. Fraga 1281b* (RSA), *Baccogalium,* D, N, B, GU357214, GU357342, GU357084, HM061053; ***G. proliferum*** A. Gray, U.S.A., Texas, *B.L. Turner 24-155* (TEX),----, H, H, N, GU357196, GU357324, GU357066, HM060989; ***G. richardianum*** Endl. ex Walp., Argentina, Corrientes, *V. Soza, J.T. Columbus, & G. Ocampo 1799* (WTU), *Relbunium,* P, N or P, B, GU357207, GU357335, GU357077, HM061019; ***G. rotundifolium*** L., Spain, Huesca, *Montserrat et al. s.n.* (JACA), *Platygalium,* H, H, N, GU357230, GU357358, GU357100, HM061059; ***G. rubioides*** L., Switzerland, Geneva Botanical Garden, *A. Natali & J.F. Manen 013* (G), *Platygalium,* H, N, N, GU357236, GU357364, GU357106, HM061063; ***G. rzedowskii*** Dempster, Mexico, Nuevo Leon, *T.F. Patterson 6149* (TEX), *Platygalium,* H, H, N, HM055799, HM055838, HM055877, HM061003; ***G. scabrum*** L., Portugal, Madeira, *Catalan & Sequeira s.n.* (MS), *Platygalium,* H, H, N, GU357231, GU357359, GU357101, HM061060; ***G. seatonii*** Greenm., Mexico, Puebla, *J. Pacheco 22* (MEXU), *Platygalium,* H, H, N, HM055800, HM055839, HM055878, HM061002; ***G. serpenticum*** Dempster **subsp. *okanoganense*** Dempster & Ehrend., U.S.A., Washington, *V. Soza, J.K. Combs, R.G. Olmstead & D.C. Tank 1718* (WTU), *Lophogalium,* D, L, N, HM055801, HM055840, HM055879, HM061029; ***G. serpenticum*** Dempster **subsp. *warnerense*** Dempster & Ehrend., U.S.A., Oregon, *V. Soza 1754a* (WTU), *Lophogalium,* D, L, N, HM055802, HM055841, HM055880, HM061028; ***G. sparsiflorum*** W. Wight, U.S.A., California,

R. Gankin 835 (WTU), *Baccogalium,* D, N, B, HM055803, HM055842, HM055881, HM061054; ***G. stellatum*** Kellogg, U.S.A., Arizona, *Messinger 274* (WTU), *Lophogalium,* D, L, N, GU357229, GU357357, GU357099, HM061045; ***G. texense*** A. Gray, U.S.A., Texas, *W. R. Carr 22860* (TEX),----, H, H, N, GU357198, GU357326, GU357068, HM060991; ***G. thunbergianum*** Eckl. & Zeyh., Africa, Kenya, *Luke 8876* (SBT), *Platygalium,* H, H, N, HM055804, HM055843, HM055882, HM061061; ***G. uncinulatum*** DC., U.S.A., Texas, *V. Soza & Y. Yaowu 1780* (WTU), *Platygalium,* H, H, N, GU357211, GU357339, GU357081, HM060999; ***G. uniflorum*** Michx., U.S.A., Texas, *S.L. Orzell & E.L. Bridges 10853* (TEX), *Bataprine,* H, N or P, B, HM055805, HM055844, HM055883, HM060995; ***G. virgatum*** Nutt. ex Torr. & A. Gray, U.S.A., Texas, *W.R. Carr 22289* (TEX),----, H, H, N, GU357197, GU357325, GU357067, HM060990; ***G. volcanense*** Dempster, Mexico, Baja California, *V. Soza, R. Arce, S. Avila Moreno, & Y. Yaowu 1786* (WTU), *Lophogalium,* P, L, N, GU357193, GU357321, GU357063, HM060985; ***G. wigginsii*** Dempster, Mexico, Baja California, *V. Soza, S. Avila Moreno, & Y. Yaowu 1790* (WTU), *Lophogalium,* P, L, N, HM055806, HM055845, HM055884, HM060986; ***G. wrightii*** A. Gray, U.S.A., Arizona, *Luckow et al. 2762* (WTU), *Lophogalium,* P, L, N, GU357191, GU357319, GU357061, HM060983; ***Valantia muralis*** L., France, Corsica, *D. Jeanmonod & A. Natali s.n.* (G),----, A, N, N, GU357241, GU357369, GU357111, HM061070.

Appendix C. Tested alternative topologies of the *Cruciata-Galium-Valantia* clade, showing polygamy as intermediate between hermaphroditism and dioecy.

Tree number, description, (topology: A. = *Asperula*, C. = *Cruciata*, G. = *Galium*, V. = *Valantia*).

Tree 1, polygamous taxon constrained as sister to remaining dioecious species in clade 1, and polygamous taxon constrained as sister to remaining dioecious species in clades 2—3,
((((((((((((((((((*G.dempsterae,G.pendulum*),*G.uncinulatum*),*G.seatonii*),*G.rzedowski i*),*G.hystricocarpum*),*G.juniperinum*),*G.carmenicola*),(((*G.pilosum,G.oresbium*),*G.hint oniorum*),*G.correllii*)),(((((*G.wrightii*,*G.wigginsii*),*G.parishii*),(*G.collomiae,G.volcanens e*)),((*G.moranii*.subsp.*aculeolatum,G.fendleri*),*G.carterae*)),*G.aschenbornii*)),((*G.proli ferum,G.virgatum*),*G.texense*)),(((((((*G.corymbosum,G.corymbosum*),*G.hypocarpium*),*G.noxium*.subsp.*valantioides*),(((*G.hirtum,G.bigeminum*),*G.megapotamicum*),*G.nigr oramosum*)),((*G.microphyllum,G.richardianum*),*G.latoramosum*)),(*G.gracilicaule,G.lill oi*)),(*G.gilliesii*.subsp.*gilliesii,G.gilliesii*.subsp.*telanthos*))),((((*G.arkansanum,G.latifoliu m*),*G.circaezans*),*G.uniflorum*),*G.bermudense*)),((((((((((*G.hypotrichium.subsp.tome ntellum,G.hypotrichium.subsp.inyoense*),*G.argense*),*G.matthewsii*),(*G.multiflorum,G. glabrescens*.subsp.*modocense*)),*G.coloradoense*),(*G.munzii*.subsp.*munzii,G.hilendi ae*.subsp.*carneum*)),(*G.grayanum,G.serpenticum*.subsp.*okanoganense*)),*G.serpenti cum*.subsp.*warnerense*),*G.glabrescens*.subsp.*glabrescens*),(((((((*G.angustifolium*.s ubsp.*angustifolium*.c1,*G.angustifolium*.subsp.*angustifolium*.c2),*G.angustifolium*.subs p.*borregoense*),*G.angustifolium*.subsp.*jacinticum*),(*G.angustifolium*.subsp.*gabrielens e*,*G.angustifolium*.subsp.*nudicaule*)),*G.jepsonii*),*G.hallii*),*G.johnstonii*),*G.stellatum*)), *G.catalinense*.subsp.*acrispum*),(*G.grande*,(((((((*G.californicum*.subsp.*flaccidum*.c1,*G .californicum*.subsp.*flaccidum*.c2),*G.californicum*.subsp.*flaccidum*.c3),*G.cliftonsmithii*),*G.martirense*),((*G.andrewsii*.subsp.*andrewsii*,*G.porrigens*),*G.sparsiflorum*)),(*G.amb iguum*.subsp.*siskiyouense*.c1,*G.ambiguum*.subsp.*siskiyouense*.c2)),*G.bolanderi*)))), *G.oreganum*),(*G.thunbergianum*,*G.kamtschaticum*)),(*G.rotundifolium,G.scabrum*)),((*G.boreale,G.rubioides*),*G.bailloni*)),((((*C.laevipes,C.taurica*),*C.glabra*),*C.pedemontan a*),*C.articulata*)),*V.muralis*),(((*A.orientalis,A.setosa*),*A.arvensis*),*A.taurina*)),*G.obtusu m*).

Tree 2, polygamous taxa of clades 8 and 9 constrained as intermediate between hermaphroditic and dioecious taxa within clades, polygamous taxon constrained as sister to remaining dioecious species in clade 1, and polygamous taxon constrained as sister to remaining dioecious species in clades 2—3,
((((((((((((((((((*G.dempsterae,G.oresbium*),(*G.carmenicola*,(*G.juniperinum,G.hystrico carpum*))),(*G.pendulum,G.uncinulatum*)),*G.seatonii*),*G.rzedowskii*),((*G.pilosum,G.hin toniorum*),*G.correllii*)),(((((*G.wrightii*,*G.wigginsii*),*G.parishii*),(*G.collomiae,G.volcanen se*)),((*G.moranii*.subsp.*aculeolatum*,*G.fendleri*),*G.carterae*)),*G.aschenbornii*)),((*G.pro liferum,G.virgatum*),*G.texense*)),(((((((*G.corymbosum,G.corymbosum*),*G.hypocarpiu m*),*G.noxium*.subsp.*valantioides*),((*G.hirtum,G.bigeminum*),*G.nigroramosum*)),(*G.mi

crophyllum,(*G.richardianum*,(*G.megapotamicum,G.latoramosum*))))),(*G.gracilicaule, G.lilloi*)),(*G.gilliesii*.subsp.*gilliesii*,*G.gilliesii*.subsp.*telanthos*))),((((*G.arkansanum,G.latifolium*),*G.circaezans*),*G.uniflorum*),*G.bermudense*)),((((((((((*G.hypotrichium*.subsp.*tomentellum*,*G.hypotrichium*.subsp.*inyoense*),*G.argense*),*G.matthewsii*),(*G.multiflorum*,*G.glabrescens*.subsp.*modocense*)),*G.coloradoense*),(*G.munzii*.subsp.*munzii*,*G.hilendiae*.subsp.*carneum*)),(*G.grayanum,G.serpenticum*.subsp.*okanoganense*)),*G.serpenticum*.subsp.*warnerense*),*G.glabrescens*.subsp.*glabrescens*),(((((((*G.angustifolium*.subsp.*angustifolium*.c1,*G.angustifolium*.subsp.*angustifolium*.c2),*G.angustifolium*.subsp.*borregoense*),*G.angustifolium*.subsp.*jacinticum*),(*G.angustifolium*.subsp.*gabrielense*,*G.angustifolium*.subsp.*nudicaule*)),*G.jepsonii*),*G.hallii*),*G.johnstonii*),*G.stellatum*)),*G.catalinense*.subsp.*acrispum*),(*G.grande*,(((((((*G.californicum*.subsp.*flaccidum*.c1,*G.californicum*.subsp.*flaccidum*.c2),*G.californicum*.subsp.*flaccidum*.c3),*G.cliftonsmithii*),*G.martirense*),((*G.andrewsii*.subsp.*andrewsii*,*G.porrigens*),*G.sparsiflorum*)),(*G.ambiguum*.subsp.*siskiyouense*.c1,*G.ambiguum*.subsp.*siskiyouense*.c2)),*G.bolanderi*)))),*G.oreganum*),(*G.thunbergianum*,*G.kamtschaticum*)),(*G.rotundifolium,G.scabrum*)),((*G.boreale,G.rubioides*),*G.bailloni*)),((((*C.laevipes,C.taurica*),*C.glabra*),*C.pedemontana*),*C.articulata*)),*V.muralis*),(((*A.orientalis,A.setosa*),*A.arvensis*),*A.taurina*)),*G.obtusum*).

Tree 3, polygamous taxa of clades 8 and 9 constrained as intermediate between hermaphroditic and dioecious taxa within clades, and polygamous taxa constrained as paraphyletic grade to dioecious species of clades 1—3,
((((((((((((((((((*G.dempsterae,G.oresbium*),(*G.carmenicola*,(*G.juniperinum,G.hystricocarpum*))),(*G.pendulum,G.uncinulatum*)),*G.seatonii*),*G.rzedowskii*),((*G.pilosum,G.hintoniorum*),*G.correllii*)),(((((*G.wrightii,G.wigginsii*),*G.parishii*),(*G.collomiae,G.volcanense*)),((*G.moranii*.subsp.*aculeolatum,G.fendleri*),*G.carterae*)),*G.aschenbornii*)),((*G.proliferum,G.virgatum*),*G.texense*)),(((((((*G.corymbosum,G.corymbosum*),*G.hypocarpium*),*G.noxium*.subsp.*valantioides*),((*G.hirtum,G.bigeminum*),*G.nigroramosum*)),(*G.microphyllum*,(*G.richardianum*,(*G.megapotamicum,G.latoramosum*))))),(*G.gracilicaule, G.lilloi*)),(*G.gilliesii*.subsp.*gilliesii,G.gilliesii*.subsp.*telanthos*))),((((*G.arkansanum,G.latifolium*),*G.circaezans*),*G.uniflorum*),*G.bermudense*)),(*G.grande*,(*G.catalinense*.subsp.*acrispum*,((((((((((*G.hypotrichium*.subsp.*tomentellum,G.hypotrichium*.subsp.*inyoense*),*G.argense*),*G.matthewsii*),(*G.multiflorum,G.glabrescens*.subsp.*modocense*)),*G.coloradoense*),(*G.munzii*.subsp.*munzii,G.hilendiae*.subsp.*carneum*)),(*G.grayanum,G.serpenticum*.subsp.*okanoganense*)),*G.serpenticum*.subsp.*warnerense*),*G.glabrescens*.subsp.*glabrescens*),(((((((*G.angustifolium*.subsp.*angustifolium*.c1,*G.angustifolium*.subsp.*angustifolium*.c2),*G.angustifolium*.subsp.*borregoense*),*G.angustifolium*.subsp.*jacinticum*),(*G.angustifolium*.subsp.*gabrielense*,*G.angustifolium*.subsp.*nudicaule*)),*G.jepsonii*),*G.hallii*),*G.johnstonii*),*G.stellatum*)),(((((((*G.californicum*.subsp.*flaccidum*.c1,*G.californicum*.subsp.*flaccidum*.c2),*G.californicum*.subsp.*flaccidum*.c3),*G.cliftonsmithii*),*G.martirense*),((*G.andrewsii*.subsp.*andrewsii,G.porrigens*),*G.sparsiflorum*)),(*G.ambiguum*.subsp.*siskiyouense*.c1,*G.ambiguum*.subsp.*siskiyouense*.c2)),*G.bolanderi*)))),*G.oreganum*),(*G.thunbergianum,G.kamtschaticum*)),(*G.rotundifolium,G.scabrum*)),((*G.boreale,G.rubioides*),*G.bailloni*)),((((*C.laevipes,C.taurica*),*C.glabra*),*C.pedemont*

ana),*C.articulata*)),*V.muralis*),(((*A.orientalis,A.setosa*),*A.arvensis*),*A.taurina*)),*G.obtusum*).

Tree 4, polygamous taxa of clades 8 and 9 constrained as intermediate between hermaphroditic and dioecious taxa within clades,
((((((((((((((((((*G.dempsterae,G.oresbium*),(*G.carmenicola*,(*G.juniperinum,G.hystricocarpum*))),(*G.pendulum,G.uncinulatum*)),*G.seatonii*),*G.rzedowskii*),((*G.pilosum,G.hintoniorum*),*G.correllii*)),(((((*G.wrightii,G.wigginsii*),*G.parishii*),(*G.collomiae,G.volcanense*)),((*G.moranii*.subsp.*aculeolatum*,*G.fendleri*),*G.carterae*)),*G.aschenbornii*)),((*G.proliferum,G.virgatum*),*G.texense*)),(((((((*G.corymbosum,G.corymbosum*),*G.hypocarpium*),*G.noxium*.subsp.*valantioides*),((*G.hirtum,G.bigeminum*),*G.nigroramosum*)),(*G.microphyllum*,(*G.richardianum*,(*G.megapotamicum*,*G.latoramosum*)))),(*G.gracilicaule,G.lilloi*)),(*G.gilliesii*.subsp.*gilliesii,G.gilliesii*.subsp.*telanthos*))),((((*G.arkansanum,G.latifolium*),*G.circaezans*),*G.uniflorum*),*G.bermudense*)),((((((((((*G.hypotrichium*.subsp.*tomentellum,G.hypotrichium*.subsp.*inyoense*),*G.argense*),*G.matthewsii*),(*G.multiflorum,G.glabrescens*.subsp.*modocense*)),*G.coloradoense*),(*G.munzii*.subsp.*munzii,G.hilendiae*.subsp.*carneum*)),(*G.grayanum,G.serpenticum*.subsp.*okanoganense*)),*G.serpenticum*.subsp.*warnerense*),*G.glabrescens*.subsp.*glabrescens*),(((((((((*G.angustifolium*.subsp.*angustifolium*.c1,*G.angustifolium*.subsp.*angustifolium*.c2),*G.angustifolium*.subsp.*borregoense*),*G.angustifolium*.subsp.*jacinticum*),(*G.angustifolium*.subsp.*gabrielense*,*G.angustifolium*.subsp.*nudicaule*)),*G.jepsonii*),*G.hallii*),*G.catalinense*.subsp.*acrispum*),*G.johnstonii*),*G.stellatum*)),(((((((*G.californicum*.subsp.*flaccidum*.c1,*G.californicum*.subsp.*flaccidum*.c2),*G.californicum*.subsp.*flaccidum*.c3),*G.cliftonsmithii*),(*G.grande,G.martirense*)),((*G.andrewsii*.subsp.*andrewsii,G.porrigens*),*G.sparsiflorum*)),(*G.ambiguum*.subsp.*siskiyouense*.c1,*G.ambiguum*.subsp.*siskiyouense*.c2)),*G.bolanderi*))),*G.oreganum*),(*G.thunbergianum*,*G.kamtschaticum*)),(*G.rotundifolium,G.scabrum*)),((*G.boreale,G.rubioides*),*G.bailloni*)),((((*C.laevipes,C.taurica*),*C.glabra*),*C.pedemontana*),*C.articulata*)),*V.muralis*),(((*A.orientalis,A.setosa*),*A.arvensis*),*A.taurina*)),*G.obtusum*).

Tree 5, polygamous taxa constrained as paraphyletic grade to dioecious species of clades 1—3,
((((((((((((((((((((*G.dempsterae,G.pendulum*),*G.uncinulatum*),*G.seatonii*),*G.rzedowskii*),*G.hystricocarpum*),*G.juniperinum*),*G.carmenicola*),(((*G.pilosum,G.oresbium*),*G.hintoniorum*),*G.correllii*)),(((((*G.wrightii,G.wigginsii*),*G.parishii*),(*G.collomiae,G.volcanense*)),((*G.moranii*.subsp.*aculeolatum,G.fendleri*),*G.carterae*)),*G.aschenbornii*)),((*G.proliferum,G.virgatum*),*G.texense*)),(((((((*G.corymbosum,G.corymbosum*),*G.hypocarpium*),*G.noxium*.subsp.*valantioides*),(((*G.hirtum,G.bigeminum*),*G.megapotamicum*),*G.nigroramosum*)),((*G.microphyllum,G.richardianum*),*G.latoramosum*)),(*G.gracilicaule,G.lilloi*)),(*G.gilliesii*.subsp.*gilliesii,G.gilliesii*.subsp.*telanthos*))),((((*G.arkansanum,G.latifolium*),*G.circaezans*),*G.uniflorum*),*G.bermudense*)),(*G.grande*,(*G.catalinense*.subsp.*acrispum*,((((((((((*G.hypotrichium*.subsp.*tomentellum,G.hypotrichium*.subsp.*inyoense*),*G.argense*),*G.matthewsii*),(*G.multiflorum,G.glabrescens*.subsp.*modocense*)),*G.coloradoense*),(*G.munzii*.subsp.*munzii,G.hilendiae*.subsp.*carneum*)),(*G.grayanum,G.serpe*

nticum.subsp.*okanoganense*)),*G.serpenticum*.subsp.*warnerense*),*G.glabrescens*.subsp.*glabrescens*),(((((((*G.angustifolium*.subsp.*angustifolium*.c1,*G.angustifolium*.subsp.*angustifolium*.c2),*G.angustifolium*.subsp.*borregoense*),*G.angustifolium*.subsp.*jacinticum*),(*G.angustifolium*.subsp.*gabrielense*,*G.angustifolium*.subsp.*nudicaule*)),*G.jepsonii*),*G.hallii*),*G.johnstonii*),*G.stellatum*)),(((((((*G.californicum*.subsp.*flaccidum*.c1,*G.californicum*.subsp.*flaccidum*.c2),*G.californicum*.subsp.*flaccidum*.c3),*G.cliftonsmithii*),*G.martirense*),((*G.andrewsii*.subsp.*andrewsii*,*G.porrigens*),*G.sparsiflorum*)),(*G.ambiguum*.subsp.*siskiyouense*.c1,*G.ambiguum*.subsp.*siskiyouense*.c2)),*G.bolanderi*))))),*G.oreganum*),(*G.thunbergianum,G.kamtschaticum*)),(*G.rotundifolium,G.scabrum*)),((*G.boreale,G.rubioides*),*G.bailloni*)),((((*C.laevipes,C.taurica*),*C.glabra*),*C.pedemontana*),*C.articulata*)),*V.muralis*),(((*A.orientalis,A.setosa*),*A.arvensis*),*A.taurina*)),*G.obtusum*).

Tree 6, Bayesian consensus tree,
((((((((((((((((((*G.dempsterae,G.pendulum*),*G.uncinulatum*),*G.seatonii*),*G.rzedowskii*),*G.hystricocarpum*),*G.juniperinum*),*G.carmenicola*),(((*G.pilosum,G.oresbium*),*G.hintoniorum*),*G.correllii*)),(((((*G.wrightii,G.wigginsii*),*G.parishii*),(*G.collomiae,G.volcanense*)),((*G.moranii.*subsp.*aculeolatum,G.fendleri*),*G.carterae*)),*G.aschenbornii*)),((*G.proliferum,G.virgatum*),*G.texense*)),(((((((*G.corymbosum,G.corymbosum*),*G.hypocarpium*),*G.noxium*.subsp.*valantioides*),(((*G.hirtum,G.bigeminum*),*G.megapotamicum*),*G.nigroramosum*)),((*G.microphyllum,G.richardianum*),*G.latoramosum*)),(*G.gracilicaule,G.lilloi*)),(*G.gilliesii*.subsp.*gilliesii,G.gilliesii*.subsp.*telanthos*))),((((*G.arkansanum,G.latifolium*),*G.circaezans*),*G.uniflorum*),*G.bermudense*)),((((((((((*G.hypotrichium*.subsp.*tomentellum*,*G.hypotrichium*.subsp.*inyoense*),*G.argense*),*G.matthewsii*),(*G.multiflorum,G.glabrescens*.subsp.*modocense*)),*G.coloradoense*),(*G.munzii*.subsp.*munzii,G.hilendiae*.subsp.*carneum*)),(*G.grayanum*,*G.serpenticum*.subsp.*okanoganense*)),*G.serpenticum*.subsp.*warnerense*),*G.glabrescens*.subsp.*glabrescens*),(((((((((*G.angustifolium*.subsp.*angustifolium*.c1,*G.angustifolium*.subsp.*angustifolium*.c2),*G.angustifolium*.subsp.*borregoense*),*G.angustifolium*.subsp.*jacinticum*),(*G.angustifolium*.subsp.*gabrielense*,*G.angustifolium*.subsp.*nudicaule*)),*G.jepsonii*),*G.hallii*),*G.catalinense*.subsp.*acrispum*),*G.johnstonii*),*G.stellatum*)),(((((((*G.californicum*.subsp.*flaccidum*.c1,*G.californicum*.subsp.*flaccidum*.c2),*G.californicum*.subsp.*flaccidum*.c3),*G.cliftonsmithii*),(*G.grande,G.martirense*)),((*G.andrewsii*.subsp.*andrewsii*,*G.porrigens*),*G.sparsiflorum*)),(*G.ambiguum*.subsp.*siskiyouense*.c1,*G.ambiguum*.subsp.*siskiyouense*.c2)),*G.bolanderi*))),*G.oreganum*),(*G.thunbergianum,G.kamtschaticum*)),(*G.rotundifolium,G.scabrum*)),((*G.boreale,G.rubioides*),*G.bailloni*)),((((*C.laevipes,C.taurica*),*C.glabra*),*C.pedemontana*),*C.articulata*)),*V.muralis*),(((*A.orientalis,A.setosa*),*A.arvensis*),*A.taurina*)),*G.obtusum*).

Appendix D. Voucher information, section, and GenBank accessions for taxa sampled in Chapter III.

Taxon, country, political subdivision, *collector & number* (herbarium), clade from Soza and Olmstead (2010b), *rpoB-trnC, trnc-psbM, trnL-trnF-ndhJ, RPB2*; NA = RPB2 accessions only, downloaded from GenBank.

Antirrhinum majus L., NA, DQ020637/DQ020642; ***Camellia japonica*** L., NA, AY566627/AY566628; ***Galium ambiguum*** W. Wight subsp. ***siskiyouense*** (Ferris) Dempster & Stebbins, U.S.A., Oregon, *V. Soza 1760* (WTU), clade 1, GU357216, GU357344, GU357086,----; ***G. andrewsii*** A. Gray, Mexico, Baja California, *V. Soza & Y. Yaowu 1792* (WTU), clade 1,----,----,----,----; ***G. andrewsii*** A. Gray subsp. ***intermedium*** Dempster & Stebbins, U.S.A., California, *V. Soza 1725b* (WTU), clade 1,----,----,----,----; ***G. angustifolium*** Nutt. ex Torr. & A. Gray subsp. ***angustifolium***, U.S.A., California, *S. Boyd 11551b* (RSA), clade 2, GU357218, GU357346, GU357088,----; ***G. aschenbornii*** S. Schauer, Mexico, Puebla, *J. Pacheco 21* (MEXU), clade 8, HM055774, HM055813, HM055852,----; ***G. bailloni*** Brandza, Romania, Arges, *F. Ehrendorfer 890821-3001* (WU), basal grade of *Platygalium p.p.,* GU357233, GU357361, GU357103,----; ***G. bolanderi*** A. Gray, U.S.A., Oregon, *V. Soza 1758a* (WTU), clade 1, GU357217, GU357345, GU357087,----; ***G. californicum*** Hook. & Arn. subsp. ***flaccidum*** (Greene) Dempster & Stebbins, U.S.A., California, *V. Soza 1731* (WTU), clade 1, HM055775, HM055814, HM055853,----; ***G. californicum*** Hook. & Arn. subsp. ***luciense*** Dempster & Stebbins, U.S.A., California, *V. Soza 1719* (WTU), clade 1,----,----,----,----; ***G. californicum*** Hook. & Arn. subsp. ***miguelense*** (Greene) Dempster & Stebbins, U.S.A., California, *S.A. Junak SR-605* (RSA), clade 1,----,----,----,----; ***G. californicum*** Hook. & Arn. subsp. ***primum*** Dempster & Stebbins, U.S.A., California, *N. Fraga 1279* (RSA), clade 1,----,----,----,----; ***G. clementis*** Eastw., U.S.A., California, *D.H. Wilken & E. Painter 16123* (RSA), clade 1,----,----,----,----; ***G. cliftonsmithii*** (Dempster) Dempster & Stebbins, U.S.A., California, *V. Soza 1720* (WTU), clade 1, HM055779, HM055818, HM055857,----; ***G. coloradoense*** W. Wight, U.S.A., New Mexico, *V. Soza & Y. Yaowu 1784* (WTU), clade 3, GU357226, GU357354, GU357096,----; ***G. correllii*** Dempster, U.S.A., Texas, *B.L. Turner 23-131* (TEX), clade 9, HM055780, HM055819, HM055858,----; ***G. grande*** McClatchie, U.S.A., California, *V. Soza 1733b* (WTU), clade 1, HM055786, HM055825, HM055864,----; ***G. hardhamae*** Dempster, U.S.A., California, *C.B. Hardham 5796* (WTU), clade 1,----,----,----,----; ***G. hypocarpium*** Endl. ex Griseb., Argentina, Corrientes, *V. Soza, J.T. Columbus, & G. Ocampo 1804* (WTU), clade 6, GU357205, GU357333, GU357075,----; ***G. martirense*** Dempster & Stebbins, Mexico, Baja California, *V. Soza, S. Avila Moreno, & Y. Yaowu 1788* (WTU), clade 1, GU357215, GU357343, GU357085,----; ***G. muricatum*** W. Wight, U.S.A., California, *L. Dempster 4098* (WTU), clade 1,----,----,----,----; ***G. nuttallii*** A. Gray subsp. ***insulare*** Ferris, U.S.A., California, *M.L. Hoefs, S.A. Junak, J. Takara, & M. Gay 2382* (RSA), clade 1,----,----,----,----; ***G. nuttallii*** A. Gray subsp. ***nuttallii***, U.S.A., California, *V. Soza 1036* (RSA), clade 1,----,----,----,----; ***G. porrigens*** Dempster, U.S.A., California, *N.*

Fraga 1281b (RSA), clade 1*,* GU357214, GU357342, GU357084,----; ***G. rotundifolium*** L., Spain, Huesca, *Montserrat et al. s.n.* (JACA), basal grade of *Platygalium p.p.,* GU357230, GU357358, GU357100,----; ***G. sparsiflorum*** W. Wight, U.S.A., California, *R. Gankin 835* (WTU), clade 1,----,----,----,----; ***Gardenia* sp**., NA, AJ558243/AJ566358; ***Linanthus californicus*** (Hook. & Arn.) J.M. Porter & L.A. Johnson, NA, DQ058636/DQ058637; ***Nicotiana sylvestris*** Speg., NA, DQ020636/DQ020640; ***Petunia* x *atkinsiana*** D. Don ex Loud., NA, DQ020638/DQ020641; ***Platanus orientalis*** L., NA, AY566618; ***Rhododendron macrophyllum*** D. Don ex G. Don, NA, DQ058627/DQ058628; ***Solanum lycopersicum*** L., NA, DQ020639.

CURRICULUM VITAE

VALERIE L. SOZA

EDUCATION

Ph.D., **Biology**, University of Washington, Seattle, WA, 12/2010

B.A., **Environmental Studies & Anthropology**, Pitzer College, Claremont, CA, 5/1997

RESEARCH EXPERIENCE

Christine Mirzayan Science & Technology Policy Fellow, Marian Koshland Science Museum, National Academy of Sciences, Washington, DC, 2010

Doctoral Student, University of Washington, Department of Biology, Seattle, WA, 2003-2010

Botanical Field Studies Coordinator & Herbarium Curatorial Assistant, Rancho Santa Ana Botanic Garden (RSABG), Claremont, CA, 1997-2003

Museum Scientist, Sweeney Granite Mountains Desert Research Center, University of California Natural Reserve System, Kelso, CA, 2000-2002

TEACHING EXPERIENCE

Guest Lecturer, Washington Native Plant Society & City of Bellevue, 2009 & 2010
- Native Plant Stewardship Program

Graduate Teaching Assistant, University of Washington, Department of Biology, 2003-2010
- Morphology & Anatomy of Land Plants
- Molecular, Cellular and Developmental Biology
- Plant Identification & Classification
- Introductory Biology

Predoctoral Lecturer, University of Washington, Department of Biology, 2006 & 2010
- Plant Identification & Classification

Guest Lecturer, University of Washington, Department of Biology, 2005-2007
- Plant Identification & Classification
- Evolution of Plant Development

AWARDS, DISTINCTIONS AND FELLOWSHIPS

Ingrith Deyrup-Olsen Distinguished Teaching Award, University of Washington, Department of Biology, 2010
Melinda Denton Writing Fellowship, University of Washington, Department of Biology, 2009
Graduate School Top Scholar Award, University of Washington Graduate School, 2008-2009
The Stroum Endowed Minority Dissertation Fellowship, University of Washington Graduate School, Graduate Opportunities & Minority Achievement Program, 2008
Graduate Student Research Award, Botanical Society of America, 2007
Graduate Student Travel Award, University of Washington, The Graduate School Fund for Excellence and Innovation, 2006 & 2008
Ford Foundation Predoctoral Diversity Fellowship Honorable Mention, National Research Council of the National Academies, 2005
Graduate Research Fellowship Honorable Mention, National Science Foundation, 2005
Graduate Student Research Grant, American Society of Plant Taxonomists, 2005
Giles Graduate Student Field Research Award, University of Washington, Department of Biology, 2004 & 2005
Molecular Phylogenetics and Systematics Graduate Fellowship, University of Washington, Department of Biology, 2003

PUBLICATIONS

Soza, V.L. and R.G. Olmstead. 2010. Evolution of breeding systems and fruits in New World *Galium* and relatives (Rubiaceae). *American Journal of Botany* 97 (10): 1630-1646.

Soza, V.L. and R.G. Olmstead. 2010. Molecular systematics of tribe Rubieae (Rubiaceae): evolution of major clades, development of leaf-like whorls, and biogeography. *Taxon* 59 (3): 755-771.

Soza, V., L. Gross, N. Fraga, and S. Boyd. (in prep). A vascular flora of the Verdugo Mountains, Los Angeles County, California. *Aliso.*

Soza, V. (in press). *Galium.* In: B.G. Baldwin, S. Boyd, B.J. Ertter, D.J. Keil, R.W. Patterson, T.J. Rosatti, & D. Wilken (eds.), *The Jepson Manual: Higher Plants of California*, 2nd edition. University of California Press, Berkeley.

Soza, V, S. Boyd and W.J. Brown, Jr. 2002. Species management guide for *Galium grande* McClatchie (Rubiaceae), San Gabriel bedstraw, Angeles National Forest. *Rancho Santa Ana Botanic Garden Occasional Publications* No. 6. 47 pp.

Soza, V., M.C. Provance, A.C. Sanders, and S. Boyd. 2000. Noteworthy collections of *Brickellia knappiana* (Asteraceae), *Brickellia multiflora* (Asteraceae), *Camissonia pterosperma* (Onagraceae), *Cornus glabrata* (Cornaceae), *Cynanchum utahense* (Asclepiadaceae), *Glyceria occidentalis* (Poaceae), and *Nicotiana acuminata* var. *multiflora* (Solanaceae) from California. *Madroño* 47 (2): 141-142.

Soza, V. 2000. Common garden study of morphological variation in Kusche's sandwort (*Arenaria macradenia* var. *kuschei*) (Caryophyllaceae), a rare plant of southern California. *Crossosoma* 26 (1): 7-11.

SELECTED PROFESSIONAL PRESENTATIONS

Soza, V. New insights from DNA studies of *Galium*, the bedstraws. Paper presentation. Washington Native Plant Society, Central Puget Sound Chapter, Washington. 2009.

Soza, V. and R. Olmstead. Systematics and evolution of sexual systems in a temperate, herbaceous tribe (Rubieae) in the coffee family (Rubiaceae). Paper presentation. Evolution 2009 Conference, Moscow, Idaho.

Soza, V. and R. Olmstead. Molecular phylogeny of tribe Rubieae, subfamily Rubioideae. Paper presentation. IV International Rubiaceae Conference, Jalapa, Veracruz, Mexico. 2008.

Soza, V. and R. Olmstead. Evolution of fleshy-fruited *Galium* (Rubiaceae) in western North America and management considerations for a number of rare taxa. Paper presentation. Botany 2008 Conference, Vancouver, B.C., Canada.

Soza, V., S. Boyd, L. Gross, and N. Fraga. The vascular flora of the Verdugo Mountains and San Raphael Hills, Los Angeles County, California. Poster presentation. Botany 2006 Conference, Chico, California.

Soza, V., S. Boyd, and A. C. Sanders. Phytogeographic "black holes" in southern California botany, a geographic information systems (GIS) model based on herbarium collections of two representative genera, *Camissonia* and *Salvia*. Poster presentation. Botany 2000 Conference, Portland, Oregon.

Soza, V. and S. Boyd. Common garden study of morphological variation in Kusche's sandwort (*Arenaria macradenia* var. *kuschei*), a rare plant of southern California. Paper presentation. Third Southwestern Rare and Endangered Plant Conference, Flagstaff, Arizona. 2000.

SERVICE ACTIVITIES

Greenhouse Docent, University of Washington, Department of Biology, 2007-2010.

Graduate Diversity Recruiter, University of Washington, The Graduate School, Graduate Opportunities & Minority Achievement Program, 2006-2010.

PROFESSIONAL AFFILIATIONS (Past & Present)

- American Society of Plant Taxonomists, Member
- Botanical Society of America, Member
- Northwest Scientific Association, Member
- Sigma Xi, Member
- Society of Systematic Biology, Member
- Southern California Botanists, Board of Directors
- Washington Native Plant Society, Member