FLAG FLOWER MORPHOLOGY AND PHYLOGENY OF HYDRANGEACEAE TRIBE HYDRANGEEAE

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of SARAH J. JACOBS find it satisfactory and recommend that it be accepted.

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Abstract

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This study examines the phylogeny of Hydrangeaceae tribe Hydrangeeae and the morphology of flag flowers that are characteristic of their inflorescences.

Phylogeny reconstructions tested previously hypothesized relationships in Hydrangeeae, a monophyletic group that consists of *Hydrangea* and allied genera. Phylogenetic analyses based on DNA sequences from nuclear ribosomal internal transcribed spacer regions and plastids indicated *Hydrangea* is paraphyletic to *Broussaisia, Decumaria, Dichroa, Pileostegia, Platycrater* and *Schizophragma* and possibly also to *Cardiandra* and *Deinanthe*. We recovered limited support for clades of Hydrangeeae that corresponded to groups circumscribed in earlier classifications.

We examined the morphology of flag flowers of Hydrangeeae that have floral dimorphism. A survey of herbarium specimens to describe variation in flag flowers across the tribe as well as histological studies revealed that all have enlarged calyces, most have a corolla, androecium and gynoecium, although structures of the latter two series are often aberrant. Reductions were common among flag flowers and these usually involved the loss of androecial

or gynoecial structures, although in taxa such as *Deinanthe* and *Schizophragma* the flag flowers consisted usually only of calyx.

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Dedication

This thesis is dedicated to Chris Bare for his enduring love, support and strength.

To my mother and father, I dedicate this thesis as a product of their inspiration and love.

To Courtney, Marlowe, Barb, Kara and Kendra, I dedicate this work as a thank you for enriching my life outside of graduate school.

Chapter One: A changing concept of *Hydrangea*: phylogeny of Hydrangeeae (Hydrangeaceae)

Abstract

Phylogenetic studies of *Hydrangea* have consistently indicated it is paraphyletic; however, those studies have sampled relatively few species and many clades had limited support. We tested further the monophyly of *Hydrangea* and the composition of its constituent clades by applying DNA sequence data from nuclear ribosomal ITS and plastid regions in phylogenetic analyses that expanded the sampling of species of *Hydrangea* and allied genera. Our results are consistent with earlier phylogenetic studies in recovering a strongly supported Hydrangeeae in which *Hydrangea* is clearly paraphyletic to *Broussaisia, Decumaria, Dichroa, Pileostegia, Platycrater* and *Schizophragma*. Placements of *Cardiandra* and *Deinanthe* as either the sister to the rest of the Hydrangeeae or nested in *Hydrangea* could not be rejected using the Shimodaira-Hasegawa test. Relationships among the deeper clades of *Hydrangea* received little support. Constituent clades of *Hydrangea*, including other genera, were largely consistent with those found in earlier phylogenetic studies.

Introduction

Since Linnaeus (1759) applied the name *Hydrangea* to the North American *H. arborescens* in *Species Plantarum*, the concept of the genus has expanded to include both Asian taxa, such as those first described as species of *Viburnum* (including *H. macrophylla* and *H. serrata*) by Thunberg (1784; see Wilson 1923), and Central and South American taxa, such as those first described as species of *Cornidia* by Ruiz and Pavon (1798). The delimitation of *Hydrangea* largely stabilized following Engler's (1891) treatment for *Die naturlichen Pflanzenfamilien*, and this concept of *Hydrangea* served as the basis of McClintock's (1957) revision of the genus, in which 23 species and 16 subspecies were recognized in two sections and eight subsections. The concept of *Hydrangea* advocated by Engler (1891, 1930) and McClintock pervaded most of the 20th century but was challenged by phylogenetic studies (Soltis et al. 1995, Hufford 1997, Hufford et al. 2001) that indicated *Broussaisia*, *Decumaria*, *Pileostegia*, *Platycrater*, *Schizophragma*, and possibly also *Cardiandra* and *Deinanthe* were nested among evolutionary lineages of *Hydrangea* species. Most of these genera had historically been considered closely related to *Hydrangea* because of morphological similarities (Engler 1928, Schulze-Menz 1964); however, they had been treated consistently as genera separate from *Hydrangea* based on distinctive floral, inflorescence, and/or seed characters.

Phylogenetic studies of *Hydrangea* have sampled relatively few species and the composition of clades has not been clear. The phylogenetic results have shown consistently that *Dichroa* is part of a clade that includes species of both *Hydrangea*

subsection *Macrophyllae* and subsection *Petalanthe* (Soltis et al. 1995, Hufford 1997, Hufford et al. 2001). Similarly, the phylogenetic results have consistently allied the Hawaiian endemic *Broussaisia* (Soltis et al. 1995, Hufford 1997, Hufford et al. 2001) with the clade consisting of *Macrophyllae* and *Petalanthe*. A clade, composed of *Decumaria, Pileostegia,* and *Schizophragma*, has received strong support in phylogenetic studies (Hufford 1995, Soltis et al. 1995, Hufford 1997, Hufford et al. 2001); however, a possible sister relationship of this clade to *Hydrangea quercifolia* has been weakly supported (Soltis et al. 1995, Hufford et al. 2001). Modest support has been found in phylogenetic studies for the placement of *Platycrater* among members of McClintock's (1957) subsection *Asperae* (Hufford 2001). Phylogenetic studies have consistently recovered a sister group relationship for *Cardiandra* and *Deinanthe*, although the placement of their clade as either the sister of *Hydrangea* s.l. (Hufford 1997, Hufford et al. 2001) or nested in *Hydrangea* s.l. (Soltis et al. 1995) has not been resolved.

Recent floristic exploration of China has also begun to alter our conception of the diversity of China. McClintock (1957), for example, recognized only 6 species of *Hydrangea* in China. In contrast, Wei (1994) recognized 46 and Wei and Bartholomew (2001) recognized 33, which calls attention to both the discovery of new diversity and differing opinions on species delimitation. Although many of the species recognized by Wei (1994) and Wei and Bartholomew (2001) were allied to sections and subsections included in McClintock (1957), their phylogenetic relationships have not been tested.

Our objective is to use molecular phylogenetics to test further the delimitation of *Hydrangea* and its constituent clades. We apply nuclear ribosomal and plastid DNA sequence data to an expanded sampling of *Hydrangea* species and allied genera to test the

following: 1) what is the least inclusive set of taxa encompassed by the monophyletic group that includes all of the species of *Hydrangea*? and 2) what are the constituent species of the clades of *Hydrangea*?

MATERIALS AND METHODS

Taxon sampling

We expanded taxon sampling of Hydrangeeae (sensu Hufford 2004) to incorporate more species than had been included in previous phylogenetic analyses. Tissue was sampled primarily from herbarium specimens or plants in botanical gardens (Table 1) if a source from a natural population could be identified. Outgroups were selected on the basis of earlier phylogenetic studies of the Hydrangeaceae (Soltis et al. 1995, Hufford et al. 2001) that placed Hydrangeeae as sister to Philadelpheae in subfamily Hydrangeoideae. Hydrangeoideae have been placed as the sister to Jamesioideae, a clade consisting only of *Fendlera* and *Jamesia*. Three outgroups were applied, including *Carpenteria californica* (Philadelpheae), *Fendlera rupicola* and *Jamesia americana* (both Jamesioideae). Because of amplification difficulties, not all taxa were sampled for each molecular marker (Table 1).

DNA Isolation and Sequencing

DNA sequences for ITS of nuclear ribosomal DNA, plastid spacer regions *accD-psa1* and *psbA-trnH* and an anonymous marker based on a microsatellite were generated for this study. The anonymous marker used in this study was based on a microsatellite developed by Rinehart et al. (2006) and corresponds with locus STAB 457. DNA

sequences for the plastid region *matK* were generated as part of this study or obtained from Hufford et al. (2001). For all accessions, total genomic DNA was isolated from approximately 10 mg silica-gel-dried or herbarium specimen leaf material using the CTAB procedure of Doyle and Doyle (1987). An ammonium acetate precipitation step was used in cases in which secondary compounds were potentially negatively affecting the quality of the DNA extraction. Both the ITS and anonymous marker were cloned to screen for possible paralogy (pGEM-T cloning kits, Promega Corp.).

Plastid and ITS PCR mixes consisted of a 25-μL sample containing 12.8 μL sterile water, 2.5 μL 10x Thermopol Reaction Buffer with 20mM Mg^{2+} (New England Biolabs, Ipswich, Massachusetts, USA), 2.5 μL 5' 5μM primer, 2.5 μL 3' 5μM primer (Table 2, including sequences for primers designed for this study), 1.5 μL 2.5 mM dNTP, 0.2 μL 5 U/μL Taq polymerase (New England Biolabs) and 2.0 μL diluted DNA template of unknown concentration. The anonymous marker PCR mixes consisted of a 15-μL sample containing 4.6 μL sterile water, 2.0 μL PCR 10x Thermopol Reaction Buffer with 20mM Mg^{2+} (New England Biolabs, Ipswich, Massachusetts, USA), 2.5 μL 25.0 mM $MgCl^{2+}$(New England Biolabs, Ipswich, Massachusetts, USA), 1 μL 5' 5μM primer, 1 μL 3' 5μM primer (Table 2), 1.6 μL 2.5 mM dNTP, 0.3 μL 5 U/μL Taq polymerase (New England Biolabs) and 2.0 μL diluted DNA template of unknown concentration. Reaction components were occasionally optimized on a case-by-case basis with changes in PCR protocol being made as one or more of the following: the amount of DNA template used, the addition of DMSO and/or the inclusion of additional Mg^{2+}.

PCR conditions for *accD-psa1* and *psbA-trnH* in a Biometra thermocycler (Whatman, Göttingen, Germany) included initial denaturation at 94˚ C for 5 min,

followed by 30 cycles at 94˚ C for 1 min, 55˚ C for 1 min and 72˚ C for 2 min, with a final extension at 72˚ C for 7 min. Optimized PCR conditions for *matK* included initial denaturation at 94˚ C for 5 min, followed by 35 cycles at 94˚ C for 1.5 min, 55.6˚ C for 2 min and 72˚ C for 3 min, with a final extension at 72˚ C for 15 min. PCR conditions for ITS included initial denaturation at 95˚ C for 2 min, followed by 35 cycles at 95˚ C for 1 min, 48˚ C for 1 min and 72˚ C for 1.5 min, with a final extension at 72˚ C for 10 min. Optimized PCR conditions for the anonymous marker included an initial denaturation at 95˚ C for 3 min, followed by two cycles at 95˚ C for 1 min and 60˚ C for 1 min. This was followed by twenty-seven cycles at 95˚ C for 30 sec, 60˚ C for 30 sec, 68˚ C for 30 sec, with a final extension at 68˚ C for 4 min. All PCR products were visualized by 1% agarose gel electrophoresis and purified using ExoSap-IT Procedure (USB Corp., Cleveland, Ohio, USA).

Sequencing reaction mixes of 10-µL contained between 3.23 and 6.23 µL ddH_2O, 0.67 µL 5 µM primer, 1.0 µL 5x sequencing buffer (Applied Biosystems, Foster City, California, USA), 1.0 µL BigDye Terminator v3.1 (Applied Biosystems) and between 1.0 and 4.0 µL clean PCR product. Cycle sequence reactions included 25 cycles of 96°C for 10 s and 50°C for 5 s, with a final extension at 60°C for 4 min. Cycle sequence products were purified using 75% isopropanol precipitation, and DNA sequences were visualized on a 48-capillary 3730 DNA Analyzer (Applied Biosystems). Sequences were assembled and edited using Sequencher version 4.6 (Gene Codes Corp., Ann Arbor, Michigan, USA). Alignments were generally unambiguous using Se-AL 2.0 (Rambaut, 2002).

Phylogenetic Analysis

Maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference

(BI) analyses were performed for a combined plastid dataset (herein called the plastid dataset), ITS data alone, the anonymous marker alone, and the combined ITS, plastid and anonymous marker data (herein called the combined dataset). The plastid and combined datasets included all taxa sampled, regardless of missing data within the individual datasets. The Partition Homogeneity Test, implemented in PAUP* v4.0b10 (Swofford 2002), was used to examine potential incongruence of phylogenetic signal among the data sets (Table 3).

Maximum parsimony analyses were performed with gaps treated as missing data. Heuristic searches were performed using random taxon addition and tree-bisection-reconnection (TBR) branch swapping. One thousand replicates were conducted in order to search for islands of equally most parsimonious trees. For analyses that did not run to completion, MaxTrees was set to 50,000.

For maximum likelihood analyses, Modeltest v 3.7 (Posada and Crandall 1998), was used to determine appropriate models of DNA substitution for our data. We used both the AIC and hLRT criterion to estimate appropriate models of molecular evolution; however, we applied only those models selected by AIC as the log likelihoods (-lnL) of these estimations were better than (or sometimes equal to) those based on the hLRT criterion (Table 4). These models of evolution were implemented in ML and BI searches of individual and combined datasets as well as in alternative phylogenetic hypothesis testing. Heuristic searches were performed using random taxon addition and branch swapping performed by tree-bisection-reconnection (TBR). One thousand replicates were conducted. For searches that did not run to completion, MaxTrees was set to 1,000.

Clade robustness of MP and ML topologies was assessed using nonparametric

bootstrap analysis (Felsenstein 1985) implemented in PAUP* v 4.0b10 (Swofford 2002). One thousand maximum likelihood and maximum parsimony bootstrap replicates were conducted. Starting trees for each replicate were obtained using random taxon addition and branch swapping was performed using tree-bisection-reconnection (TBR).

Bayesian analyses were conducted in Mr.Bayes v 3.1 (Huelsenbeck and Ronquist 2001). The DNA substitution models described above (Table 4) were applied in analyses; model parameters were estimated in Mr.Bayes (Hulsenbeck and Ronquist 2001). Analyses were initiated using random starting trees and run for a minimum of 2,000,000 generations using two sets of four Markov Chains (three heated, one cold) that were sampled every 1,000 generations. The number of trees required to reach stationarity was estimated using Tracer v 1.4.1 (Rambaut et al. 2008) to visualize the burn-in. The first approximate 25% of trees were discarded as burn-in and excluded from the tree set. Posterior probabilities at each node were estimated by creating a 50% majority rule consensus tree calculated from the remaining trees.

Alternative Phylogenetic Hypotheses

Alternative hypotheses of relationships among members of the Hydrangeeae were examined by constructing topologies that modeled alternative relationships. In order to test whether 1) *Hydrangea* is monophyletic and 2) *Cardiandra* and *Deinanthe* are sister to the *Hydrangea* clade, we searched for the best topologies under these constraints in phylogenetic analyses using ML under the same search parameters mentioned above. Additionally, we estimated log likelihood scores for a pool of most parsimonious trees. Utilizing the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa 1999) as implemented in PAUP 2.0, with the resampling-estimated log-likelihood (RELL)

optimization, log likelihood scores from a pool of MP trees, the unconstrained ML trees and the constrained ML tree were compared.

Results

Phylogenetic data

The lengths and variation of the aligned DNA sequence data for Hydrangeeae are summarized in Table 3. The *psbA-trnH, accD-psa1,* and ITS alignments had numerous indels. Ambiguous regions, composing almost 28% of the total aligned sequences, especially in *accD-psa1*, *psbA-trnH* and ITS data, were removed prior to analyses. Cloning did not reveal any intraspecific ITS polymorphism or paralogy in the anonymous marker.

Plastid data regions

Maximum likelihood analyses of the plastid dataset resulted in a single most likely tree (Table 3, tree not shown) that was similar to the topologies obtained using MP and BI (Table 3, trees not shown). Differences in reconstructions among the analyses were centered at deeper nodes in the tree as well as in the placement of *Broussaisia arguta*, *H. arborescens*, and *H. quercifolia*.

Nuclear and anonymous marker data regions

ML analyses of neither ITS nor the anonymous marker ran to completion and the 1,000 best trees for each marker had log likelihood scores of 3597.9423 and 547.06967, respectively (Table 3). The strict consensus trees for both ITS and the anonymous marker based on ML, MP, and BI were unresolved polytomies (not shown).

Combined data

The Partition Homogeneity Test revealed the *accD-psa1* data region was statistically incongruent with all other data regions and *matK* was statistically incongruent with the anonymous marker data region (Table 4). Additionally, the plastid dataset was found to be statistically incongruent with the ITS dataset but not with the anonymous marker dataset. We note, however, that provisional phylogenetic analyses of individual markers resulted largely in unresolved trees; thus, the sources of incongruent signal may be largely random variation in markers that have low phylogenetic signals. In order to examine whether for consistent phylogenetic signals among the data regions, we combined the markers for phylogenetic analysis.

MP analysis of the combined dataset resulted in 302 most parsimonious trees of 1375 steps (Fig. 1, Table 3). ML analyses of the combined dataset resulted in two most likely trees with a log likelihood score of 11925.95445 (Fig. 2, Table 3). BI analyses converged on similar log likelihood values after about 2,000 generations and resulted in a topology (Fig. 3) that differed from the ML results only in the placements of *Broussaisia arguta*, *Hydrangea quercifolia*, and *H. zhewanensis* as well as in the relationships found in subsection *Asperae*.

All three analyses recovered a strongly supported Hydrangeeae that included *Broussaisia*, *Cardiandra*, *Decumaria*, *Deinanthe*, *Dichroa*, *Pileostegia*, *Platycrater* and *Schizophragma* nested among or in a basal polytomy with species of *Hydrangea* (Fig. 1: MPbs = 100; Fig. 2: MLbs = 100, pp = 100%; Fig. 3, pp = 100%). The placement of *B. arguta*, *H. arborescens*, *H. quercifolia* and *H. zhewanensis* were not resolved in the MP

tree (Fig. 1), and in the ML and BI analyses their placements differed. *Broussaisia arguta* was placed as sister to the *Petalanthe* in the ML topology (Fig. 2) and as sister to the *Schizophragma* clade in the BI topology (Fig. 3). *Hydrangea arborescens* and *H. quercifolia* were allied to the *Schizophragma*, *Calyptranthe* and *Asperae* clades in ML and BI topologies; however, BI analyses further resolved the placement of *H. quercifolia* as sister to the *Schizophragma* clade (Fig. 3). *Hydrangea zhewanensis* was placed as sister to the *Schizophragma* clade in ML topologies (Fig. 2) and sister to the *Heteromallae* in BI topologies (Fig. 3). Finally, the BI reconstruction of the *Asperae* differed from the ML reconstructions in the placement of the sister taxa *H. strigosa* and *H. coacta* (Figs. 2, 3), although without robust support for either alternative. *Petalanthe* were strongly supported (MLbs = 100; pp = 100%); however, their placement as sister to the rest of the Hydrangeeae had limited support (Fig. 2). The sister relationship of *Cardiandra* and *Deinanthe* received strong support (MLbs = 98; pp = 100%), and their clade was placed among species of *Hydrangea*, although with limited (MLbs = < 50%) support. The *Heteromallae* (MLbs = 98; pp = 100%) consisted of *H. heteromalla*, *H. bretschneideri*, *H. paniculata*, and *H. xanthoneura*. The *Schizophragma* clade (MLbs = 97; pp 100%) included *Decumaria*, *Pileostegia*, and *Schizophragma*. A sister relationship between the *Schizophragma* clade and the *Asperae* + *Calyptranthe* was recovered, although, with weak support. The *Asperae* were strongly supported (MLbs = 90, pp = 100%) to include *H. sargentiana, H. aspera, H. longipes, H. caudatifolia, H. strigosa, H. coacta, H. sikokiana, H. involucrata* and *Platycrater arguta*. The *Calyptranthe* was moderately supported (MLbs = 66, pp = 97%) and includes *H. peruviana, H. asterolasia, H. serratifolia, H. seemannii, H. integrifolia, H. anomala* and *H. petiolaris*. The sister

relationship of the *Calyptranthe* and *Asperae* clades were moderately supported (MLbs = 72, pp = 100%).

Alternative Phylogenetic Hypotheses.

An SH test indicated that the best topology constrained to have a monophyletic *Hydrangea* was significantly worse than our best ML tree, in which *Hydrangea* was paraphyletic ($P = < 0.05$). An SH test indicated that the best topology constrained to place the *Cardiandra* + *Deinanthe* clade as the sister to the rest of the Hydrangeeae was not significantly worse that our best ML trees ($P = 0.768$).

Discussion

Hydrangea paraphyly

Our results are consistent with earlier phylogenetic studies that found *Broussaisia, Decumaria, Pileostegia, Platycrater* and *Schizophragma* nested in a paraphyletic *Hydrangea* (Soltis et al. 1995, Hufford 1997, Hufford et al. 2001). Earlier phylogenetic studies differed in the placement of *Cardiandra* and *Deinanthe* relative to *Hydrangea* species, finding them as a monophyletic group placed either among species of *Hydrangea* (Soltis et al. 1995) or as the sister of all other Hydrangeeae (Hufford et al. 2001, Hufford 1997). Although our best trees placed *Cardiandra* and *Deinanthe* as a clade nested among species of *Hydrangea*, the SH test could not reject an alternative topology in which *Cardiandra* and *Deinanthe* were placed as the sister of all other Hydrangeeae. Thus, additional data will be needed to test further the placement of *Cardiandra* and *Deinanthe*. Our results, however, do provide strong support for the monophyly of

Cardiandra, Deinanthe, and *Hydrangea* s.l., which includes also *Broussaisia, Decumaria, Dichroa, Pileostegia, Platycrater* and *Schizophragma*.

***Hydrangea* sections**

Many classifications of *Hydranea* have been proposed, several of which are reviewed in Table 6. The most recent revision of *Hydrangea* by McClintock (1957), following the classifications of Engler (1891, 1930), treated the genus as consisting of two sections: *Hydrangea* and *Cornidia*. The latter was based on the genus *Cornidia* described by Ruiz and Pavon (1798) and consisted of hydrangeas centered in Mexico, Central- and South America and *H. integrifolia* from Taiwan. Our results, like those of Soltis et al. (1995), Hufford (1997), and Hufford et al. (2001), found a monophyletic *Cornidia* sensu McClintock (1957) nested within section *Hydrangea* as the sister of *H. anomala* and *H. petiolaris*. We recommend that *Cornidia* be treated in the future at the same rank as its sister clade, which was McClintock's subsection *Calyptranthe*.

Major clades

The name *Calyptranthe*, applied first by Macimowicz (1867), refers to the calyptrate corolla of *H. anomala*, which has petals that are postgenitally fused laterally and abscise as a unit (Hufford 2001). McClintock recognized two subspecies of *H. anomala*, which differed in stamen number and distribution, as composing *Calyptranthe*. Others have treated *H. anomala* and *H. petiolaris* as separate species (e.g., Zoku 1965).

Our results recovered support for the sister group relationship of the *Calyptranthe-Cornidia* clade with *Asperae* (Fig. 2) as was also found previously by Soltis et al. (1995), Hufford (1997), and Hufford et al. (2001). Rehder (1911) circumscribed *Hydrangea* subsection *Asperae* to include *H. aspera, H. sikokiana* and *H. involucrata*,

and this treatment was followed by McClintock (1957). Three subspecies that McClintock (1957) recognized as part of *H. aspera* were elevated by Wei (1994) and Wei and Bartholomew (2001) to species status as *H. robusta*, *H. sargentiana* and *H. strigosa* (Table 5). Our results recovered *H. aspera, H. sikokiana*, *H. involucrata*, and *H. sargentiana* (*H. robusta* and *H. strigosa* were not sampled) as well as *H. longipes*, *H. caudatifolia*, *H. coacta* and *Platycrater arguta* in a monophyletic *Asperae*. *Hydrangea longipes* was recognized by Wei and Bartholomew (2001) but treated as a synonym of *H. aspera* subspecies *robusta* by McClintock (1957). Bartholomew, in Wei and Bartholomew (2001), considered *H. caudatifolia* to be conspecific with *H. chungii*; however, our results placed the later among *Petalanthe*.

We found strong support for the placement of *Platycrater arguta* among the *Asperae*, although it differs from species of *Hydrangea* in having flag flowers that have synorganized calyces, fewer and larger flowers in its inflorescences, and flowers that have numerous stamens and ovules (Hufford 2001). These morphological differences may have earlier obscured understanding the relationships of *Platycrater*, but we can now recognize its unique inflorescence and floral states as derived from an ancestor that had attributes much like those of extant *Hydrangea*.

Maximowicz (1867) described series *Petalanthe* for taxa with persistent, reflexed petals at anthesis. The *Petalanthe* originally consisted of both Asian and North American taxa. Rehder (1911) later moved the North American *Petalanthe* to the *Americanae*, for which it was named. McClintock's *Petalanthe* consisted of *H. hirta* and *H. scandens*. She recognized the latter as consisting of the following four subspecies: *chinensis*, *kwangtungensis*, *liukiuensis*, and *scandens* (Table 5). Subspecies *chinensis* and

kwangtungensis were elevated to species level by Wei (1994), and this was followed by Wei and Bartholomew (2001). *Hydrangea davidii* (recognized by McClintock as a synonym of *H. scandens*) has also been elevated to specific ranking by Wei (1994). McClintock (1957) removed *H. macrophylla* (and its subspecies *chungii*, *macrophylla*, *serrata* and *stylosa*) from the *Petalanthe* sensu Rehder (1911) and placed it in a novel subsection *Macrophyllae* (Table 5). Our results recovered McClintock's subsection *Macrophyllae* as paraphyletic to her subsection *Petalanthe*.

Hydrangea macrophylla subspecies *macrophylla*, *chungii* and *stylosa* sensu McClintock (1957) were recognized as separate species by Wei (1994) and/or Wei and Bartholomew (2001). We recovered strong support for a sister relationship between *H. stylosa* and *Dichroa febrifuga*. *Dichroa* was allied to *Hydrangea* by Engler (1928) and Schulze-Menz (1964), and recent phylogenetic work has consistently shown it to be nested in *Hydrangea* (Soltis et al. 1995, Hufford 1997, Hufford et al. 2001). *Dichroa* is similar to many other members of *Petalanthe* (except *H. macrophylla, H. stylosa*, and *H. chungii*) in having urceolate seeds (Hufford 1995, Hufford 1997). Our results find *H. chungii* well supported as sister to a clade that consists of two relatively recently described species, *H. linkweiensis* (Chun 1954) and *H. lingii* (Hoo 1951). Bartholomew (in Bartholomew and Wei 2001) suggested *H. linkweiensis* to be a variant of *H. chinensis*, but this is not consistent with our results (Fig. 2) that found *H. chinensis* to be more closely related to *H. scandens* and *H. luteovenosa* than to *H. linkweiensis*.

Broussaisia, an endemic of the Hawaiian Islands, and *Dichroa* are among the few Hydrangeaceae known to inhabit wet, tropical forests. A close relationship between *Broussaisia* and *Dichroa* has been suggested (Gray 1842, Forbes 1938), and the two

genera are unique among Hydrangeaceae in having fleshy fruits. *Broussaisia* has previously been allied with *H. macrophylla* in molecular and morphological phylogenetic studies (Soltis et al. 1995, Hufford 1995, Hufford 1997, Hufford et al. 2001). Although our ML results recovered a placement of *Broussaisia* as sister to *Petalanthe*, this was weakly supported, and our BI results recovered it as sister to the *Schizophragma* clade. Our phylograms place *Broussaisia* on a long branch (Fig. 4) relative to other clades of Hydrangeeae. *Broussaisia* appears to have experienced accelerated molecular evolution, which may be a consequence of founder effects faced in its colonization of Hawaii and/or the intense selection it faced in its evolution in a tropical forest, which included vegetative, floral, and fruit modifications.

Heteromallae were circumscribed first by Rehder (1911) to accommodate the Chinese species *H. paniculata* and *H. heteromalla*. McClintock (1957; Table 5) followed Rehder's treatment of the group. In a phylogenetic analysis based on morphological characters, Hufford (1997) had recovered *H. heteromalla* and *H. paniculata* as sister species. Our results recover *H. xanthoneura* + *H. bretschneideri* as the sister of *H. heteromalla*, and they, in turn, as the sister of *H. paniculata* (Fig. 2). Both *H. bretschneideri* (Dippel 1893) and *H. xanthoneura* (Diels 1900) were treated by McClintock (1957) as a part of *H. heteromalla*, but were elevated to specific ranking by Wei (1994; Table 5; see also Wei and Bartholomew 2001). Although our results found strong support for a monophyletic *Heteromallae*, consisting of *H. heteromalla*, *H. xanthoneura*, *H. bretschneideri*, and *H. paniculata*, we recovered little support for the placement of this clade.

Decumaria, Pileostegia, and *Schizophragma* (Fig. 1) have been allied consistently to *Hydrangea* (Engler 1928, Hutchinson 1927, Schulze-Menz 1964) and phylogenetic studies have previously supported both their monophyly and their placement in a paraphyletic *Hydrangea* (Hufford 1995, Soltis et al. 1995, Hufford 1997, Hufford et al. 2001). Our results placed *Decumaria, Pileostegia,* and *Schizophragma* in *Hydrangea*, although we did not find robust support for the sister group of the clade.

Conclusions

Our results are largely consistent with those of earlier phylogenetic studies and demonstrate the paraphyly of *Hydrangea* and the value of treating *Broussaisia, Cardiandra*, *Decumaria, Deinanthe*, *Pileostegia, Platycrater* and *Schizophragma* as congeneric with *Hydrangea*. We recovered several of the same deeper clades in *Hydrangea* s.l. that had been recovered by earlier phylogenetic studies, and these demonstrate that section and subsection delimitations in *Hydrangea* warrant revision. The relationships among the deeper clades of *Hydrangea* s.l. remain difficult to resolve. It is possible that *Hydrangea* s.l. experienced rapid diversification early in the history of the lineage, resulting in deep clades supported by few DNA sequence synapomorphies, and, if true, this may continue to confound phylogeny reconstructions.

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APPENDIX

Fig. 1. Strict consensus of 302 most parsimonious trees from the maximum parsimony analysis of data from the *accD-psa1*, *matK*, *psbA-trnH*, ITS, and the anonymous marker regions. Numbers above branches indicate maximum parsimony bootstrap proportions .

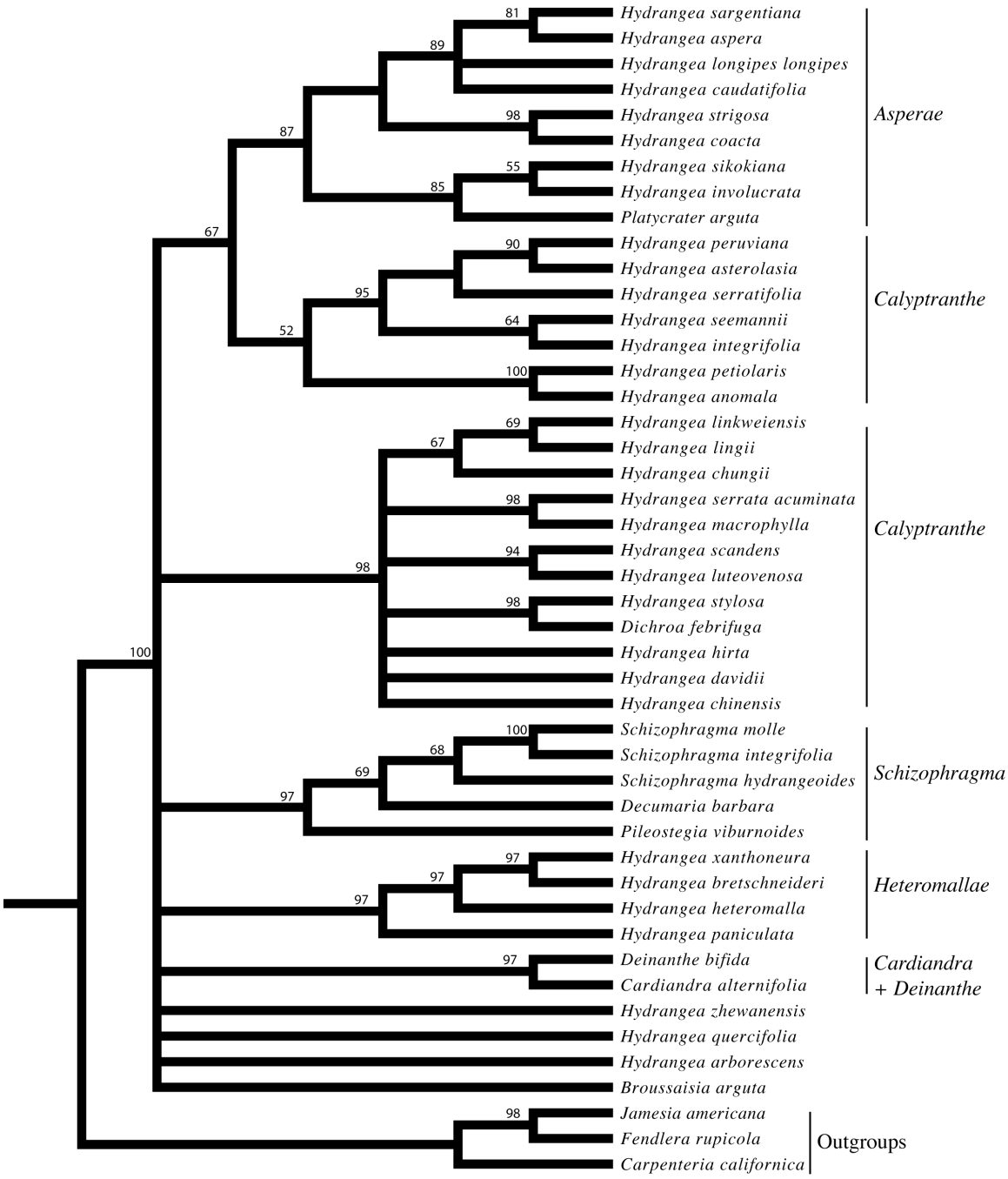
Hydrangea sargentiana
Hydrangea aspera
Hydrangea longipes longipes
Hydrangea caudatifolia
Hydrangea strigosa
Hydrangea coacta
Hydrangea sikokiana
Hydrangea involucrata
Platycrater arguta
Asperae
Hydrangea peruviana
Hydrangea asterolasia
Hydrangea serratifolia
Hydrangea seemannii
Hydrangea integrifolia
Hydrangea petiolaris
Hydrangea anomala
Calyptranthe
Hydrangea linkweiensis
Hydrangea lingii
Hydrangea chungii
Hydrangea serrata acuminata
Hydrangea macrophylla
Hydrangea scandens
Hydrangea luteovenosa
Hydrangea stylosa
Dichroa febrifuga
Hydrangea hirta
Hydrangea davidii
Hydrangea chinensis
Calyptranthe
Schizophragma molle
Schizophragma integrifolia
Schizophragma hydrangeoides
Decumaria barbara
Pileostegia viburnoides
Schizophragma
Hydrangea xanthoneura
Hydrangea bretschneideri
Hydrangea heteromalla
Hydrangea paniculata
Heteromallae
Deinanthe bifida
Cardiandra alternifolia
Cardiandra + Deinanthe
Hydrangea zhewanensis
Hydrangea quercifolia
Hydrangea arborescens
Broussaisia arguta
Jamesia americana
Fendlera rupicola
Carpenteria californica
Outgroups

Fig. 2. Strict consensus of two most likely trees from the maximum likelihood analysis of the combined data from *accD-psa1*, *matK*, *psbA-trnH*, ITS, and the anonymous marker regions. Numbers above branches are ML bootstrap values; numbers below branches are BI posterior probabilities. Bold taxon labels indicate non-*Hydrangea* genera recovered nested within the *Hydrangea* lineage. Colored branches and clade labels indicate affiliations based on McClintock (1957). Clades indicated under the Hydrangeeae heading are proposed following our results.

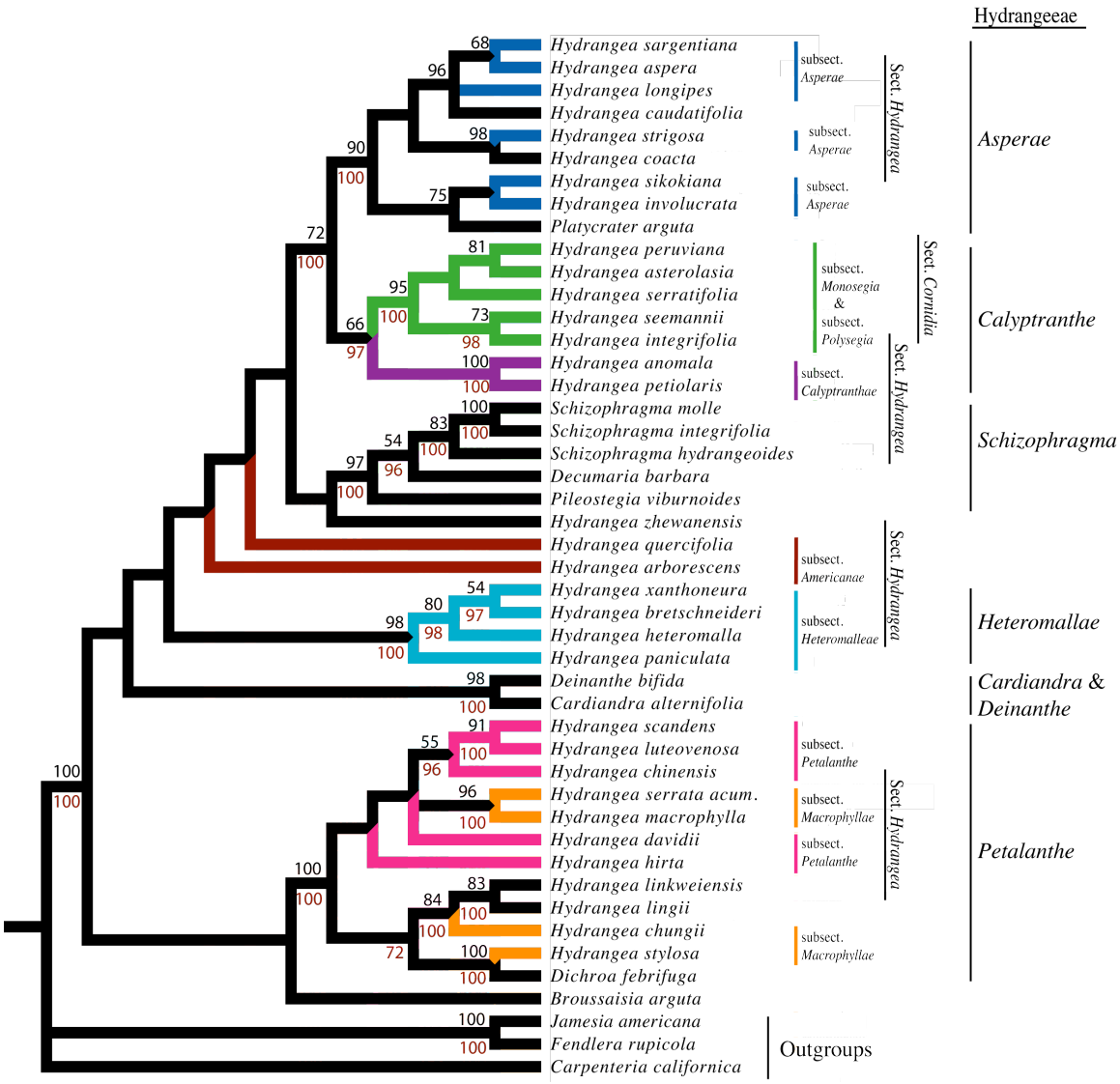
Hydrangeeae
Hydrangea sargentiana
Hydrangea aspera
Hydrangea longipes
Hydrangea caudatifolia
Hydrangea strigosa
Hydrangea coacta
Hydrangea sikokiana
Hydrangea involucrata
Platycrater arguta
Hydrangea peruviana
Hydrangea asterolasia
Hydrangea serratifolia
Hydrangea seemannii
Hydrangea integrifolia
Hydrangea anomala
Hydrangea petiolaris
Schizophragma molle
Schizophragma integrifolia
Schizophragma hydrangeoides
Decumaria barbara
Pileostegia viburnoides
Hydrangea zhewanensis
Hydrangea quercifolia
Hydrangea arborescens
Hydrangea xanthoneura
Hydrangea bretschneideri
Hydrangea heteromalla
Hydrangea paniculata
Deinanthe bifida
Cardiandra alternifolia
Hydrangea scandens
Hydrangea luteovenosa
Hydrangea chinensis
Hydrangea serrata acum.
Hydrangea macrophylla
Hydrangea davidii
Hydrangea hirta
Hydrangea linkweiensis
Hydrangea lingii
Hydrangea chungii
Hydrangea stylosa
Dichroa febrifuga
Broussaisia arguta
Jamesia americana
Fendlera rupicola
Carpenteria californica
subsect. Asperae
subsect. Asperae
subsect. Asperae
Sect. Hydrangea
subsect. Monosegia & subsect. Polysegia
Sect. Cornidia
subsect. Calyptranthae
Sect. Hydrangea
subsect. Americanae
subsect. Heteromalleae
Sect. Hydrangea
subsect. Petalanthe
subsect. Macrophyllae
subsect. Petalanthe
Sect. Hydrangea
subsect. Macrophyllae
Outgroups
Asperae
Calyptranthe
Schizophragma
Heteromallae
Cardiandra & Deinanthe
Petalanthe

Fig. 3. Majority rule consensus of topologies recovered from BI analyses of all data from the *accD-psa1*, *matK*, *psbA-trnH*, ITS, and the anonymous marker regions. Numbers above branches indicate posterior probabilities.

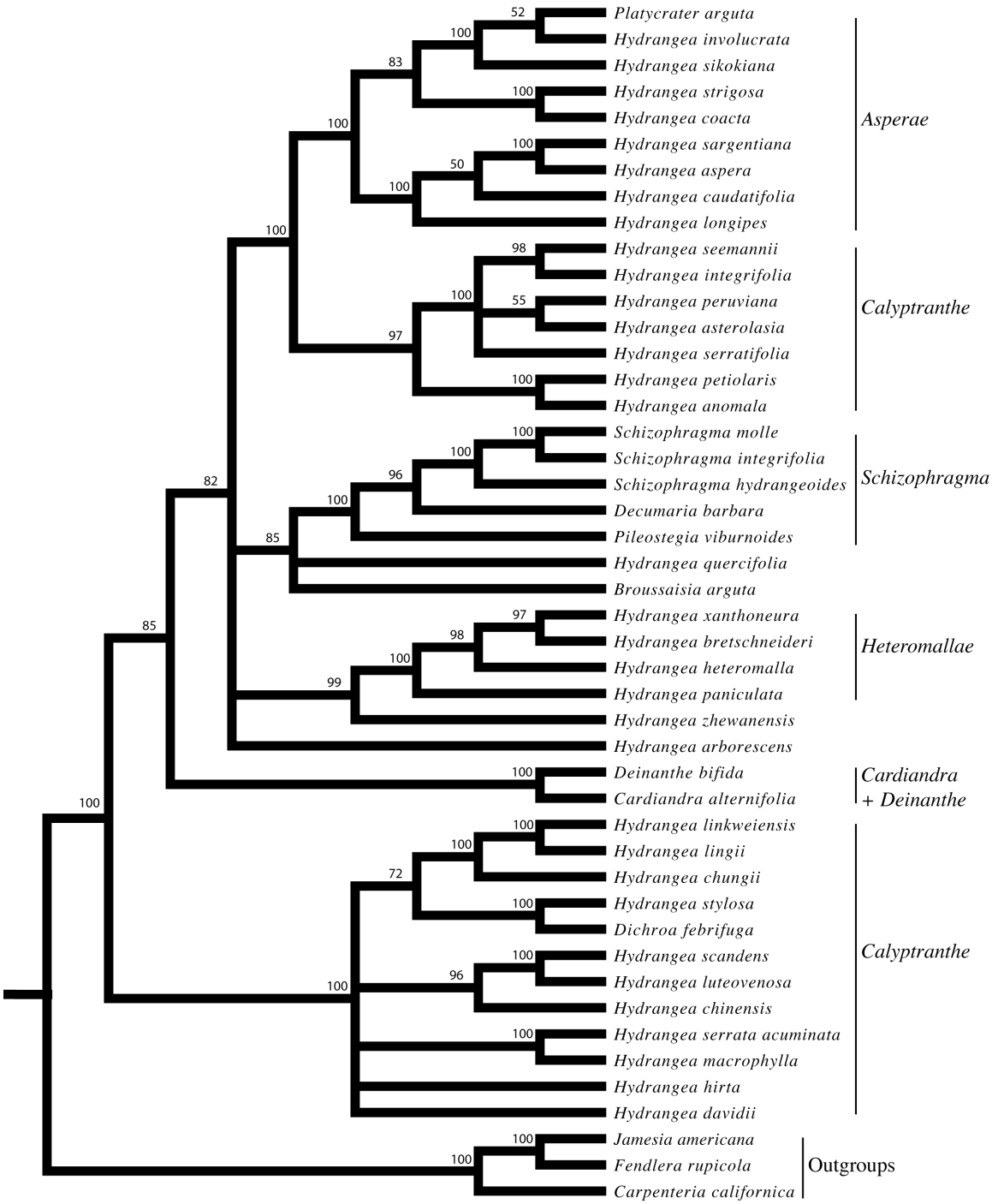
Platycrater arguta
Hydrangea involucrata
Hydrangea sikokiana
Hydrangea strigosa
Hydrangea coacta
Hydrangea sargentiana
Hydrangea aspera
Hydrangea caudatifolia
Hydrangea longipes
Asperae
Hydrangea seemannii
Hydrangea integrifolia
Hydrangea peruviana
Hydrangea asterolasia
Hydrangea serratifolia
Hydrangea petiolaris
Hydrangea anomala
Calyptranthe
Schizophragma molle
Schizophragma integrifolia
Schizophragma hydrangeoides
Decumaria barbara
Pileostegia viburnoides
Schizophragma
Hydrangea quercifolia
Broussaisia arguta
Hydrangea xanthoneura
Hydrangea bretschneideri
Hydrangea heteromalla
Hydrangea paniculata
Heteromallae
Hydrangea zhewanensis
Hydrangea arborescens
Deinanthe bifida
Cardiandra alternifolia
Cardiandra + Deinanthe
Hydrangea linkweiensis
Hydrangea lingii
Hydrangea chungii
Hydrangea stylosa
Dichroa febrifuga
Hydrangea scandens
Hydrangea luteovenosa
Hydrangea chinensis
Hydrangea serrata acuminata
Hydrangea macrophylla
Hydrangea hirta
Hydrangea davidii
Calyptranthe
Jamesia americana
Fendlera rupicola
Carpenteria californica
Outgroups

Fig. 4. Phylogram of one of two most likely trees from ML analyses of the combined data from the *accD-psa1*, *matK*, *psbA-trnH*, ITS, and the anonymous marker regions.


Hydrangea sargentiana
Hydrangea aspera
Hydrangea longipes
Hydrangea caudatifolia
Hydrangea strigosa
Hydrangea coacta
Hydrangea sikokiana
Hydrangea involucrata
Platycrater arguta
Hydrangea peruviana
Hydrangea asterolasia
Hydrangea serratifolia
Hydrangea seemannii
Hydrangea integrifolia
Hydrangea petiolaris
Hydrangea anomala
Schizophragma molle
Schizophragma integrifolia
Schizophragma hydrangeoides
Decumaria barbara
Pileostegia viburnoides
Hydrangea zhewanensis
Hydrangea quercifolia
Hydrangea arborescens
Hydrangea xanthoneura
Hydrangea bretschneideri
Hydrangea heteromalla
Hydrangea paniculata
Deinanthe bifida
Cardiandra alternifolia
Hydrangea scandens
Hydrangea luteovenosa
Hydrangea chinensis
Hydrangea serrata acuminata
Hydrangea macrophylla
Hydrangea davidii
Hydrangea hirta
Hydrangea linkweiensis
Hydrangea lingii
Hydrangea chungii
Hydrangea stylosa
Dichroa febrifuga
Broussaisia arguta
Jamesia americana
Fendlera rupicola
Carpenteria californica
0.02 changes

Table 1. Specimens sampled for molecular phylogenetic study. Marker 1 = *psbA-trnH*; Marker 2 = *accD-psa1*; Marker 3 = *matK*; Marker 4 = ITS; Marker 5 = anonymous marker.

Taxon	Collection	Marker sampled				
		1	2	3	4	5
Broussaisia arguta	Hufford 592, Hufford unmounted collection, Hawaii		√		√	
	Flynn 5060			√		
Cardiandra alternifolia	Hufford 671, WS, Japan	√				
	Hufford 674, WS, Japan			√		
Carpenteria californica	Winbauer 93105, WS333522, California	√	√		√	
	Gard s.n., RSA			√		
Decumaria barbara	Hufford 1289, K 69.50409	√	√		√	√
	WSU Greenhouse			√		
Deinanthe bifida	Jacobs 454, University of British Columbia Botanical Garden, HC0678 33657-598-98		√		√	
	Hufford 689, WS335477, Japan	√		√		
Dichroa febrifuga	Bartholomew 1972, China	√	√		√	√
	Bohm s.n.			√		
Fendlera rupicola	Winbauer 94207	√	√	√	√	
Hydrangea anomala	Hufford 3315, K 1994-821	√	√		√	√
	MOBOT 821779			√		
Hydrangea arborescens	Hufford 3312, K 1961-29104 CPHG	√	√		√	√
	Ware s.n.			√		
Hydrangea aspera	Hufford 1296, KEW	√	√	√	√	√
Hydrangea asterolasia	Grantham & Parsons, University of California Botanical Garden 90.2599, Costa Rica	√	√	√	√	√
Hydrangea bretschneideri	Liu & Zeng 284, MO 4714074, China	√	√	√	√	
Hydrangea caudatifolia	Miao H10064, MO, China	√	√	√		
Hydrangea chinensis	Shu-Hui 1264, CAS 999801, Taiwan	√	√	√	√	
Hydrangea chungii	Guo-Sheng 9766, MO 4726816, China	√	√	√	√	√
Hydrangea coacta	Kelly et.al 98-98, CAS 1002807, Taiwan	√		√	√	
Hydrangea davidii	Smith 10107, MO	√	√	√		
Hydrangea heteromalla	Hufford 3424, K 1910-65049 AARB	√	√		√	
	Boufford et.al 30098, MO 4866609, China			√		√
Hydrangea hirta	Sugawara 3082316, CAS 1055511, Japan	√	√			
	Takasu s.n. 17.6.94			√		
	Hufford 637				√	

Table 1. Continued

Taxon	Collection	Marker sampled				
		1	2	3	4	5
Hydrangea integrifolia	Bartholomew 7731, CAS 967646, Taiwan		√		√	√
	Jacobs 457, University of British Columbia Botanical Garden 30945-610-93 (4/6)	√				
	Leonardo 3244, A, Phillipines			√		
Hydrangea involucrata	Hufford 1308, K	√	√		√	√
	(from Hufford et al. 2001 study)			√		
Hydrangea lingii	He Guosheng 535P, MO 4538003	√				
Hydrangea linkweiensis	Guangzhao 13953, MO 4741752, China	√	√	√		
Hydrangea longipes longipes	Hufford 3425, K 1973-14659	√	√	√		√
	Liu Miao H40082, MO				√	
Hydrangea luteo venosa	University of California Botanical Garden 92.0321, Japan	√	√	√	√	√
Hydrangea macrophylla	Hufford 1319, K 73-14602	√	√	√	√	√
Hydrangea paniculata	Hufford 1297, K 251-7105052	√	√	√	√	√
Hydrangea peruviana	Grantham & Parsons 0013-90, CAS 932803, Costa Rica	√	√	√	√	√
Hydrangea petiolaris	Sugawara 3082305, CAS 1055379, Japan		√		√	√
	Jacobs 449, University of British Columbia Botanical Garden	√		√		
Hydrangea quercifolia	Fishbein 4731, WS359826, Mississippi, USA	√	√		√	√
	Moody 44, WS361670, WSU Greenhouse			√		
Hydrangea sargentiana	Wilson 772, CAS 1012878, China (collected from E 19081032*A)	√	√	√	√	√
Hydrangea scandens	Hufford 3418, K 1999-3918 WAHO 834	√	√	√	√	√
Hydrangea seemannii	Fishbein 3685, WS350626, Sonora, Mexico	√	√	√	√	
Hydrangea serrata acuminata	University of California Botanical Garden 2001.0337, South Korea	√	√	√	√	√
Hydrangea serratifolia	University of California Botanical Garden 82.0597, Chile	√	√		√	√
	(from Hufford et al. 2001 study)			√		
Hydrangea sikokiana	Murata 27062, US2409853, Japan	√	√	√	√	
Hydrangea strigosa	S.A.G.B. 116, CAS 773465, China	√	√	√	√	√
Hydrangea stylosa	Lin-Dong 3325, MO, China	√	√	√		√
Hydrangea xanthoneura	Heng et.al 26331, MO, China	√	√	√	√	
Hydrangea zhewanensis	Chenyaodong 2358, MO, China	√	√	√		√
Jamesia americana	1096 HOWK 440	√	√		√	
	Moody 42, WS361663, Arizona, USA			√		
Pileostegia viburnoides	Hufford 3317, K 1968-2907	√	√		√	√
	Qiu s.n., 1992			√		
Platycrater arguta	Jacobs 475, Japan	√	√		√	√
	Ogawa 05522			√		

Table 1. Continued

Taxon	Collection	Marker sampled				
		1	2	3	4	5
Schizophragma hydrangeoides	Jacobs 448, University of British Columbia Botanical Garden 3AC7 022120-0068-1983	√	√		√	
	Hufford 1689, WS, WSU Greenhouse			√		
Schizophragma integrifolia	Jacobs 461, University of British Columbia Botanical Garden 0237-0437-1984	√	√	√	√	√
Schizophragma molle	Jacobs 442, University of British Columbia Botanical Garden 3A 037903.09-0437.2005, China	√	√	√	√	

CAS - California Academy of Sciences
MO - Missouri Botanic Garden
E - Royal Botanic Garden, Edinburgh
RSA - Rancho Santa Ana Botanic Garden
US - United States National Herbarium
K – Royal Botanic Gardens, United Kingdom, England
A – Arnold Arboretum Herbarium, Harvard University
WS - Marion Ownbey Herbarium, Washington State University

Table 2. Primers used for PCR and cycle sequencing. Sources of previously published primer sequences and sequences for new primers developed for this study are provided.

Region	Primers used	Reference/primer sequence (5' to 3')
matK	matK 710-F, K2-R	(Johnson and Soltis 1994)
	HYDMATK-F	CTGAGCTATCCCGACCATTC
	HYDMATK-R	TGAGCCAAATCCTGTTTTCC
	MATK 85F	CCAGACCGGCTTACTAATGG
	MATK 796R	TCGTCACTCGGTAAAAGATGC
	MATK 559F	TCAAGAAGGGCTCCAGAAGA
	MATK 1358R	CGAACTAGATAGATATCAACAACACGA
trnH-psbA	trnH(GUG), psbA	(Sang et al. 1997, Tate and Simpson 2003)
	PSBA 380R	CAAATGGATAAGACTTTGGTSTT
accD-psa1	accD, psa1	(Small et al. 1998)
	HYDPSA1-F	TCATTATTGCCGAACCCAAT
	HYDACCD-R	TGGGGTACCTCAATTTACTAGTTG
	HYDaccdIP-R	CAAAATGGGGATTCTCGGTA
	HYDaccdIP-F	TCATTATTGCCGAACCCAAT
Anonymous marker	corresponds with locus STAB457_458	(Rinehart et al. 2006)
ITS	Nnc18s10, C26A	(Wen and Zimmer 1996)

Table 3. Results of 100 Partition Homogeneity Test replicates as implemented in PAUP* to examine sequence congruence between combined plastid, ITS and A.M. datasets. A setting of 2,000 maximum trees was enacted. Asterisks next to *P*-values indicate significance at the $\alpha = 0.05$ level.

	P-value		
Markers	plastid	ITS	anonymous marker
plastid	-	0.02*	0.03*
ITS	0.02*	-	0.78
anonymous marker	0.03*	0.78	-

* Indicates *P*-values that are significantly incongruent

Table 4. Statistics for datasets used, including results from MP and ML searches, and likelihood model parameters.

	accD-psa1	*matK*	*psbA-trnH*	plastid	ITS	A.M.	Complete dataset
Number of accessions sampled (of 46)	43	45	45	43	40	27	43
Number of characters (after alignment)	723	1135	324	2182	490	143	2815
Parsimony							
Variable sites (%)	137 (19%)	110 (10%)	57 (18%)	304 (14%)	63 (13%)	21 (15%)	388 (14%)
Parsimony-informative sites (%)	77 (10%)	70 (6%)	60 (19%)	203 (9%)	126 (26%)	21 (15%)	350 (12%)
Max tree setting	50,000	50,000	50,000	50,000	50,000	50,000	50,000
Number of MP trees	50,000	23,846	50,000	2784	50,000	50,000	302
Length of MP tree	283	231	186	732	542	63	1375
Likelihood							
Model of molecular evolution (hLRT criterion)	K81uf+G	TVM+G	F81+G	TVM+I+G	TRN + I + G	JC + G	GTR + I + G
log likelihood score	2545.0391	3062.0745	1819.3354	6684.3193	3618.2646	578.9992	12136.1562
Model of molecular evolution (AIC criterion)	GTR+G	GTR+G	TIM+G	GTR+I+G	TRN + I + G	TVM + G	GTR + I + G
log likelihood score	2541.4592	3058.9458	1801.5189	6681.7422	3618.2646	565.6913	12136.1562
Proportion of invariant sites (I)	0	0	0	0.3941	0.3819	0	0.4675
Gamma distribution shape parameter	1.0795	0.5839	0.7352	1.0864	0.8351	0.5321	0.6693
Max tree setting	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Number of ML trees	1,000	101	422	1	1,000	1,000	2
Likelihood score (-lnL)	2541.4593	3058.94591	1472.19547	7487.72687	3597.9423	547.06967	11925.95445

Table 5. Comparison of the most recent classification of *Hydrangea* and recent taxonomies of *Hydrangea* in China.

McClintock (1957) New World and Old World species	Wei (1994) Chinese species	Wei and Bartholomew (2001) Chinese species
Section *Hydrangea*	Section *Petalanthae*	*H. anomala*
Subsect. *Calyptranthe*	*H. candida*	*H. aspera*
H. anomala subsp. *anomala*	*H. caudatifolia*	*H. bretschneideri*
H. anomala subsp. *petiolaris*	*H. chinensis*	*H. candida*
Subsect. *Asperae*	*H. chungii*	*H. caudatifolia*
H. aspera subsp. *aspera*	*H. coenobialis*	*H. chinensis*
H. aspera subsp. *robusta*	*H. davidii*	*H. chungii*
H. aspera subsp. *Sargentiana*	*H. gracilis*	*H. coacta*
H. aspera subsp. *strigosa*	*H. kwangsiensis*	*H. coenobialis*
H. involucrata	*H. kwangtungensis*	*H. davidii*
H. sikokiana	*H. lingii*	*H. dumicola*
Subsect. *Americanae*	*H. linkweiensis*	*H. gracilis*
H. arborescens subsp. *arborescens*	*H. macrophylla*	*H. heteromalla*
H. arborescens subsp. *discolor*	*H. macrosepala*	*H. hypoglauca*
H. arborescens subsp. *radiata*	*H. mangshanensis*	*H. integrifolia*
H. qurecifolia	*H. obovatifolia*	*H. kawakamii*
Subsect. *Heteromallae*	*H. shaochingii*	*H. kwangsiensis*
H. heteromalla	*H. stenophylla*	*h. kwangtungensis*
H. paniculata	*H. taronensis*	*H. lingii*
	H. vinicolor	*H. linkweiensis*
	H. zhewanensis	*H. longifolia*

McClintock (1957) New World and Old World species	Wei (1994) Chinese species	Wei and Bartholomew (2001) Chinese species
Subsect. *Petalanthe*	Section *Heteromallae*	*H. longipes* var. *fulvescens*
H. hirta	*H. bretschneideri*	*H. longipes* var. *lanceolata*
H. scandens subsp. *chinensis*	*H. dumicola*	*H. longipes* var. *longipes*
H. scandens subsp. *kwangtungensis*	*H. heteromalla*	*H. macrocarpa*
H. scandens subsp. *liukiuensis*	*H. hypoglauca*	*H. mangshanensis*
H. scandens subsp. *scandens*	*H. macrocarpa*	*H. paniculata*
Subsect. *Macrophyllae*	*H. mandarinorum*	*H. robusta*
H. macrophylla subsp. *Chungii*	*H. molle*	*H. sargentiana*
H. macrophylla subsp. *macrophylla*	*H. paniculata*	*H. stenophylla*
H. macrophylla subsp. *serrata*	*H. sungpanensis*	*H. strigosa*
H. macrophylla subsp. *stylosa*	*H. xanthoneura*	*H. stylosa*
Section *Cornidia*	Section *Hydrangea*	*H. sungpanensis*
Subsect. *Monosegia*	*H. aspera*	*H. xanthoneura*
H. asterolasia	*H. coacta*	*H. zhewanensis*
H. diplostemona	*H. discocarpa*	
H. integrifolia	*H. glabripes*	
H. oerstedii	*H. kawakamii*	
H. peruviana	*H. longialata*	
H. preslii	*H. longifolia*	
H. seemannii	*H. longipes*	
H. Steyermarkii	*H. rosthornii*	
Subsect. *Polysegia*	*H. rotundifolia*	
H. Jelskii	*H. sargentiana*	
H. Mathewsii	*H. strigosa*	
H. serratifolia	*H. villosa*	
H. tarapotensis	Section *Cornidia*	
	Subsect. *Monosegia*	
	H. integrifolia	
	Section *Calyptranthe*	
	H. anomala	
	H. glaucophylla	

Table 6. A comparison of *Hydrangea* descriptions. * indicates Cultivated species; ** indicates Central and South American species; *** Chinese species. ‘s’ indicates Series; ‘*g*’ indicates Groups; ‘*s*’ indicates Subsections.

Maximowicz (1867)	Engler (1891)	Rehder (1927) *	Briquet (1919) **	Engler (1930)	McClintock (1957)	Wei (1994) ***
Euhydrangea	*Euhydrangea*	*Euhydrangea*	*Cornidia*	*Euhydrangea*	*Hydrangea*	*Petalanthe*
s. *Petalanthe*	s. *Petalanthe*	*g. Petalanthe*	*s. Monosegia*	*s. Petalanthe*	*s. Americanae*	*Heteromallae*
s. *Piptopetalae*	s. *Piptopetalae*	*g. Heteromallae*	*s. Polysegia*	*s. Heteromallae*	*s. Asperae*	*Hydrangea*
Calyptranthe	*Calyptranthe*	*g. Asperae*		*s. Asperae*	*s. Calyptranthe*	*Cornidia*
s. *Americanae*	s. *Americanae*	*g. Americanae*		*s. Americanae*	*s. Heteromallae*	*Calyptranthe*
s. *Japonico-sinensis*	s. *Japonico-sinensis*	*Calyptranthe*		*Calyptranthe*	*s. Macrophyllae*	
	Cornidia			*Cornidia*	*s. Petalanthe*	
				s. Monosegia	*Cornidia*	
				s. Polysegia	*s. Monosegia*	
					s. Polysegia	

Chapter Two

Flag flowers of *Hydrangea* and its allies (Hydrangeaceae tribe Hydrangeeae)

Abstract

Floral dimorphism in Hydrangeaceae tribe Hydrangeeae, which consists of *Hydrangea* and allied genera, is manifest most noticeably in inflorescences through the production of numerous small, conventional flowers that have inconspicuous calyces and less numerous flag flowers that have especially prominent calyces. Our study addressed the structure and variation of flag flowers among Hydrangeeae. A survey of herbarium specimens revealed that flag flowers typically have a perianth that includes a corolla as well as a calyx, although the corolla was usually absent in *Deinanthe* and *Schizophragma*. Flag flowers of most Hydrangeeae also have androecial and/or gynoecial structures, and these structures were observed to form pollen and ovules, respectively. Slightly fewer than half of the examined species had inflorescences in which flag flowers reached the fruit stage.

Introduction

Many species of flowering plants are characterized by the possession of more than one form of flower (Darwin 1896). Sexual systems such as monoecy, dioecy, and heterostyly are common expressions of floral polymorphism. Floral polymorphism is typically associated with reproductive strategies that promote outcrossing (Marshall and Abbott 1982, Abbott and Irwin 1988), and the evolution of floral polymorphism often results in changes to the perianth as well as in the androecium and gynoecium (Darwin 1896, Bell 1985). Perianth reductions occur in some instances of polymorphisms, notably in cases of monoecy and dioecy that involve shifts to wind pollination (Endress 1996), but elaborations of the perianth can also be a consequence of the evolution of floral polymorphism. The elaboration of the corolla in peripheral flowers of some Apiaceae, Asteraceae, and Dipsacaceae are well known (Weberling 1989, Bell 1985). In some other flowering plants, such as *Viburnum* (Weberling 1989) and *Hydrangea*, so-called flag flowers, which have particularly heightened floral displays compared to other flowers of the inflorescence are present.

Among Hydrangeaceae, flag flowers are present not only in *Hydrangea* but also in allied genera that include *Cardiandra*, *Deinanthe*, *Platycrater*, and *Schizophragma* (McClintock 1957). We have observed flag flower diversity within *Hydrangea* as well as between it and allied taxa. In this study, we survey the flag flowers of Hydrangeaceae tribe Hydrangeeae, which includes *Hydrangea* and its allies, to determine their morphology and the variations that are present.

A second objective of our study is to test for the presence of pollen and ovules among flag flowers, which have often been termed 'sterile flowers' (Darwin 1896, McClintock 1957, Weberling 1989, among others). Uemachi et al. (2004) demonstrated flag flowers of *H. macrophylla* cultivars were capable of pollen and seed production. We examine for the presence of pollen and ovules in structural studies of Hydrangeeae.

Methods and Materials

To distinguish between the two forms of flowers found in inflorescences of most Hydrangeeae, we apply the term 'flag flower' for the large, conspicuous flowers positioned typically around the periphery of the inflorescence and the term 'nonflag flower' to indicate the more conventional but smaller and inconspicuous flowers found in large numbers in the center of the inflorescences. Hufford (2001) described the morphology of nonflag flowers of Hydrangeeae and we use that study to compare flag forms.

Herbarium survey

The morphological diversity of flag flowers was assessed in a survey of intraplant, intraspecific, and interspecific variation using herbarium specimens. Nine hundred specimens encompassing 49 species were examined for presence of flag flowers, number of flag flowers per inflorescence, calyx merosity, presence and opening of corolla, presence of stamens, presence of styles, and presence of flag flower as fruits.

Species varied in their availability for this survey; thus, the phenological states and number of specimens available varied widely among the sampled specimens (Table 1).

Microscopic study

Selected species, representing different lineages of Hydrangeeae recognized in phylogenetic studies (Soltis et al. 1995, Hufford 1995, Hufford 1997, Hufford et al. 2001), were sampled for additional morphological study. We sampled *Schizophragma hydrangeoides*, *Deinanthe bifida*, *Cardiandra alternifolia*, *Hydrangea anomala*, *H. quercifolia*, and *H. macrophylla* with both light and scanning electron microscopy (SEM). Open flowers and available developmental stages of these species were collected either from natural populations or from botanical gardens (Table 2). Specimens were preserved in formalin-acetic alcohol. Specimens for SEM were dehydrated in a graded ethanol series, critical point dried, mounted on metal stubs, sputter coated with gold, and examined on a Hitachi scanning electron microscope using accelerating voltages between 10-12 kV. Specimens for histological study were preserved in formalin-acetic alcohol, dehydrated in a graded tertiary butyl alcohol series, infiltrated and embedded in Paraplast®, and sectioned at 12 µm using a rotary microtome. Sections were stained using safranin and fast green for light microscopy.

Ancestral state reconstruction.

A phylogenetic tree from chapter one was used to infer the evolution of flag flower states in the Hydrangeeae. Taxa were coded as having inflorescences without flag flowers (Absent) or inflorescences in which both non-flag and flag flowers were present. Flag flowers were distinguished as possessing either two-four separate calyx lobes, two-four extensively connate calyx lobes, or only a single calyx lobe. Mesquite (Maddison

and Maddison, 2009) was used for parsimony and maximum likelihood reconstructions of ancestral states. For the parsimony reconstruction, character states were treated as unordered. The likelihood reconstruction used the module StochChar (Maddison and Maddison, 2006) which was implemented using a one parameter Mk model in which all states were equally probable and the rate of change between states was estimated from the data.

Results

Herbarium survey

Of the 49 species of Hydrangeeae surveyed here, 37 had inflorescences with flag flowers (Table 1). Most species that possessed flag flowers had 1-10 flag flowers per inflorescence (five species had 1-20 flag flowers; only H. *quercifolia* consistently had more than 20 flag flowers per inflorescence). Flag flowers were typically located at the distal ends of primary and secondary inflorescence branches (Fig. 2), except in *Deinanthe bifida*, which had flag flowers inserted below the terminal flower (Fig. 3). In expanded inflorescences, flag flowers were typically deployed around the periphery (Fig. 2). Flag flowers tended to have relatively long pedicels, allowing these display-enhancing flowers to hang or droop slightly (Fig. 2) and serving to position flag flowers in the same plane (or slightly below) as that of non-flag flowers on the same branch of the inflorescence.

The calyx and corolla of flag flowers, when present, form in conventional positions, as demonstrated by *H. anomala* in which flag flowers are similar to non-flag flowers through organogenesis (Fig. 4). Flag flowers have a calyx that extends at a

greater rate than the corolla during early floral development (Fig. 4). The calyx of the flag flowers loosely encloses the corolla, androecium and style of the epigynous bud until the calyx lobes spread, approximating the timing of anthesis.

The presence of a calyx was the only common element among flag flowers of examined Hydrangeeae. Calyces were typically tetramerous (Fig. 1); however, lobe numbers ranged from 1-5 both among and within species. *Schizophragma* species had flag flowers characterized typically by a single calyx lobe (Fig. 5), although rarely two lobes were present (Fig. 6). The flag flowers of *Platycrater arguta* typically had connate calyx lobes, creating a synorganized unit (Fig. 7). Both *Cardiandra alternifolia* and *Deinanthe bifida* had typically three calyx lobes per flag flower. Calyx lobe shape ranged commonly from ovate to obovate to orbicular, although some species had lobes that were additionally elliptic or deltoid in shape, as in *Schizophragma* and *C. alternifolia*, respectively.

Flag flowers of most species had appendages of floral whorls other than the calyx also present (Table 1, Fig. 1). A corolla was present in 30 of 37 sampled species, although only 22 of these clearly had a corolla that opened (others retained a bud-like, closed corolla as in Fig. 8). Stamens and styles were observed in the majority of species (24 and 28 of 37 species, respectively). Fifteen of the 37 species were represented by at least one specimen in which the flag flower had continued development to the fruiting stage (e.g., Fig. 9).

Flag flowers had typical radial (poly-) symmetry. *Schizophragma*, with its shift to a single calyx lobe, is the most prominent departure from radial to bilateral symmetry in

flag flowers (Fig. 4). Shifts to bilateral (bi-) symmetry were also manifest in flag flowers in which calyx lobes differed in size and shape (e.g., Figs. 10, 11).

We observed also a phenomenon that could be term 'sub-flag flowers.' These were instances in which flowers at nodes near flag flowers had calyces in which only one (Fig. 11) or two lobes of the calyx were enlarged (Fig. 12).

Microscopic survey

Morphological and structural studies of *H. macrophylla* and *H. quercifolia* (Fig. 13-16, Fig. 18-20) demonstrated that flag flowers of these two species had a corolla, androecium, and gynoecium that were similar to those of the non-flag flowers (*H. macrophylla*, Fig. 13-15; *H. quercifolia*, Fig. 18, 19). Both of these species had flag flowers that produced pollen and ovules (*H. macrophylla*, Fig 16, 17; *H. quercifolia*, Fig. 20).

Both *H. anomala* (Fig. 21-26) and *C. alternifolia* (Fig. 27-31) had flag flowers that had enlarged calyces and relatively conventional appearing corollas and androecia (*H. anomala*, Fig. 21, 22; *C. alternifolia*; Fig. 27, 28); however, their gynoecia diverged from the forms found in non-flag flowers. Examined *H. anomala* had aberrant stylar forms (Figs. 22-24), and *C. alternifolia* had either aberrant styles or styles in which development had been curtailed early (Fig. 29). Pollen was found in anthers of flag flowers of both of these species (*H. anomala*, Fig. 26; *C. alternifolia*, Fig. 31). Ovules were observed in the ovaries of *H. anomala* (Fig. 25), but not in those of *C. alternifolia* (Fig. 30).

Flag flowers were highly differentiated in *Schizophragma* (Fig. 32-36) and *Deinanthe* (Fig. 38-40). Studies of *S. hydrangeoides* and *D. bifida* revealed the formation

of an enlarged calyx (*S. hydrangeoides*, Fig. 32; *D. bifida*, Fig. 37, 39) in flag flowers, but neither species typically formed any other floral appendages (*S. hydrangeoides*, Fig. 33, 34; *D. bifida*, Fig. 38). Rarely, we did observe flag flowers of *S. hydrangeoides* that had floral appendages other than a calyx, and, when present, these other appendages appeared aberrant (Fig. 35, 36).

Ancestral state reconstruction

The parsimony reconstruction of flag flower evolution indicates that the ancestral state for Hydrangeeae was either inflorescences in which flag flowers were absent or inflorescences in which flag flowers have two-four separate lobes (Fig. 40). The maximum likelihood reconstruction shows a high proportional likelihood for inflorescences that lack flag flowers for the basal node of Hydrangeeae (Fig. 41). Both methods of ancestral state reconstruction indicate that flag flowers could have originated independently more than once, especially in the *Petalanthe*. Both optimization approaches indicate that flag flowers have been lost multiple times in the Hydrangeeae. Changes between floral dimorphism and monomorphism are concentrated in the *Calyptranthe*, *Petalanthe*, and the *Schizophragma* clade.

Discussion

In various clades of angiosperms, intra-inflorescence floral dimorphism has evolved to result in inflorescences in which flowers differ in appearance. Flowers that have an embellished appearance, here termed flag flowers, in these florally dimorphic inflorescences often possess an enlarged corolla, as can be seen in *Viburnum* and some

Apiaceae, such as *Heracleum* (Weberling 1989). In contrast, flag flowers of the Hydrangeeae have an enlarged calyx that differs from the miniscule, green calyx of the non-flag flower (Hufford 2001) by being much larger than the corolla and white or colored (ranging from blue to red or yellow).

The calyces of flowers of most eudicots serve to protect the corolla, androecium and gynoecium over the course of pre-anthetic bud expansion (Endress 1994). In contrast, calyces of non-flag flowers of many Hydrangeaceae are involved in bud protection only in the earliest phases of floral development with a shift of protective function to the corolla for later stages of development and bud expansion (Roels et al. 1996; Hufford 2001). Indeed, it is the valvate petals that serve to protect the developing stamens and style over the course of bud expansion in nonflag flowers of Hydrangeeae. Notably, the flag flowers of dimorphic Hydrangeeae have the calyx as the protective structure for the flower during bud development (e.g., Fig. 4). The shift of protective function during floral bud development from the calyx to the corolla in Hydrangeeae inflorescences may have relaxed one aspect of selection on the calyx, allowing directional selection to enhance overall inflorescence showiness by acting on natural variants that expressed hyper-enlargement of the calyx.

Flowers with showy calyces are not uncommon, as can be seen in some Verbenaceae and *Holmskioldia* (Weberling 1989). An expansion of calyx function from bud protection to encompass display is often associated with the reduction of the corolla as is evident in *Gnidia* (Weberling 1989). Many Hydrangeeae have non-flag flowers in which the corolla abscises concomitant with anther dehiscence or soon after (Hufford 2001). Thus, at the time of peak male function in some non-flag flowers, the corolla may

be absent, which would diminish floral display if flag flowers were also absent. It is unclear whether early abscission of the corolla was a force in the origin of flag flowers and floral dimorphism in Hydrangeeae or whether the evolution of flag flowers has simply allowed for the early abscission of corollas in nonflag flowers.

Little research has addressed the biological functions of flag flowers, which are assumed to enhance pollinator attraction in wild Hydrangeaceae. Plateau (1898) observed insects visiting only the non-flag flowers of *Hydrangea* and continued to observe their visitation after he removed the flag flowers from an inflorescence, which indicates that flag flowers may not be essential for pollinator attraction (although they may enhance visitation rates). The loss of flag flowers in several clades of Hydrangeeae (Figs 40, 41) also calls attention to evolutionary ease of this transformation. It may indicate also that selection on male fitness regularly involves modulation of inflorescence states in Hydrangeeae, particularly variants for presence or absence of flag flowers.

Position

Diggle (2003) has demonstrated that both inflorescence architecture and development play a role in the evolution and deployment of floral polymorphisms. Significantly, she noted that floral function could shift dramatically over the course of inflorescence development. She especially called attention to the last flowers to develop on the inflorescence branches, where resources for fruit maturation may be most limited. We noted that flag flowers of Hydrangeeae are positioned typically as the last flowers of inflorescence branches (Fig. 2) and note the possibility that intra-inflorescence resource deployment as well as opportunities for the peripheral positioning of flag flowers could have been involved in the evolution of floral dimorphism in this clade.

Reproductive functions of flag flowers

Uemachi et al. (2004) demonstrated that flag flowers of cultivars of *H. macrophylla* were capable of both pollen and seed production; however, this has not been tested in wild species. Our results confirm that development of flag flowers proceeds through fruit production in several species (Table 1; for example, *H. chinensis*, Fig. 9). Furthermore, our structural studies verify that pollen and ovules are produced in *H. anomala* (Fig. 25, 26), *H. macrophylla* (Fig. 16, 17), and *H. quercifolia* (Fig. 20). *Cardiandra alternifolia* produced pollen but no ovules (Fig. 30, 31), although all specimens observed of this species had nonopening corollas. The survey of herbarium specimens indicated that nearly 65% of the examined species produced both androecial and gynoecial structures, although these were sometimes malformed.

Our results demonstrate also a range of variations in the forms of flag flowers. This includes species that are very similar to non-flag flowers aside from an enlarged calyx (such as *H. macrophylla*, Fig. 13-17, and *H. quercifolia*, Fig. 18-20), those that nearly always lack androecial and gynoecial structures in their flag flowers (such as *Deinanthe*, Fig. 37-39, and *Schizophragma*, Fig. 32-36), and others that had a complete perianth but androecial and/or gynoecial structures that tended to be aberrant (such as *C. alternifolia*, Fig. 29, and *H. anomala*, Fig. 22-24).

We observed variation in the flag flowers of the Hydrangeeae at the intraplant, intraspecific, and interspecific levels. Meristic variation among non-flag flowers in inflorescences is common among members of the Hydrangeaceae (Hufford 2001), and, thus, intraplant variation in flag flowers should perhaps also be expected (for example, Fig. 11, 12). Although flag flowers of several species of Hydrangeeae can function in

pollen dispersal and possibly also in seed production, insect visits to these flowers may play a minor role in fitness, and, thus, many of the variations we observe, such as size differences among calyx lobes and symmetry shifts (Fig. 10, 11), may play little role in the reproductive ecology of individuals.

A range of variation in the flag flowers may indicate that strong selection maintains a large calyx, but the other floral features of flag flowers may not face strong selection. For example our data do not show biased retention of androecial over gynoecial functions.

The origin of flag flowers

Flag flowers may have originated multiple times in the Hydrangeeae (Figs 40, 41). Parsimony state reconstructions are equivocal about the presence of flag flowers at the origins of the Hydrangeeae and *Hydrangea* section *Petalanthe*, at which the common ancestor either lacked flag flowers or had flag flowers that were characterized by two-four separate calyx lobes. The origin of floral dimorphism in Hydrangeeae is, thus, unclear. Parsimony reconstructions also indicate both loss and regain of floral dimorphism in the *Schizophragma* lineage, in which unique flag flowers characterized by enlargement of a single calyx lobe and lack of other calyx lobe expression evolved. The likelihood reconstruction supports a monomorphic ancestor, and this requires at least four gains of dimorphism, including two in *Hydrangea* section *Petalanthe* (in the clade containing *H. linkweiensis, H. chungii* and *H. stylosa* and in the clade containing *H. scandens, H. luteovenosa, H. chinensis, H. serrata, H. macrophylla* and *H. davidii*), once in the ancestor of the rest of the Hydrangeeae and once in the *Schizophragma* lineage. If *Cardiandra* and *Deinanthe* are sister to all other Hydrangeeae (as recovered by Hufford

2001), then the ancestor of Hydrangeeae would be reconstructed as possessing floral dimorphism and only two origins of flag flowers would have occurred, once at the origin of the Hydrangeeae and second in the *Schizophragma* lineage.

Flag flowers have been lost from inflorescences in multiple lineages of the Hydrangeeae (Figs 40, 41). At least three independent losses can be inferred from both parsimony and likelihood reconstructions including the *Schizophragma* clade (in *Pileostegia* and *Decumaria*) and *Hydrangea* section *Calyptranthe* (*H. serratifolia*). *Hydrangea* section *Petalanthe* is also characterized by lineages that lack flag flowers; however state reconstructions in this clade are equivocal and the order at which transformations occurred are unclear. For example, the common ancestor of *Petalanthe* may have been dimorphic and flag flowers may have been lost independently in *H. hirta*, *H. lingii,* and *Dichroa* (e.g., Fig. 40); conversely, if the ancestor of *Petalanthe* lacked flag flowers, they were gained multiple times (e.g., Fig. 41).

Phylogenetic diversification

Among Hydrangeaceae, flag flowers have evolved only in Hydrangeeae. Within Hydrangeeae, most members of the clade have largely similar flag flowers that may vary subtly in the sizes and shapes of the calyx lobes and in the degree to which stamens and carpels develop normally. *Cardiandra* and *Deinanthe*, which phylogenetic studies have shown to be sister taxa, share the possession of flag flowers that typically have three calyx lobes. Calyces of flag flowers have been uniquely synorganized in *Platycrater* and *Schizophragma* uniquely has only a single enlarged calyx lobe. A possible loss of flag flowers in *Broussaisia* is especially interesting because it has occurred concomitant with a shift to dioecy, another form of floral dimorphism. Shifts to dioecy have often been

associated with decreased investment in floral attractiveness (Vamosi et al. 2003), which is true of *Broussaisia*.

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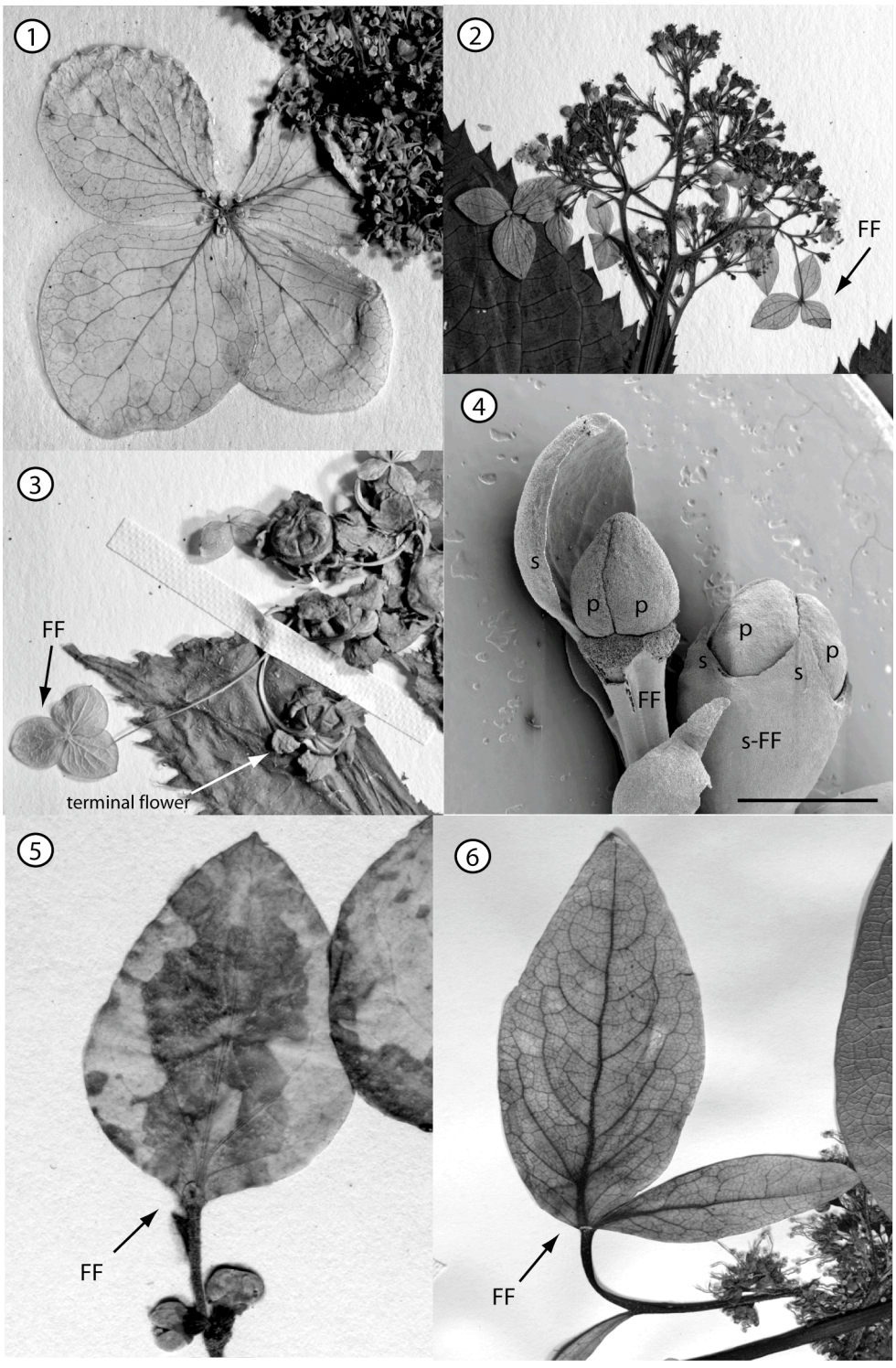
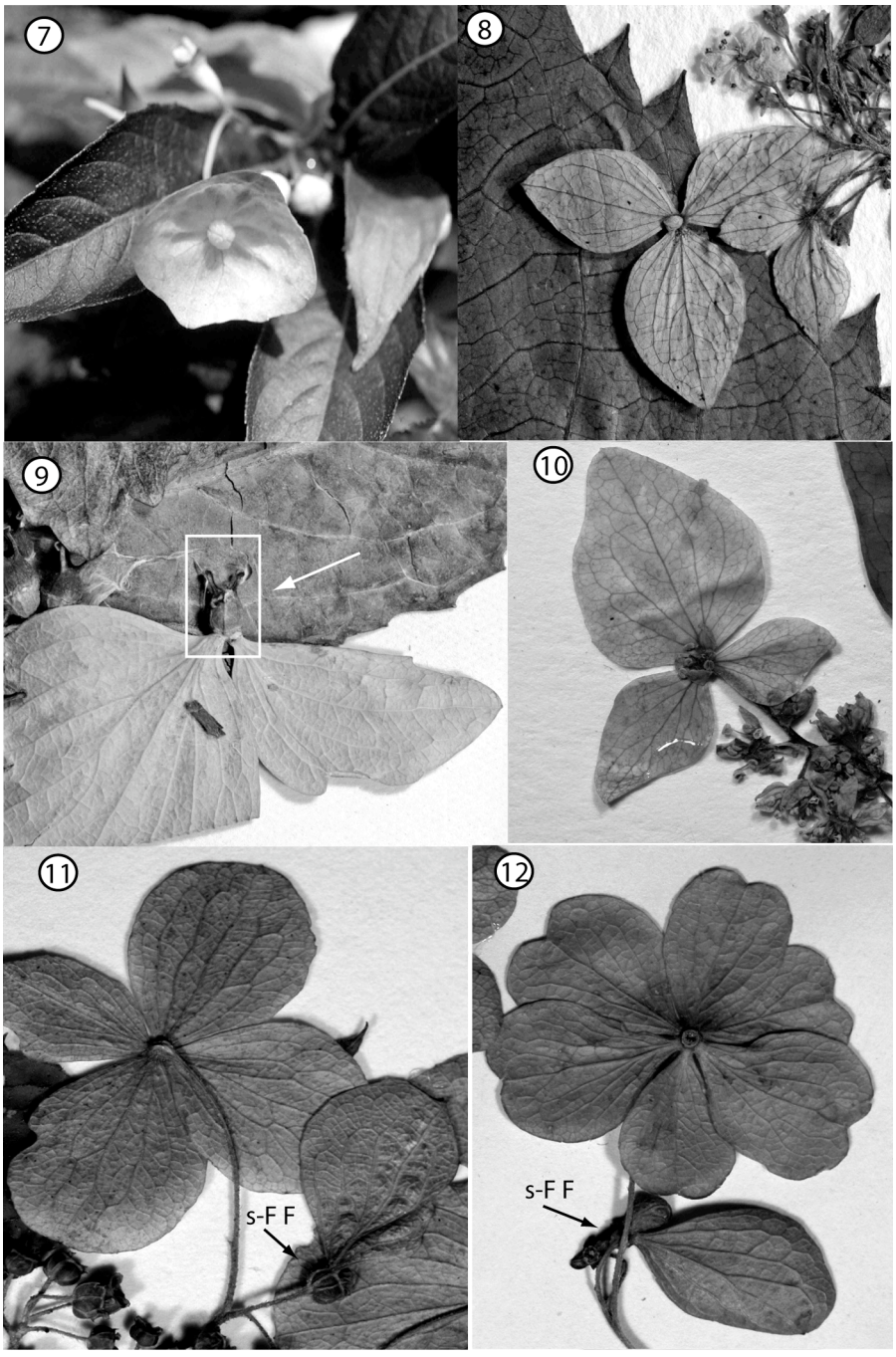
APPENDIX

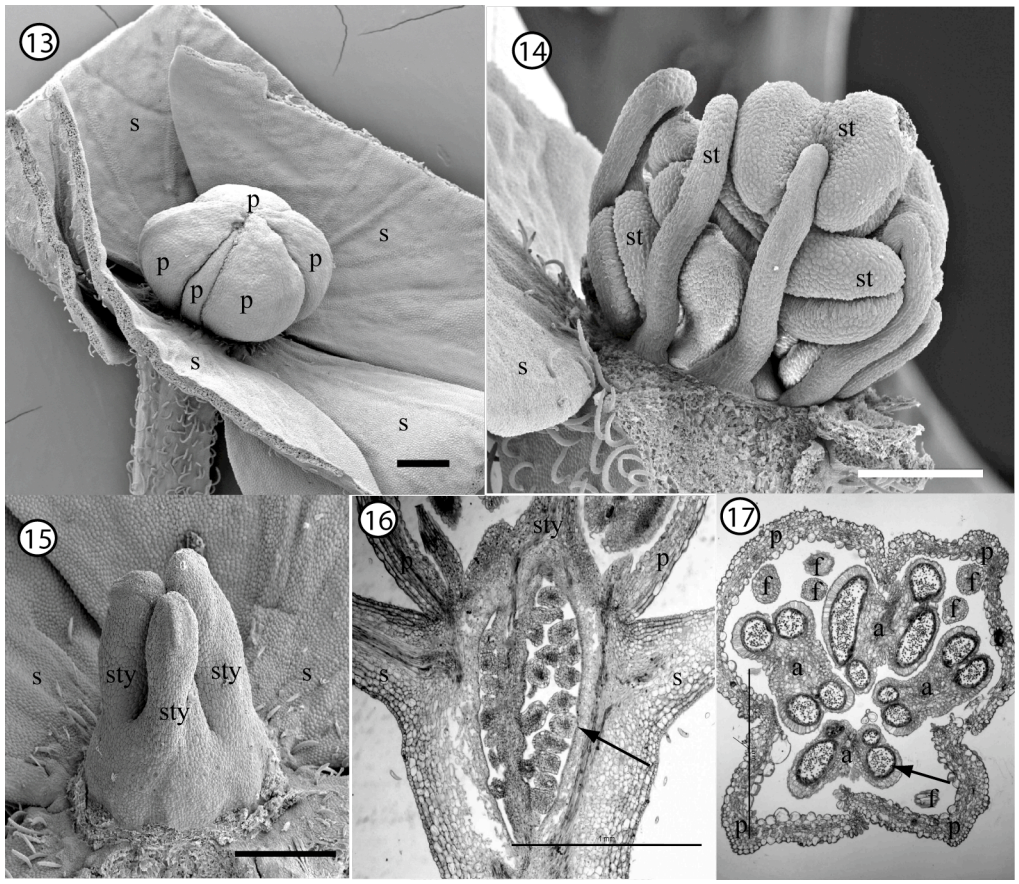
Figures 1-6. Flag flower forms of the Hydrangeeae. Fig. 1. Flag flower of *Hydrangea quercifolia* indicating the presence of floral series. Fig. 2. Inflorescence of *Cardiandra alternifolia* showing peripheral positioning of the flag flower within the inflorescence and terminal position within inflorescence branches. Fig. 3. Inflorescence of *Deinanthe bifida* indicating the insertion of the flag flower below that of the terminal flower. Fig. 4. Flag flower and sub-flag flower of *Hydrangea anomala*. Fig. 5. Flag flower of *Schizophragma hydrangeoides* with one enlarged calyx lobe. Fig. 6. Flag flower of *Schizophragma hydrangeoides* with two enlarged calyx lobes. Scale bars = 1.0 mm in Fig. 3. Abbreviations: FF = flag flower, s-FF = sub-flag flower, s = sepal, p = petal.


1
2
FF
3
FF
terminal flower
4
s
p
p
p
s
s
p
FF
s-FF
5
FF
6
FF

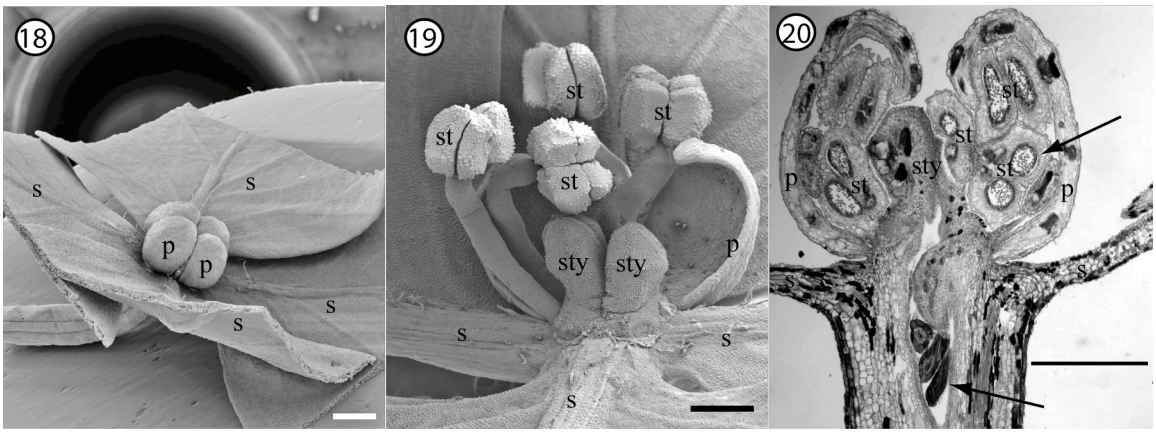
Figures 7-12. Flag flower forms of the Hydrangeeae. Fig. 7. Flag flower of *Platycrater arguta*. Fig. 8. Flag flower of *Cardiandra alternifolia* exhibiting an unopened corolla. Fig. 9. Flag flower of *Hydrangea chinensis*. Arrow and box highlight evidence of continued development through fruiting. Fig. 10. Flag flower of *Hydrangea chinensis* exhibiting bilateral symmetry. Fig. 11. Flag flower of *Hydrangea strigosa*. Arrow indicates sub-flag flower with one enlarged calyx lobe. Fig. 12. Flag flower of *Hydrangea strigosa*. Arrow indicates sub-flag flower with two enlarged calyx lobes. Abbreviations: s-FF = sub-flag flower.


7
8
9
10
11
s-F F
12
s-F F

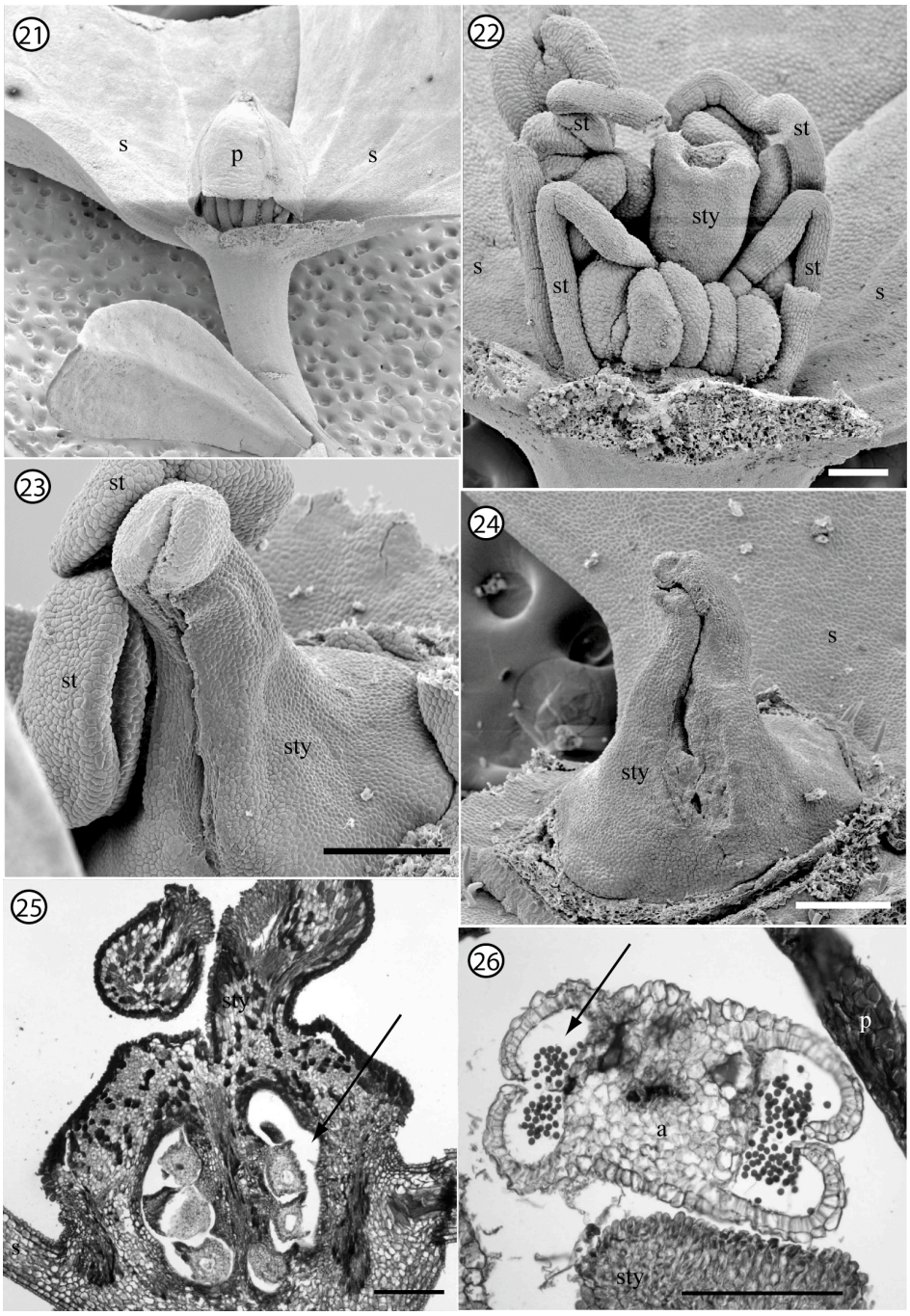
Figures 13-17. Flag flowers of *Hydrangea macrophylla*. Fig. 13. Flag flower exhibiting the position of the perianth components. Fig. 14. Flag flower where corolla lobes have been removed exhibiting morphology of the stamens. Fig. 15. Flag flower where corolla lobes and anthers have been removed exhibiting form of the style. Fig. 16. Longitudinal section of flag flower. Arrow points to ovules. Fig. 17. Cross-section of flag flower. Arrow points to pollen grains. Scale bars = 0.50 mm in Figs 13-15; 1.0 mm in Figs 16, 17. Abbreviations: s = sepals, p = petals, st = stamens, sty = styles, a = anthers, f = filaments.


13
s
p
p
p
p
p
s
s
s
14
st
st
st
st
s
15
s
sty
sty
sty
s
16
sty
p
p
s
s
17
p
f
f
f
f
p
p
a
a
a
a
f
p
p

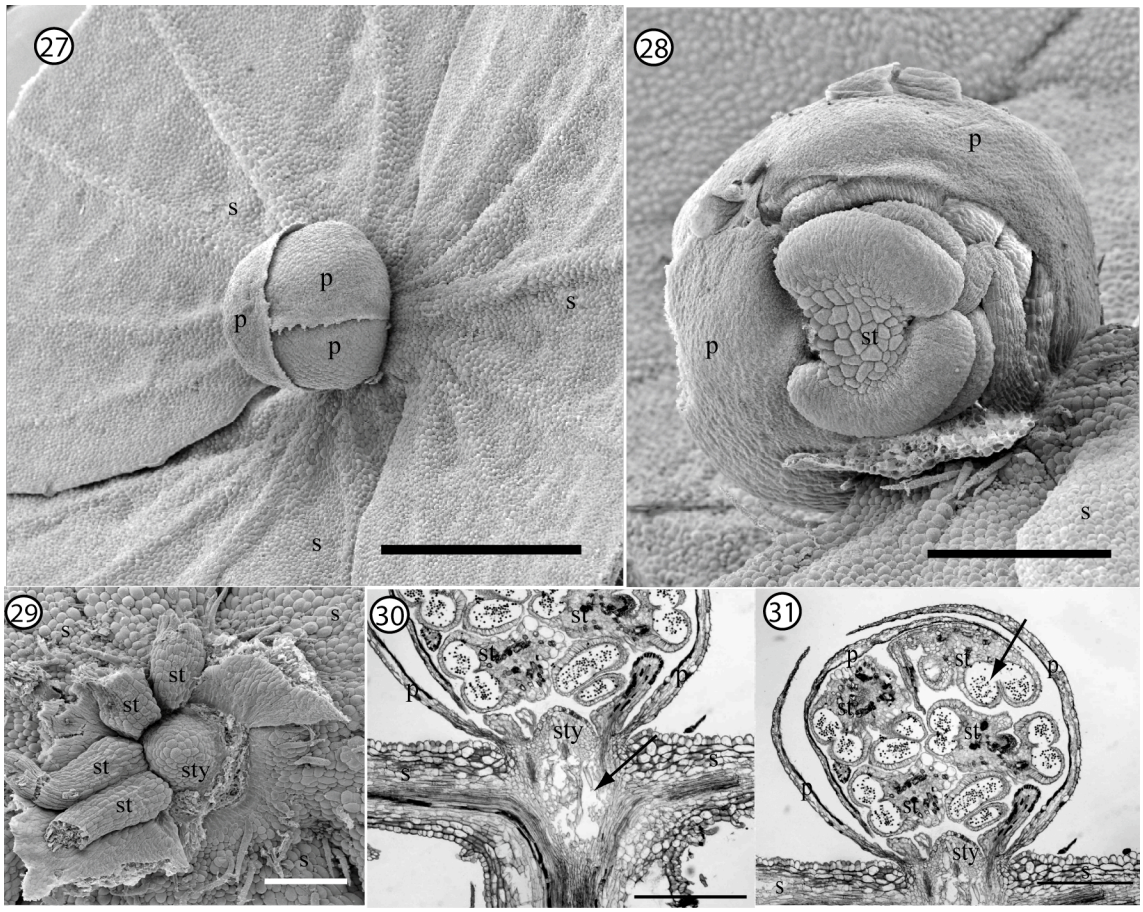
Figures 18-20. Flag flowers of *Hydrangea quercifolia*. Fig. 18. Flag flower indicating insertion of perianth components. Fig. 19. Flag flower where select petals and stamens have been removed to exhibit the forms of the stamens and styles. Fig. 20. Longitudinal section of *Hydrangea quercifolia*. Top-most arrow points to pollen; bottom-most arrow points to ovules. Scale bars = 0.50 mm in Figs 18, 19; 1.0 mm = Fig. 20. Abbreviations: s = sepals, p = petals, st = stamens, sty = styles.


18
s
s
p
p
s
s
19
st
st
st
st
sty
sty
p
s
s
s
20
st
st
st
sty
st
p
p
st
s
s

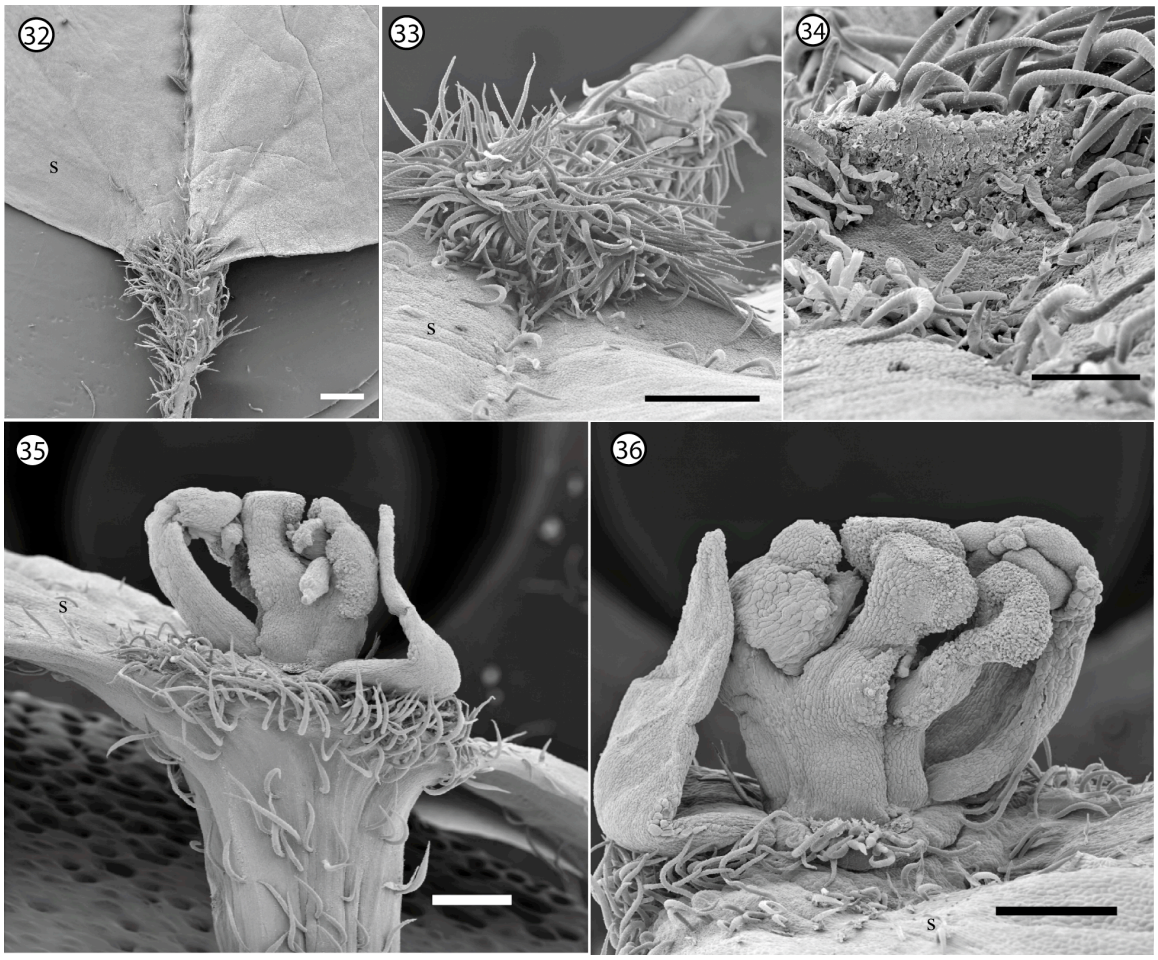
Figures 21-26. Flag flower of *Hydrangea anomala*. Fig. 21. Flag flower indicating position of perianth components. Petals are fused to form a calyptra. Fig. 22. Flag flower where calyx, corolla, and select stamens have been removed to exhibit form of stamens and style. Fig. 23. Style of flag flower. Fig. 24. Style of flag flower where calyx, corolla, and stamens have been removed. Fig. 25. Longitudinal section of flag flower. Arrow points to ovules. Fig. 26. Cross-section of flag flower. Arrow points to pollen. Scale bars = 1.75 mm in Fig. 21; 0.25 mm in Figs 23-26. Abbreviations: s = sepal, p = petal, st = stamen, sty = style.


21
s
p
s
22
st
st
sty
s
st
st
s
23
st
st
sty
24
s
sty
25
sty
s
26
p
a
sty

Figures 27-31. Flag flowers of *Cardiandra alternifolia*. Fig. 27. Flag flower indicating position of perianth components. Fig. 28. Flag flower where a petal was removed to exhibit form of stamen. Fig. 29. Flag flower where corolla and select stamens have been removed (some filaments remain) to exhibit form of style. Fig. 30. Longitudinal section of flag flower. Arrow points to empty ovary. Fig. 31. Longitudinal section of flag flower. Arrow points to pollen. Scale bars = 1.0 mm in Fig. 27; 0.5 mm in Figs 28, 43, 44; 0.25 mm in Fig. 29. Abbreviations: s = sepals, p = petals, st = stamens, sty = style.


27
s
p
p
p
s
s
28
p
p
st
s
29
s
s
st
st
st
sty
st
s
30
st
st
p
p
sty
s
s
31
p
st
p
st
st
p
st
sty
s
s

Figures 32-36. Flag flowers of *Schizophragma hydrangeoides*. Fig. 32. Adaxial view of flag flower. Fig. 33. Looking toward the floral meristem; distal to proximal view down the enlarged calyx lobe. Fig. 34. Looking at the floral meristem after removal of trichomes; distal to proximal view down the calyx lobe. Fig. 35. Adaxial view of aberrantly formed flag flower. Fig. 36. Abaxial view of same aberrant flag flower in Fig. 35. Scale bars = 0.5 mm in Figs 32, 33, 35, 36; 0.25 mm in Fig. 34. Abbreviations: s = sepal.


32
S
33
S
34
35
S
36
S

Figures 37-39. Flag flowers of *Deinanthe bifida*. Scale bars = 1.0 mm in Figs 37, 39; 0.25 mm in Fig. 38. Abbreviations: s = sepal.

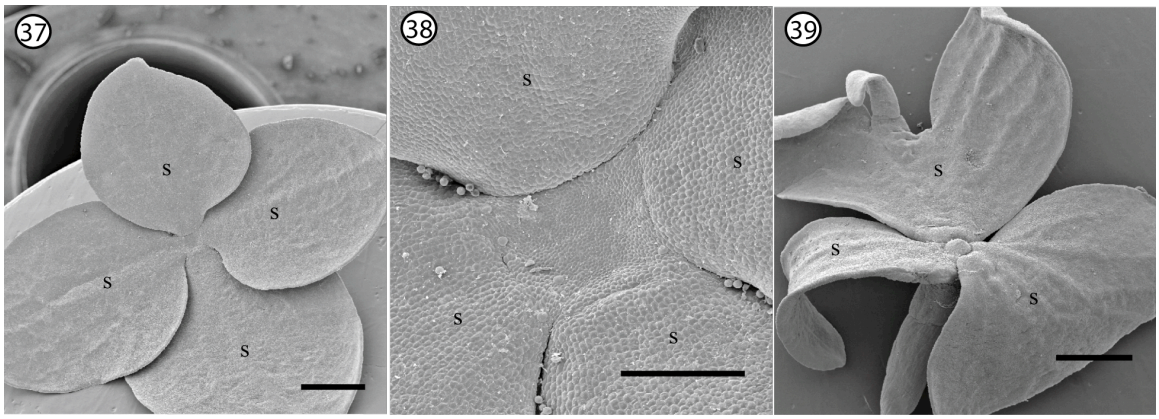

37
S
S
S
S
38
S
S
S
S
39
S
S
S

Fig. 40. Reconstruction of floral dimorphism in the Hydrangeeae based on parsimony optimization using the strict consensus topology from chapter one.

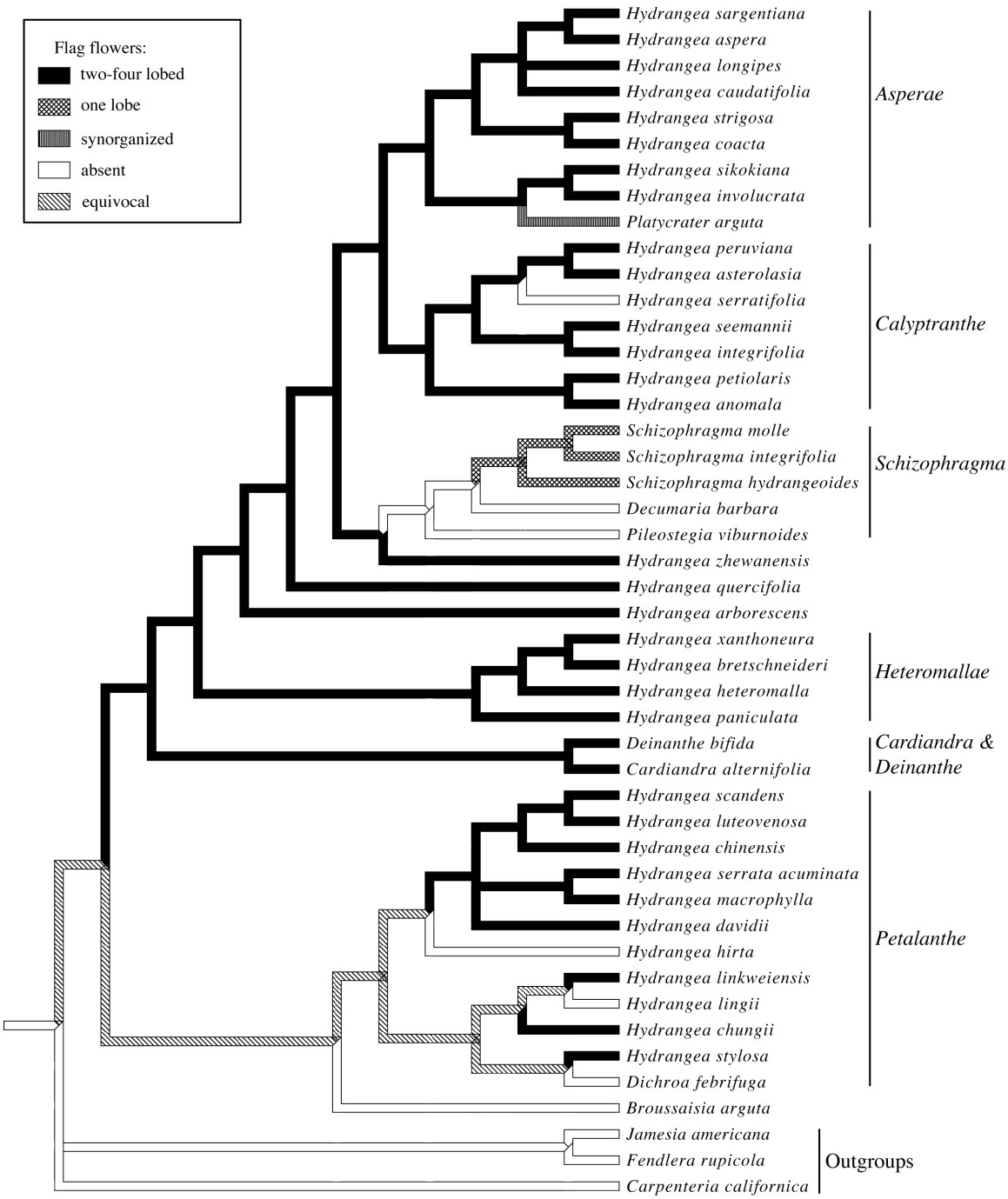

Flag flowers:
two-four lobed
one lobe
synorganized
absent
equivocal
Hydrangea sargentiana
Hydrangea aspera
Hydrangea longipes
Hydrangea caudatifolia
Hydrangea strigosa
Hydrangea coacta
Hydrangea sikokiana
Hydrangea involucrata
Platycrater arguta
Asperae
Hydrangea peruviana
Hydrangea asterolasia
Hydrangea serratifolia
Hydrangea seemannii
Hydrangea integrifolia
Hydrangea petiolaris
Hydrangea anomala
Calyptranthe
Schizophragma molle
Schizophragma integrifolia
Schizophragma hydrangeoides
Schizophragma
Decumaria barbara
Pileostegia viburnoides
Hydrangea zhewanensis
Hydrangea quercifolia
Hydrangea arborescens
Hydrangea xanthoneura
Hydrangea bretschneideri
Hydrangea heteromalla
Hydrangea paniculata
Heteromallae
Deinanthe bifida
Cardiandra alternifolia
Cardiandra & Deinanthe
Hydrangea scandens
Hydrangea luteovenosa
Hydrangea chinensis
Hydrangea serrata acuminata
Hydrangea macrophylla
Hydrangea davidii
Hydrangea hirta
Hydrangea linkweiensis
Hydrangea lingii
Hydrangea chungii
Hydrangea stylosa
Dichroa febrifuga
Petalanthe
Broussaisia arguta
Jamesia americana
Fendlera rupicola
Carpenteria californica
Outgroups

Fig 41. Reconstruction of floral dimorphism in the Hydrangeeae based on a maximum likelihood optimization using the strict consensus topology from chapter one. Pie charts indicate a proportional likelihood of character states at each node.

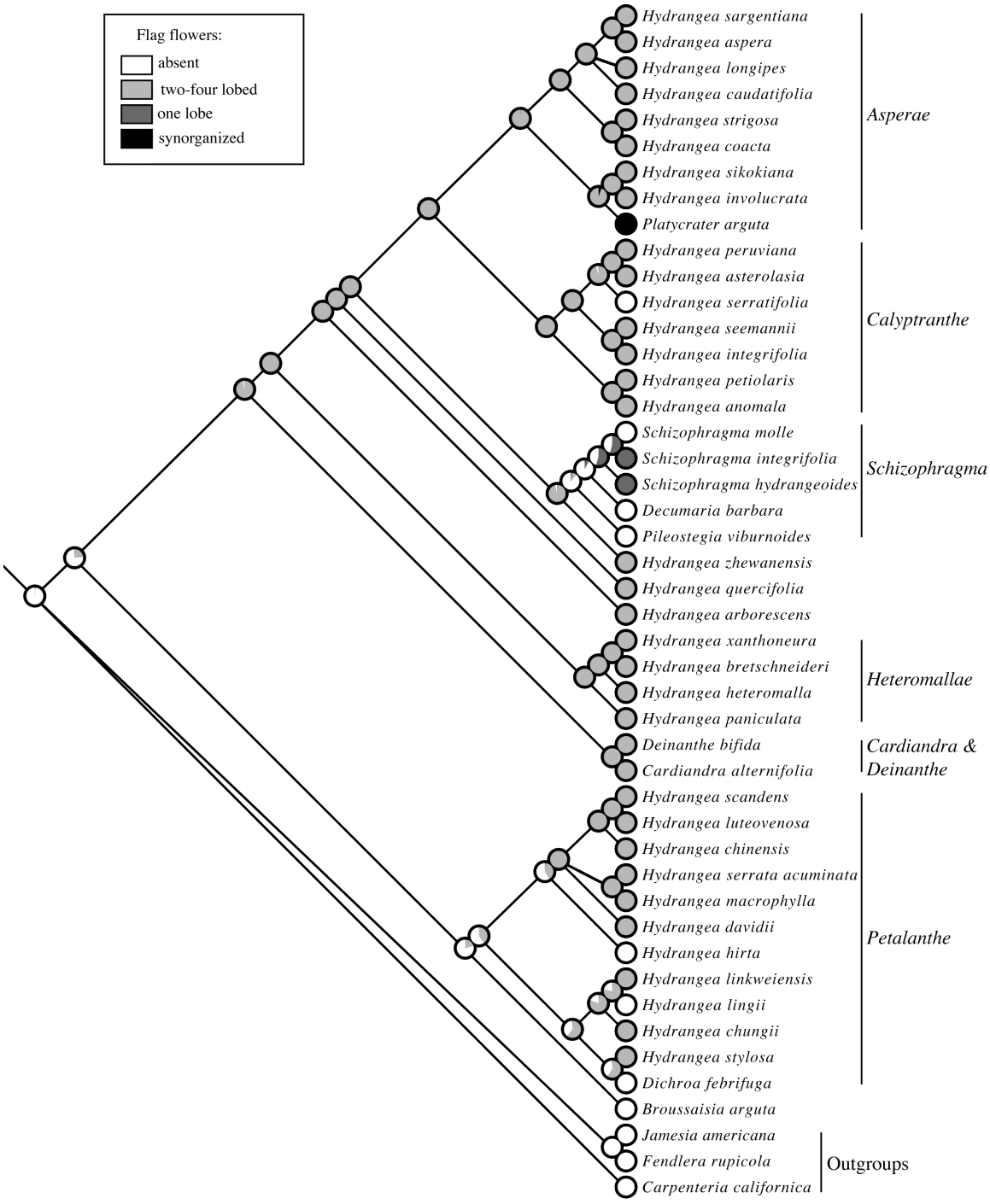

Flag flowers:
absent
two-four lobed
one lobe
synorganized
Hydrangea sargentiana
Hydrangea aspera
Hydrangea longipes
Hydrangea caudatifolia
Hydrangea strigosa
Hydrangea coacta
Hydrangea sikokiana
Hydrangea involucrata
Platycrater arguta
Asperae
Hydrangea peruviana
Hydrangea asterolasia
Hydrangea serratifolia
Hydrangea seemannii
Hydrangea integrifolia
Hydrangea petiolaris
Hydrangea anomala
Calyptranthe
Schizophragma molle
Schizophragma integrifolia
Schizophragma hydrangeoides
Schizophragma
Decumaria barbara
Pileostegia viburnoides
Hydrangea zhewanensis
Hydrangea quercifolia
Hydrangea arborescens
Hydrangea xanthoneura
Hydrangea bretschneideri
Hydrangea heteromalla
Hydrangea paniculata
Heteromallae
Deinanthe bifida
Cardiandra alternifolia
Cardiandra & Deinanthe
Hydrangea scandens
Hydrangea luteovenosa
Hydrangea chinensis
Hydrangea serrata acuminata
Hydrangea macrophylla
Hydrangea davidii
Hydrangea hirta
Hydrangea linkweiensis
Hydrangea lingii
Hydrangea chungii
Hydrangea stylosa
Dichroa febrifuga
Petalanthe
Broussaisia arguta
Jamesia americana
Fendlera rupicola
Carpenteria californica
Outgroups

Table 1. Taxa and characteristics observed in herbarium survey.

Species	Flag flower present	Number flag flower/inflorescence	Shape of calyx	Number calyx lobes	Corolla present	Corolla observed open or always closed	Stamens present	Styles present	Flag flower in fruit	Number of specimens examined
Broussaisia arguta	no	-	-	-	-	-	-	-	-	51
Cardiandra alternifolia	yes	4 (1-7)	ovate, deltoid	3 (2-4)	yes	no	no	no	no	35
Decumaria barbara	no	-	-	-	-	-	-	-	-	36
Decumaria sinensis	no	-	-	-	-	-	-	-	-	13
Deinanthe bifida	yes	3 (1-4)	ovate, orbicular	3 (2-4)	no	no	no	no	no	15
Deinanthe caerulea	yes	2 (1-3)	ovate, obovate, orbicular	4 (3-5)	no	no	no	no	no	8
Dichroa febrifuga	no	-	-	-	-	-	-	-	-	87
Hydrangea angustipetala	yes	13	ovate, orbicular	4	no	no	no	yes	yes	1
Hydrangea anomala	yes	3 (0-7)	ovate, obovate, orbicular	4 (3-4)	yes	yes	yes	yes	yes	19
Hydrangea arborescens	yes	1-10 (0-10)	oval, ovate, obovate, orbicular	4 (3)	yes	yes	yes	yes	yes	14
Hydrangea aspera	yes	3 (0-11)	oval, ovate, obovate, orbicular	4 (4-5)	yes	yes	yes	yes	yes	21
Hydrangea asterolasia	yes	2-7	obovate, orbicular	3	no	no	no	no	no	3
Hydrangea bretschneideri	yes	4 (1-16)	oval, obovate, orbicular	4 (3-5)	yes	yes	yes	yes	no	22
Hydrangea chinensis	yes	4 (0-9)	obovate, orbicular	4 (2-5)	yes	yes	yes	yes	yes	7
Hydrangea chungii	yes	4-5	oval, orbicular	4	yes	no	unknown	unknown	no	2
Hydrangea cinerea	yes	1-10 (0-20)	oval, obovate, orbicular	4 (3-4)	yes	yes	yes	yes	no	1
Hydrangea coacta	yes	6 (1-10)	orbicular	4	yes	yes	yes	yes	yes	2
Hydrangea davidii	yes	2-8	orbicular	3-4	yes	yes	yes	yes	no	2
Hydrangea diplostemona	yes	7-16	ovate, obovate	3 to 4	yes	no	yes	yes	no	34
Hydrangea dumicola	yes	6-7	oval, ovate	4-5	yes	yes	yes	yes	no	11
Hydrangea gracilis	no	-	-	-	-	-	-	-	-	1
Hydrangea heteromalla	yes	7 (1-18)	oval, ovate, obovate	4 (3-5)	yes	yes	yes	yes	no	16
Hydrangea hirta	no	-	-	-	-	-	-	-	-	28
Hydrangea integrifolia	yes	4 (0-18)	oval, orbicular	2-4	yes	yes	yes	yes	no	1
Hydrangea kwangtungensis	no	-	-	-	-	-	-	-	-	2
Hydrangea lingii	no	-	-	-	-	-	-	-	-	1
Hydrangea linkweiensis	yes	5	ovate, orbicular	3-4	yes	yes	yes	yes	no	8
Hydrangea longipes	yes	3 (2-14)	obovate, orbicular	4 (3-5)	yes	yes	yes	yes	no	7
Hydrangea luteovenosa	yes	2 (1-3)	obovate, orbicular	3-4	yes	yes	yes	yes	no	55
Hydrangea macrophylla	yes	1-5 (1-20)	oval, obovate, ovate, orbicular	4 (3-5)	yes	yes	yes	yes	yes	47
Hydrangea oerstedii	yes	4-10	obovate	3-4	yes	no	unknown	unknown	no	10
Hydrangea paniculata	yes	1-5 (1-25)	oval, ovate, obovate, orbicular	4 (3-5)	yes	yes	yes	yes	yes	37

Table 1. Continued

Species	Flag flower present	Number flag flower/inflorescence	Shape of calyx	Number calyx lobes	Corolla present	Corolla observed open or always closed	Stamens present	Styles present	Flag flower in fruit	Number of specimens examined
Hydrangea peruviana	yes	2 (0-11)	obovate, orbicular	4 (3-5)	yes	no	yes	yes	yes	49
Hydrangea quercifolia	yes	30 (20-50)	orbicular	4 (4-5)	yes	yes	yes	yes	yes	24
Hydrangea radiata	yes	1-10 (1-20)	oval, ovate, obovate	3 (2-5)	yes	yes	yes	yes	yes	18
Hydrangea sargentiana	yes	8	obovate, orbicular	4	no	no	no	yes	no	1
Hydrangea scandens	yes	2 (0-15)	obovate, orbicular	4 (3-5)	yes	yes	yes	yes	yes	39
Hydrangea serratifolia	no	-	-	-	-	-	-	-	-	10
Hydrangea sikokiana	yes	3 (0-10)	orbicular	4	yes	no	unknown	yes	no	26
Hydrangea strigosa	yes	1-5 (1-25)	obovate, orbicular	4 (3-6)	yes	yes	yes	yes	yes	10
Hydrangea styermarkii	no	-	-	-	-	-	-	-	-	8
Hydrangea stylosa	yes	2-7	oval	4	yes	yes	yes	yes	no	30
Hydrangea xanthoneura	yes	7 (2-16)	oval, ovate, orbicular	4 (3-5)	yes	yes	yes	yes	no	3
Pileostegia viburnoides	no	-	-	-	-	-	-	-	-	33
Platycrater arguta	yes	1-3	lobed	2-4	yes	no	unknown	unknown	no	15
Schizophragma crassum	no	-	-	-	-	-	-	-	-	4
Schizophragma hydrangeoides	yes	1-20	ovate, orbicular, deltoid	1**	no*	no	no	no	yes	18
Schizophragma integrifolia	yes	5-15	elliptic, ovate, oval, orbicular	1**	yes*	no	no	yes	yes	13
Schizophragma molle	yes	10-11	elliptic	1	no	no	no	no	no	2
									total number of specimens:	900

* unidentifiable structures found at base of flag flower

** additional calyx lobes occasionally observed

Table 2. Collections used for histological and scanning electron microscopy investigations.

Scientific name	Collector name/number, Locality, Date Collected
Cardiandra alternifolia	Hufford 671, Tatayama River, Japan
	Yamamura Prefix, Japan, 13-Aug-93
	Soltis 2529
Deinanthe bifida	Tokushima Prefix, Japan, 12-Aug-93
	Nikko, Japan
Hydrangea anomala subsp. *petiolaris*	UMD Greenhouse, 15-Apr-95, 1-Apr-93
Hydrangea anomala	Hufford 18269-A
	Hufford, KEW 65.75408, 21-Apr-95
	Jacobs, University of British Columbia Botanic Garden, 3-Jul-08
	Hufford, KEW 1994-821, 21-Apr-98
	Jacobs 468, University of British Columbia Botanic Garden 011414-0117-1972, 3-Jul-08
	Jacobs 472, University of British Columbia Botanic Garden 34023-598-98, 3-Jul-08

Table 2. Continued

Scientific name	Collector name/number, Locality, Date Collected
Hydrangea macrophylla var. *normalis*	Hufford, KEW 000-73.10410, 26-Jun-95
Hydrangea macrophylla subsp. *serrata*	Jacobs 471 #1/10, University of British Columbia Botanic Garden 029609.01-5402-1991, 3-Jul-08
	Jacobs 471 #2/10, University of British Columbia Botanic Garden 029609.01-5402-1991, 3-Jul-08
	Jacobs 471 #3/10, University of British Columbia Botanic Garden 029609.01-5402-1991, 3-Jul-08
	Jacobs 471 #4/10, University of British Columbia Botanic Garden 029609.01-5402-1991, 3-Jul-08
	Jacobs 471 #6/10, University of British Columbia Botanic Garden 029609.01-5402-1991, 3-Jul-08
	Jacobs 471 #7/10, University of British Columbia Botanic Garden 029609.01-5402-1991, 3-Jul-08
	Jacobs 471 #9/10, University of British Columbia Botanic Garden 029609.01-5402-1991, 3-Jul-08
	Jacobs 471 #10/10, University of British Columbia Botanic Garden 029609.01-5402-1991, 3-Jul-08
Hydrangea quercifolia	UMD Greenhouse, 8-Apr-94, 23-Apr-93
	Hufford, WSU Greenhouse, 26-Apr-94, 27-Apr-95
	Hufford, KEW 000-73-14607, 5-Apr-95
Schizophragma hydrangeoides	Hufford, WSU Greenhouse, 31-Mar-98, 15-Apr-94, 26-Apr-94, 1-May-94, 19-May-94
	Nikko, Japan, 1994
	92-45
	Jacobs 462, University of British Columbia Botanic Garden, 3-Jul-08