EVOLUTION OF BREEDING SYSTEMS AND FRUITS IN NEW WORLD GAILUM AND RELATIVES (RUBIACEAE)1

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• Premise of the study: 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 traditionally defined by fruit morphology occur within this group. We tested the monophyly of sections defined by fruit morphology and sought to determine origins and pathways of breeding systems within this group.

• Methods: 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.

• Key results: 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 arisen at least twice from dioecy and at least six times from hermaphroditism.

• Conclusions: 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.

Key words: breeding systems; Cruciata; ETS; fruits; Galium; phylogeny; rpoB-trnC; trnC-psbM; trnl-ndkJ; Valantia.
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 determination 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, 2010).

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, 2010), 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 it has been characterized as dioecious and bearing fleshy fruits lacking specialized hairs (Fig. 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. 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 have hooked (Fig. 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. 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 1), 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 1; Manen et al., 1994; Natali et al., 1995, 1996; Soza and Olmstead, 2010). 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, 2010) 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, 2010).

**Molecular methods**—DNA samples were obtained from field-collected, silica gel-dried tissue; herbarium specimens; or other Rubiaceae researchers (Appendix 1). We extracted DNA using the 2% CTAB procedure (Doyle and Doyle, 1987). DNA from field-collected, silica gel-dried tissue was purified with 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 2× 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 trnCCA-R primers (Shaw et al., 2005). For DNA of lower quality, we amplified this region in two overlapping fragments using the c  and f primers (Taberlet et al., 1991) and ndhJ primers. The 3′ region in two overlapping fragments using the c  (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 primer and the 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 10× 30 mM MgCl2 reaction buffer, 2.5 μL 10× 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 H2O. 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 (Stratagene, 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 H2O. 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 min 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+” (5′-GTAAACACGAGCGCCAGTGAAT-3′) and “M13+” (5′-CAGGAAACAGCTATG-ACCATTG-3′; primers modified by K. Karol, New York Botanical Garden) in 20-μL volumes: 2.0 μL 10× 30 mM MgCl2 reaction buffer, 2.0 μL 10× Taq diluent, 2.0 μL dNTPs (10 mM), 1.2 μL each primer (5 μM), 0.1 μL Taq, and 11.5 μL H2O. 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). 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 2 μL 5× sequencing reagent premix, 0.25 μL primer (5 μM), 0.5–2.75 μL template, and 2 μL 5× sequencing reagent premix, 0.25 μL primer (5 μM), 0.5–2.75 μL template.

Table 1. Sections sampled among genera of the Cruciat-Galium-Valantia clade.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Section</th>
<th>No. taxa sampled</th>
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<tbody>
<tr>
<td>Cruciat Mill.</td>
<td>“Baccogalium” 5</td>
<td></td>
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<tr>
<td>Galium L.</td>
<td>Bataprine Nwd. 2</td>
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<td></td>
<td>Lophogalium K. Schum. 39</td>
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<tr>
<td></td>
<td>Platygalium W. Koch 16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Relbunium Endl. 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unassigned taxa 7</td>
<td></td>
</tr>
<tr>
<td>Valantia L.</td>
<td>Note: Classification follows Ehrendorfer et al. (2005) and F. Ehrendorfer (University of Vienna, personal communication).</td>
<td></td>
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</table>
remaining volume of H₂O. 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 weight 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. The alignment was ambiguous were excluded from the analyses. 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/TB2:10364).

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 Akaiake information criterion (Akaiake, 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 invariant sites and gamma shape parameter for among-site rate variation was an 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 30383 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 herbarium specimens (Appendix 1). Breeding systems were categorized as andromonecous, dioecious, hermaphrodite, or polygamous. Fruit types were categorized as nutlets (i.e., dry; Fig. 1C–F) or berries (i.e., fleshy; Fig. 1A, B, G, H). Fruit hairs were categorized as none (Fig. 1A, H), long (Fig. 1C, D), hooked (including curved; Fig. 1E, F), or pubescent (i.e., minute; Fig. 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.

Polytomous taxa are not allowed under likelihood reconstructions in Mesquite. Therefore, five taxa with both glabrous and pubescent fruits (Appendix 1) 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 2). 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-wide log-likelihoods for all six trees were obtained from PAUP* version 4.0b10 (Swoford, 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 test (Shimodaira, 2002). Topologies with P values less than 0.05 were rejected as candidate trees.

**RESULTS**

**Sampling and molecular results—** Summarized in Table 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 region.

**Phylogenetic results—** We examined the majority rule consensus trees from the cp and nr data sets (Appendixes S1–S2, see Supplemental Data at http://www.amjbot.org/cgi/content/full/ajb.100130/DC1) 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 data sets 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 data sets (Fig. 2), the CGV clade was found to contain two major clades that were strongly supported with pp = 1.00. One clade corresponded to Cruciatia 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). Two strongly supported groups are derived from this grade of sect. Platygalium: clades 1–3 and clades 4–9 (Fig. 2). 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. gracili-caule) and two unassigned species (G. latoramosum, G. liloi). 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 1), 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. 3). Likelihood reconstruction of ancestral breeding systems (−ln L = 68.979) reconstructed a hermaphroditic origin of the CGV clade with proportional ancestral breeding systems (−ln L = 68.979) reconstructed a hermaphroditic origin of the CGV clade with proportional ancestral breeding systems required 15 transformations and reconstructed an equivocal origin of the CGV clade as andromonoecious or hermaphroditic (Fig. 3).

Hermaphroditism in clade 9 (Fig. 3).

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. 4). Fruits with long-straight hairs have arisen at least five times in clades 2–3, 5–6, and 8–9 (Fig. 4). Fruits with hooked hairs have arisen at least four times in clades 2 and 4 and at the base of the Galium clade (Fig. 4). Pubescent fruits have arisen at least twice in clades 1 and 6 (Fig. 4).

Parimony 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. 4). 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. 4).

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 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) corresponds to Galium sect. Baccogalium, the fleshy-fruit 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-fruit 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). Monophyly has been confirmed for the fleshy-fruit group from Oregon to Baja California only.

Clade 2 (Fig. 2) 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,
Fig. 2  Phylogeny of Crucista-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.
Fig. 3. 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.
Fig. 4. 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 (C), and outgroup.
Table 4. Five candidate trees of evolution of polygamy in the Crucitata-Galium-Valantia clade and $P$ values from approximately unbiased test.

<table>
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<tr>
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Note: See Appendix 2 for tree form. $P$ values rounded off to 3 decimal places.

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) corresponds to 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) 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. 4). Ploidy levels are unknown for this group.

Clade 5 (Fig. 2) 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) 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. nigroramous 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, 2010). 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) 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. 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) 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. cartereae 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) 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. 4). 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). These results conflict with the Rubieae phylogeny based entirely on cpDNA sequences presented by Soza and Olmstead (2010), 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 (Appendix S1) shows a strongly supported CGV clade, but relations among Cruciata, Galium, and Valantia are not well supported. The ETS phylogeny (Appendix S2), 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. 3). 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; Wellera and Saeki, 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 long-tongued bees (Batra, 1984). This association of dioecy with small flowers pollinated by specialized insects has been found in other cases of dioecy as well (Bawa and Opler, 1975; Bawa, 1980; Ibarra-Manriquez 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. 3). However, Cruciata and Valantia may form a clade together, as prior studies have shown (Soza and Olmstead, 2010). 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. 3). All known cases in the genus occur in this clade of Galium (Soza and Olmstead, 2010). 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 we perform a weighted parsimony reconstruction of ancestral states, it is equally possible that dioecious G. latoramamas in clade 6 (Fig. 3) arose from a hermaphroditic 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. 3), 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. 4) 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 latu), with fleshy fruits inferred in the common ancestors of both groups.

We found in examining the evolution of fruit types and breeding systems in the CGV clade (Fig. 5), 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-Manriquez 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
Fig. 5. 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 (C), and outgroup.
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. 5).

Fruit hairs also are evolutionarily labile in this clade of Galium (Fig. 4). 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. 4). 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 (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. 4). 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).

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.

LITERATURE CITED


Soza and Olmstead — New World Galium


Appendix 2. Tested alternative clades of the Crucia-Galium-Valantioida clade, showing polygamy as intermediate between hermaphroditism and dioecy.
