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Evolutionary developmental genetics of floral monosymmetry in *Cleome violacea* (Cleomaceae)

by

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Abstract

Cleomaceae is an ideal system in which to investigate evolutionary transitions between monosymmetric flowers from polysymmetric ancestors. Previous studies have not produced a resolved phylogeny or explored the role of the candidate gene, *TCP1*, in the evolution of monosymmetric flowers. Here, I use phylogenetic analysis of chloroplast genes *matK*, *ndhF*, and *ycf1*, in addition to the mitochondrial gene *rps3* and nuclear ribosomal gene *ITS1*, to generate the first support for relationships that constitute the backbone of the phylogeny. I then explore *TCP1* homologues in Cleomaceae by isolating two paralogues from three species (*Cleome spinosa*, *C. violacea*, and *C. viridiflora*) and correlating expression domain of one copy, *ClevioTCP1.1*, in *C. violacea* to floral development using *in situ* hybridization and scanning electron microscopy. These results provide a phylogenetic framework in which to interpret patterns of evolution and are the first steps towards understanding floral symmetry evolution at the molecular level in Cleomaceae.

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Chapter 1: Introduction and Objectives

The role of evolutionary developmental genetics in the study of floral evolution.

Floral structure directly affects the reproductive success of angiosperms by facilitating pollen transfer between members of the same species. Specifically, in flowers pollinated by animal vectors, characteristics of the corolla, particularly the number of planes of symmetry about a central axis, have been shown to be a key character directing pollinator specificity and thus efficiency of pollen transfer (Giurfa et al. 1999; Rodriguez et al. 2004). Types of floral symmetry are generally split into two broad groupings: polysymmetric (actinomorphic; radial), or monosymmetric (zygomorphic; bilateral). Polysymmetric flowers have three or more planes of symmetry about the central axis, while monosymmetric flowers have a single plane of symmetry (Endress 2001). In monosymmetric species, symmetry is considered a key adaptation to insect pollination capable of communicating nectar availability (Moller and Eriksson 1995) and orienting the pollinator for efficient pollen transfer (Johnson et al. 1998). Furthermore, monosymmetry is observed in distantly related lineages and dominates many of the most diverse, species-rich families such as Lamiaceae, Orchidaceae, and Scrophulariaceae (Johnson et al. 1998; Endress 1999). Thus monosymmetry is an evolutionary and developmentally labile trait with important implications for understanding angiosperm diversification.

Morphological variation in floral symmetry may be approached from an evolutionary developmental genetic perspective, which includes characterization of phylogenetic relationships, description of early developmental trajectories, and determining underlying molecular regulatory pathways. At the molecular level, numerous transitions from polysymmetry to monosymmetry in unrelated angiosperm lineages reflect differences in spatiotemporal expression of conserved genetic modules (Howarth and Donoghue 2006), in addition to sequence divergence. Thus, candidate genes with a putative role in developmental genetics can be identified based on homology to genes with an established functional role in model organisms. Consistently, asymmetrical expression of homologues of

TCP1, a member of TCP family of transcription factors, across the floral meristem is correlated with adaxial/abaxial differentiation of floral meristems (Martin-Trillo and Cubas 1999). Applying these hypotheses to non-model groups is necessary to establish the diversity of gene interactions, but first requires documentation of evolutionary history: phylogenetic analysis identifies a framework which, when combined with early developmental data, is used to interpret evolution of morphological diversity across taxa. Phylogenetic analysis is also applied to evolution of candidate genes to assess sequence evolution by duplication and subsequent divergence.

The high degree of diversity in the small - approximately 200-300 species (Sanchez-Acebo 2005; Hall 2008) - pantropical family Cleomaceae facilitates the investigation of processes with considerable ecological importance, including evolution of floral monosymmetry. Furthermore, Cleomaceae is the most closely related family to Brassicaceae (Hall et al. 2002, 2004; Hall 2008), which includes the model organism *Arabidopsis thaliana*. Morphologically, Cleomaceae is distinguished from the closely Brassicaceae on the basis of palmately compound leaves and monosymmetric, rather than disymmetric, flowers in the former (Judd et al. 1994; Hall et al. 2002; Iltis et al. 2011). Cleomaceae flowers are monosymmetric due to upward curvature of the petal and stamen bases; however, the most highly monosymmetric species also show adaxial-abaxial differentiation of organs in the sepal and petal whorls (Patchell et al. 2011). Although floral symmetry cannot be studied directly in *A. thaliana* because this species does not exhibit monosymmetric flowers, studies of diversity within Cleomaceae have the potential to provide insight into co-option of the candidate gene *TCP1* for novel function in the development of monosymmetric flowers.

Currently, advances are being made to refine Cleomaceae as a model group, including genome sequencing and phylogenetic study. The transcriptome of *Cleome violacea* has been sequenced, with proposal for the full genome to follow as part of a broader comparative genomics study in Brassicaceae (personal communication J. C. Pires; E. Schranz). In addition, the genome of *C. spinosa* is currently being sequenced (personal communication E. Schranz) which will

complement published transcriptome (Barker et al. 2009) and BAC libraries of this species (Schranz and Mitchell-Olds 2006). *Cleome gynandra* and other species are actively pursued as models for investigating C_4 photosynthesis (Brown et al. 2005; Koteyeva 2011). Phylogenetic analyses have been conducted using sequence data: chloroplast regions *trnH-psbA* (Sanchez-Acebo 2005); *matK* and *ndhF* (Hall 2008), *trnL-trnF* and *ndhF* (Hall et al. 2002); and nrDNA *ITS* (Inda et al. 2008; Feodorova et al. 2010). These analyses established familial boundaries, highlighted that the type genus, *Cleome* L.*,* is not monophyletic, and identified major clades in the family. However, the evolutionary relationships between these clades remain poorly understood. Thus, more extensive taxon and genome sampling is required to resolve relationships between clades (Cummings and Meyer 2005). In the following introduction, I briefly review recent work in evolutionary developmental genetics of Cleomaceae and provide background information to provide context for the purpose of this thesis.

Introduction to Cleomaceae

Cleomaceae has long been recognized as a close relative of Capparaceae and Brassicaceae. Rollins 1993 shows that these three groups form a monophyletic clade united by a biosynthetic pathway that converts methionine to mustard oils (glucosinolates). Formerly Cleomaceae was considered a subfamily of Capparaceae (subfamily Cleomoideae; Pax and Hoffman 1936), but it is now recognized as the sister family to Brassicaceae (Hall et al. 2002, 2004; Iltis et al. 2011). Separation of these three groups is well established by molecular data and is supported by morphology (Hall et al. 2002; Hall 2008; Iltis and Cochrane 2011; Iltis et al. 2011). Cleomaceae includes four major clades: the Western North American cleomoids (Western N.A. cleomoids), *Cleome* s. s. including the type species *C. ornithopodioides*, a *C. droserifolia* clade, and a large *Polanisia Clade* (Hall 2008). However, evolutionary relationships among these clades remain unresolved (Sanchez-Acebo 2005; Hall 2008; Inda et al. 2008; Feodorova et al. 2010)

The genus *Cleome* (common name "spider flower, or "bee plants" in North America) is the most species rich genus (about 200-250 species) in the Cleomaceae (approximately 300 species) (Hall et al. 2002; Hall 2008). It is a taxonomically complicated genus that is paraphyletic with related genera *Cleomella* D.C., *Dactylaena* Schrader ex Schult f., *Haptocarpum* Ule, *Oxystylis* Torr. et Frem., (Hall et al. 2002) Sanchez-Acebo 2005; Hall 2008; Inda et al. 2008). Although changes in nomenclature are in progress, agreement on genus assignment has not yet been reached (Sanchez-Acebo 2005; Hall 2008; Inda et al. 2008). Cleomaceae is represented in Alberta by two species, *Peritoma serrulata* (= *Cleome serrulata*; beeflower) and *Polanisia dodecandra* (clammyweed) (Moss 2008).

Floral symmetry development in Cleomaceae: at least two distinct patterns of early development underlie monosymmetry of mature flowers.

Floral monosymmetry was formerly proposed as a synapomorphy delimiting the subfamily Cleomoideae (Judd et al. 1999). However, this trait has been shown to be homoplasious within the Brassicales (Hall et al. 2002). Within Cleomaceae, floral symmetry is morphologically diverse and can be assessed separately in the sepals, petals, androecium and gynoecium. In a typical cleomoid flower, monosymmetry is due to upward curvature of the petal bases in the corolla. However, highly monosymmetric species, such as *Cleome violacea*, also show differentiation in shape and colour between the adaxial and abaxial petals, an enlarged abaxial sepal, and prominent nectar gland. Features of the androecium such as stamen abortion (*Dactylaena*) or proliferation (*Polanisia*) as well as curvature (some *Cleome*) also impart monosymmetry to mature flowers (Hall et al. 2002). Furthermore, two distinct developmental trajectories are involved in generating monosymmetric flowers in Cleomaceae: early monosymmetry and early disymmetry (Patchell et al. 2011). Early disymmetry is exhibited in buds with four equally sized sepal primordia and a square shaped floral apex, while early monosymmetry is characterized by an enlarged abaxial sepal and a trapezoidal shaped floral apex (Patchell et al. 2011). Thus,

morphology of mature flowers and pattern of early floral development are both considerations in assessing evolution of monosymmetry in Cleomaceae. Although floral development has been described in some species (Koevenig 1973; Endress 1992; Erbar and Leins 1997; Patchell et al. 2011), development of late stages of development, including curvature and gland proliferation, remains poorly documented. The few studies that have investigated pollination biology in Cleomaceae show that flowers attract a range of diurnal bees, wasps, and butterflies, (Cane 2008**)**, while *Cleome spinosa* is pollinated at least in part by bats (Sperr et al. 2011).

Candidate gene: TCP1 homologues implicated in differentiation of abaxial and adaxial regions of developing flowers.

Homologues of *TCP1* are proposed as a candidate gene underlying development of floral monosymmetry in *Cleome*. TCP is a large family of plant transcription factors with diverse functions in plant growth and development (*teosinte branched1*, *CYCLOIDEA* and *PROLIFERATING CELL FACTORS 1* and *2*; Cubas et al. 1999). Homologues have been implicated with a role in development of monosymmetric flowers in phylogenetically distant angiosperms: *Antirrhinum majus* (Plantaginaceae; Luo et al. 1996); *Iberis amara* (Brassicaceae; Busch and Zachgo 2007); and *Pisum sativum* (Fabaceae; Wang et al. 2008). Importantly, a TCP1 homologue, *AtTCP1*, is briefly expressed in floral buds of *Arabidopsis thaliana* (Cubas et al. 2001), suggesting that this gene is involved in establishment of adaxial –adaxial patterning even in actinomorphic flowers (Rosin and Kramer 2009). These data combined indicate the TCP family is a promising candidate gene for establishing monosymmetry in Cleomaceae. The timing and pattern of expression correspond to events in early floral development that generate differential growth rates of organs along an adaxial-abaxial axis.

Ecology: habitat and C_4 photosynthesis.

Cleomaceae is recently diverged from the Brassicaceae (personal communication W. Cardinal-McTeague 2010; Schranz and Mitchell-Olds 2006;

Feodorova et al. 2010) with a putative center of origin in tropical Africa and Southeast Asia, and subsequent diversification throughout the tropics (Iltis 1957; Feodorova et al. 2010). There is limited diversity in temperate regions (Iltis 1957). Plants are herbs or small subshrubs (Hall et al. 2002; Iltis et al. 2011). Several species such as *Cleome gynandra* and *C. viscosa* are pantropical weeds of disturbed habitats (McCormack 2007).

In addition to floral symmetry, Cleomaceae are also ideal candidates for investigation of C_4 photosynthesis (Brown et al. 2005). Under high light conditions C_4 photosynthesis concentrates carbon dioxide in bundle sheath cells to limit damaging photorespiration reactions and functionally increases carbon dioxide fixation rates (Hopkins and Huner 2004). The Cleomaceae includes several C_4 taxa: *Cleome allamanii, C. angustifolia, C. gynandra*, *C. luderitziana*, *C. oxalidea, C. siliculifera*, and *C. sparsifolia* (Voznesenskaya et al. 2007; Marshall et al. 2007), as well as physiologically intermediate species *C. paradoxa* (Voznesenskaya et al. 2007). Perhaps more importantly, Cleomaceae also represents five independent transitions to C_4 photosynthesis (Feodorova et al. 2011).

Economic: valued for food, pharmaceuticals, and aesthetics.

Several species within the Cleomaceae are being developed for commercial production. Seeds of *Cleome lutea* and *Peritoma serrulata* are used in rehabilitation efforts in western rangelands in North America (Cane 2008). These annual species flower rapidly from seed and support pollinator populations, including agricultural pollinators *Apis mellifera* and *Megachile rotudata* prior to establishment of perennial species (Cane 2008). Cleome seeds are also consumed for food. In rural India, seeds of *Cleome viscosa* are ground and used a locally available substitute for cumin (*Cuminum cyminum*) and in preparation of pickles, sausages, curries (Maikhuri et al. 2000). *Cleome gynandra* (= *Gynandropsis gynandra*) is known locally as African cabbage where it is grown in semi-cultivation in eastern and southern Africa (Mnzava 1990). It is often cheaper to produce than contemporary crops, has comparatively similar nutritional qualities,

and has fatty acid content similar to leguminous oilseeds, although its bitter taste is unpalatable (Mnzava 1990).

The ethnobotanical properties of members of the Cleomaceae are still being explored. *Cleome viscosa* is known locally as Jakhiya in India and grows as a weed in agricultural and abandoned crop fields (Maikhuri 2000). *Cleome viscosa* has antipyretic (Devi et al. 2003), anti-diarrhoeal (Devi et al. 2002), anti-inflammatory (Parimaladevi et al. 2003), immunomodulatory (Tiwari et al. 2004), local anasthetic activities (Singh and West 1991), and anti-malarial activity (Saxena et al. 2000). *Cleome gynandra* is also used to repel ticks and mites (Malonza 1992). *Taraneya* (= *Cleome) hassleriana* and *Taraneya* (= *Cleome*) *spinosa* are used as a florist species (personal observation).

Purpose of this thesis

The purpose of this thesis is to characterize evolutionary developmental genetics of floral monosymmetry evolution in the plant family Cleomaceae with emphasis on *Cleome violacea*. The first data chapter addresses phylogenetic relationships within Cleomaceae based on chloroplast genes *matK*, *ndhF*, and *ycf1*, in addition to the nuclear intragenic spacer *ITS* and mitochondrial gene *rps3*. This study is the first to include sampling from nuclear, chloroplast, and mitochondrial genomes. I also present two new phylogenetic data sets for Cleomaceae: *rps3* and *ycf1*. These data combined resolve relationships between clades within the family. In Chapter 3, I examine the role of TCP1 homologues in floral monosymmetry of Cleomaceae using *in situ* hybridization. Development of *Cleome violacea* is documented in twelve stages that parallel stages described for *A. thaliana* (Smyth et al. 1990). These developmental stages are then assessed for correlation to expression of a candidate gene, *ClevioTCP1.1*. I have confirmed that *TCP1* homologues are expressed in floral meristems, although expression patterns do not clearly correlate with developmental events that generate monosymmetry in mature flowers. Furthermore, while only one copy of *TCP1*

has been detected in the sister family Brassicaceae, at least two copies occur in *C. violacea*, *C. spinosa*, and *C. viridiflora*.

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Chapter 2: Floral Symmetry of Cleomaceae in a Phylogenetic Framework

Introduction

Cleomaceae is a small (13 genera and approximately 180-200 species), pantropical plant family that is the recent focus of investigations into evolution of floral monosymmetry (zygomorphy), C_4 photosynthesis, and comparative genomics (Hall et al. 2002; Brown et al. 2005; Shranz et al. 2006; Marshall et al. 2007; Barker et al. 2009; Feodorova et al. 2010; Koteyeva et al. 2011). Furthermore, investigations of these intriguing biological phenomena are facilitated by the sister relationship between Cleomaceae and Brassicaceae (Hall et al. 2002, 2004; Hall 2008), one of the most morphologically cohesive angiosperm families (Rollins 1993) that also includes the model organism, *Arabidopsis thaliana*. Both Cleomaceae and Brassicaceae are generally herbaceous in habit, and have fruits with a persistent replum. Cleomaceae is recognized primarily on the basis of bracteate inflorescences and strongly incurved testa forming highly curved, reniform to horseshoe shaped seeds (Iltis et al. 2011). Notably, the flowers of Cleomaceae are monosymmetric and morphologically diverse in comparison to the almost invariant cruciform flowers of Brassicaceae. Since floral morphology is an important factor determining degree of reproductive isolation in angiosperms (Sargent 2004), characterizing floral morphology within Cleomaceae in a phylogenetic context has potential to illuminate diversification of an evolutionarily significant trait.

Monosymmetric flowers in Cleomaceae have the same disymmetric ground plan as Brassicaceae: four sepals, four petals, a bicarpellate gynoecium, and usually six stamens. However, monosymmetric flowers are the dominant and plesiomorphic state in Cleomaceae, with at least one reversal to polysymmetry (Hall et al. 2002). In a typical cleomoid flower, the petals, stamens, and gynoecium are directed upwards (Endress, 1992; Patchell et al. 2011). Furthermore, size, shape, and colour between adaxial and abaxial petals and sepals may differ, while some taxa also show prominent adaxial nectar gland and variation in organ number (Table 2.1). Some of the most unusual floral morphologies are due to reduced number of organs within each whorl. For

example *Dactylaena* produces flower with a single adaxial stamen, four abaxial staminodes, and four linear petals (Kers 2003) and the vine *Haptocarpum* has four stamens and two petaloid staminodes, but only abaxial petals (Kers 2003).

A strong phylogenetic hypothesis is required for evaluation of floral symmetry evolution in Cleomaceae and to provide framework for other inquiries. Although phylogenetic placement of Cleomaceae is resolved at the family level (Hall et al. 2002, 2004; Hall 2008), intrageneric relationships remain subject to interpretation (Hall et al. 2002; Sanchez-Acebo 2005; Hall 2008; Inda et al. 2008; Feodorova et al. 2010). Several classification systems have been proposed based on morphology (Pax and Hoffman 1936; Iltis 1952). However, these groupings are not consistent with clades identified by analysis of sequence data (Sanchez-Acebo 2005; Hall 2008; Inda et al. 2008; Feodorova et al. 2010). Notably, *Cleome*, the largest genus in the family, is not monophyletic (Hall 2008) and a number of major lineages have been identified (Hall 2008; Inda et al. 2008; Feodorova et al. 2010). Despite a number of previous studies using both nuclear (Inda et al. 2008; Feodorova et al. 2010) and chloroplast markers (Hall et al. 2002; Sanchez-Acebo 2005; Hall 2008), the backbone of the Cleomaceae phylogeny remains unresolved. Importantly, the placement of the root of the family is unclear (Feodorova et al. 2010). The long branches of these early diverging clades are connected by short backbone internodes, which is characteristic of rapid speciation events implicated in other observed "bushes of life" (Jian et al. 2008; Rothfels et al. 2012; Whitfield and Lockhart 2007). Furthermore, several early diverging lineages may have accumulated many base pair changes during the course of evolution and may be subject to long-branch attraction (Feodorova et al. 2011), where misidentification of homoplasious characters as homologous confounds pairing of closely related taxa (Heath et al. 2008).

Increasing taxon and genome sampling improves resolution between clades (Cummings and Meyer 2005; Rothfels et al. 2012). Character sampling can be increased by amassing sequence data for each taxon, whether by increasing the number of gene regions sampled or by increasing the length of sequence reads available for a single gene (Rothfels et al. 2012). However the entire genetic

complement of each taxonomic unit is the sum of chloroplast, mitochondrial, and nuclear genomes, each of which have the potential to reflect different evolutionary histories due to the effects of hybridization and polyploidy events (Rieseberg et al. 1990; Rieseberg and Soltis 1991; Baldwin 1992; Kim and Jansen 1994; Baldwin et al. 1995; Wendel et al. 1995). Analysis of any one of these genomes in isolation can lead to potentially misleading species phylogenies, which can be overcome by including sequence data from all three genomes. This approach has been successfully applied using whole genomes for a small number of taxa (Pereira and Baker 2006; Regier et al. 2010). To reduce the possibility of increasing support for erroneous relationships due to susceptibility of increasing character data to branch-length variation and rate heterogeneity characteristics when clade sampling is limited (Gaut and Lewis 1995; Soltis et al. 2004; Whitfield and Lockhart 2007), taxonomic sampling of Cleomaceae was also pursued. Used in conjunction with increased genome sampling, large taxonomic sampling moderates the effects of long-branch attraction that otherwise complicate analysis of rapidly evolving genes or rapidly evolving lineages (Zwickl and Hillis 2002). The combination of increased character and taxon sampling has greatly improved phylogenetic hypotheses in other eudicot lineages (e.g., Euphorbiaceae (Horn et al. 2012); Rosaceae (Lo and Donoghue 2012); Saxifragales (Jian et al. 2008); Malpighiales (Wurdack and Davis 2009**).**

Interpreting patterns of floral evolution in Cleomaceae requires characterizing diversity in a phylogenetic framework. The purpose of this investigation is to resolve the Cleomaceae phylogeny and explore patterns of floral symmetry evolution within this framework. Towards these ends, I compiled a five-gene data set (three chloroplast, one nuclear and one mitochondrial) for 95 species of Cleomaceae and generated a well-supported phylogenetic hypothesis using Bayesian inference. This represents the most thorough taxon and character sampling of the family to date; previous studies based on a single marker sampled 38 species (Inda et al. 2008) to 81 species of Cleomaceae (Feodorova et al. 2010) whereas studies based on only two chloroplast markers included a maximum of 32 species (Hall 2008).

Materials and Methods

Character and taxon sampling

Five loci were sampled from all three genomes: chloroplast (*matK*, *ndhF*, and *ycf1*), mitochondrial (*rps3*), and nuclear ribosomal (*ITS1*). Nuclear ribosomal internal transcribed spacer (*ITS1*), *ndhF* (encodes a subunit of the chloroplast NADP deydrogenase enzyme), and *matk* (encodes a maturase K gene within the intron of *trnK*) were chosen because they have been shown to be phylogenetically informative and published data sets are available from Genbank (Hall 2008; Feodorova et al. 2010). Taxon sampling for the *matK*, *ndhF*, and *ITS1* data sets was increased by addition of sequence data from *de novo* DNA extractions. New data sets were generated for the rapidly evolving chloroplast gene *ycf1* (hypothetical chloroplast open reading frame 1) and slower evolving mitochondrial gene *rps3* (encodes the ribosomal small subunit protein 3). An accession table, including taxa all taxa sampled and corresponding Genbank accession numbers, is available in Table 2.2.

Taxa were broadly sampled from across the family, including 8 out of 9 genera (89%) and 95 out of 180 species (52%). When possible, multiple species were sampled from newly segregated genera of *Cleome* (Iltis and Cochrane 2007; Tucker and Vanderpool 2010). Particular emphasis was made to include species from all described lineages (Hall 2008; Inda et al. 2008; Feodorova et al. 2010) in addition to species that have not previously been sampled in molecular analyses. Despite considerable effort to acquire DNA of *Haptocarpum*, this genus was not included in the taxonomic sampling. Thirteen taxa from Brassicaceae were included as outgroups (Hall et al. 2002, 2004; Hall 2008) (Table 2.2). Because sequence data are not available for all taxa across all five genes, partial sequences were included when available. Uncertainty introduced to the analysis due to inclusion of partial sequence data is not expected to obscure relationships between taxa (Galtier and Daubin 2008; Burleigh et al. 2009; Sanderson et al. 2010).

DNA extraction, amplification, and sequencing

Total DNA was extracted from fresh or herbarium specimens using Qiagen DNeasy Minikits (Qiagen, Germantown, Maryland, USA) or a modified CTAB method (Doyle and Doyle 1987; Smith et al. 1991). Standard PCR methods were used (Hall et al. 2002; Hall 2008). PCR reactions with a total volume of 20 μl: 2.5 microL of 10X Extaq Buffer (Takara; Otsu, Shiga, Japan), sterilized distilled water, 2.5 mM of each dNTP, 0.2-1.0 microM of each primer, 0.625U *Extaq* polymerase and less than 250 ng of genomic DNA. Primers used in this study are listed in Table 2.4. Polymerase chain reactions (PCR) were implemented in an Eppendorf Mastercycler Pro, gradient thermal cycler (Eppendorf Canada). Amplification conditions were specific to the region amplified: 1) *matK*, initial denaturation for 10 min. at 94 °C, followed by 36 cycles of denaturation at 94 °C for 0.5 min, annealing at 55°C for 1 min, and extension at 72 °C for 2 min, followed by a final extension of 72°C for 10 min; 2) *ndhF*, initial denaturation for 10 min. at 94 °C, followed by 36 cycles of denaturation at 94 °C for 0.5 min, annealing at 48°C for 1 min, and extension at 72 °C for 2 min, followed by a final extension of 72°C for 10 min.; 3) *rps3*, initial denaturation for 10 min. at 94 °C, followed by 36 cycles of denaturation at 94 °C for 0.5 min, annealing at 55°C for 1 min, and extension at 72 °C for 45 sec., followed by a final extension of 72°C for 10 min.; 4) *ITS1*, initial denaturation for 5 min. at 94 °C, followed by 36 cycles of denaturation at 94 °C for 0.5 min, annealing at 58°C for 1 min, and extension at 72 °C for 45 sec., followed by a final extension of 72°C for 10 min. and 5) *ycf1*, initial denaturation for 10 min. at 94 °C, followed by 36 cycles of denaturation at 94 °C for 0.5 min, annealing at 54°C for 1 min, and extension at 72 °C for 2 min, followed by a final extension of 72°C for 10 min. Problematic extractions required separate amplification of shorter contiguous fragments using different primer pairs (Table 2.4). PCR products were visualized using 1% gel electrophoreses then cleaned with QIAquick PCR purification columns (Qiagen, Inc.). Both strands were cycle sequenced using a range of primers (Table 2.4). Reactions were cleaned with

Performa DTR V3 96-well Short Plate Kit (Edge BioSystems, Gaithersburg, MD), and sequenced using an ABI-3730 DNA Analyzer (Applied Biosystems).

Sequences were edited and initially aligned using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, Mich.). Sequences were Clustal aligned using MacVector v.12.0.2 using default settings then manually codon aligned using *Arabidopsis thaliana* (L.) Heynh sequence in Mesquite v. 2.75 (Maddison and Maddison 2009).

Phylogenetic analysis

Separate maximum parsimony bootstrap analyses were conducted on each gene region, including separate analysis of coding and noncoding regions of *ycf1*, to assess congruence between data sets using the following search parameters in PAUP* v.4.0b10 (Swofford 2000): tree bisection-reconnection (TBR) branch swapping, simple taxon addition, and saving no more than 1000 trees per replicate. The individual topologies were then considered similar based on visual comparison of clades with greater than 70% maximum parsimony bootstrap values (data not shown).

Phylogenetic relationships were determined using Bayesian inference implemented in MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001) for four data sets: chloroplast, mitochondrial, nuclear ribosomal, and total evidence (chloroplast, mitochondrial, and nuclear ribosomal). The following regions were subsequently treated as separate partitions in the total evidence analysis: *ITS1*, *matk*, *ndhF*, *rps3*, *ycf1*-coding, and *ycf1* non-coding. The most suitable model of evolution was determined independently for each partition using the Akaike information criterion (AIC) implemented in MrModelTest ver 2.3 (Nylander 2004). Bayesian analysis were run with default priors for one million generations for the combined chloroplast and mitochondrial data sets, two million generations for the combined chloroplast, and 6 million generations for the total combined analysis. Model parameters for each partition were estimated separately. The number of chains was increased to eight (four is default) and temperature lowered to 0.1 (default 0.2) after initial runs indicated these data were slow to converge.

Runs were stopped when the average standard deviation of split frequencies was less than 0.01, with the exception of total evidence (average deviation of split frequencies circa 0.1 after 6 million generations). Convergence was also confirmed by a potential scale reduction factor (PSRF) value approaching 1.0. Stationarity was achieved when a large effective sample size (ESS values >200) was reach as determined in Tracer 1.4.1 (Rambaut and Drummond 2007). The first 25% of trees recovered were discarded as burnin (trees produced prior to convergence). Clade support was also determined using maximum parsimony bootstrapping (BS) (Felsenstein 1985) with 1000 replicates of heuristic searching using the following parameters implemented in PAUP* v.4.0b10 (Swofford 2000): TBR branch swapping, simple taxon addition, and saving no more than 1000 trees per replicate.

Morphological characters and ancestral state reconstruction

Evolution of floral morphology was evaluated in a subset of taxa (70 species; 73%) for the purpose of ancestral state reconstruction (Appendix 2.1). The majority of species were scored from examination of available herbariums specimens (Table 2.2), while *C. violacea* was scored directly from fresh material. Characters were scored to reflect degree of monosymmetry: 1) visably enlarged abaxial sepal - present, absent; 2) differentiation of size and shape between abaxial and adaxial petal pairs - present, absent; 3) prominent adaxial nectar gland - present, absent; and 4) differentiation of colour between abaxial and adaxial petal pairs - present, absent. Ancestral state reconstruction was assessed using Maximum Likelihood (ML) criterion, specifically the one-parameter Markov *k*-state model, in Mesquite v. 2.75 (Maddison and Maddison 2009). Ancestral states at each node were reconstructed over the last 500 topologies recovered from the Bayesian analysis of the combined chloroplast data set to account for phylogenetic uncertainty (e.g., Reeb et al. 2004; Arnold et al. 2009). Using the trace characters-over-trees command, reconstructions were then summarized on the majority-rule consensus tree by counting trees with a uniquely best state, taking into account some branches are not present on all topologies.

Results

Sequence data

The aligned length of the data matrix including *matK* (1589 bp), *ndhF* (1109 bp), *ycf1* (2050 bp), *rps3* (1558 bp), and *ITS1* (1230 bp) was combined for a total length of 7536 bp for the 95 taxa included in this data set. A total of 2301 characters were parsimony informative. The most appropriate model of evolution was assessed for each gene separately and applied in partitioned Bayesian analysis: GTR + I + Γ (*ndhF*, *ycf1* coding, *rps3*, and *ITS1*) and GTR + Γ (*matK* and *ycf1* noncoding). These results are summarized far more succinctly in Table 2.4.

Phylogenetic reconstructions

Bayesian analysis of the chloroplast matrix retrieved the four major clades identified in Hall 2008: (1) Western North American cleomoids (Western N.A. cleomoids), (2) *Cleome* s. s. including the type species *C. ornithopodioides*, (3) *C. droserifolia* clade, and (4) a large *Polanisia clade* (Figure 2.1). Importantly, the relationships among these clades have strong support. The Western North American cleomoids are sister to all other clades (86 PP/ 99 BS). The C. *droserifolia* clade, including *C. droserifolia*, *C. fimbriata* and *C. quinquinervia*, is sister to all Cleomaceae excluding the Western N. A. cleomoids (100 PP/ 91 BS). The remaining two clades, *Cleome* s. s. clade and the *Polanisia* clade, are sister (100 PP/ 99 BS). The *Polanisia* clade can be subdivided into several smaller clades, which have been previously identified (Sanchex-Acebo 2005; Feodorova et al. 2010): a combined *Angustifolia* and Australian clade that includes *C. viscosa* (100 PP/ 90 BS), Clade 6 that includes *Dipterygium* (100 PP/ 97 BS), a *Gynandropsis* clade that includes *Gynandropsis gynandra* (96 PP/ 0 BS), a *Cleome* s. s. clade (99 PP/ 0 BS), a *Dactylaena* clade (forms a polytomy with *C. hemsleyana* clade), and the Andean clade (99 PP/ 63 BS) and *Tarenaya* clades (99 PP/ 62 BS). The *ITS1* analysis recovers well-supported clades, with low

support for backbone relationships comparable to Feodorova et al. 2010 (Figure 2.3). Analysis of *rps3* data supports a basal postition of a clade that includes *Dactylaena microphylla*, *Cleome monophylla*, *C. paludosa* (100 PP/99 BS), with no support for relationships among other clades (Figure 2.2). These results are not consistent with chloroplast or nuclear ribosomal data, and did not generate the same degree of resolution as the chloroplast data, even in the combined analysis (Figure 2.4). Subsequently, only the combined chloroplast phylogeny will be referenced in the discussion.

Floral evolution

Patterns of ancestral states across Cleomaceae are unclear because of limited sampling of taxa. Enlarged abaxial sepals occur in *C. hemsleyana*, Clade 6, *Angustifolia* clade, and *Cleome* s. s. clade (Figure 2.5). Differentiation of petal pairs occurs in *Cleome* s. s., and the African clade (sect. *Rutidosperma*) (Figure 2.6, and Figure 2.7). Differentiation in the stamen whorl is observed in the *Angustifolia* clade, Australian clade, Clade 6, the African clade, and *C. hemsleyana* (Figure 2.8). Adaxial glands are observed in the *Cleome* s. s. clade (Hall 2008), the Andean clade, and *Tarenaya* clade (Figure 2.9). None of the species observed within the Western N.A. cleomoids clade or the *C. droserifolia* clade show differentiation between adaxial and abaxial regions of any whorls. These results are summarized graphically in Figure 2.10.

Discussion

Increased genome and taxon sampling resolved relationships within Cleomaceae. First, the root of the family is between Western N.A. cleomoids and the rest of the family, which is consistent with Hall 2008, but not Feodorova et al. 2010. Second, the analyses presented resolved relationships amongst previously unplaced lineages: the *C. droserifolia* clade, the *C. ornithopodioides*, and the Western N.A. cleomoids. The phylogeny presented here is sufficient to discuss

evolutionary relationships between clades and reveals that the clade with the least monosymmetric flowers, the Western North American cleomoids, is basal in Cleomaceae. Furthermore, elaboration of floral monosymmetry due to abaxial/ abaxial differentiation within whorls is only observed in more derived clades.

Phylogenetic relationships in Cleomaceae

Difficulties resolving basal relationships within Cleomaceae, specifically the western N.A. cleomoids, *C. droserifolia* clade, and *Cleome* s. s. clade, have confounded efforts to examine evolution of ecologically fascinating novelties such as C_4 photosynthesis (Feodorova et al. 2010) and floral monosymmetry (Patchell et al. 2011). At the morphological level, the close relationship among these clades is supported by similarities in ornamentation of the pollen grains and shape of the seed cleft (Inda et al. 2008). At the molecular level, previous phylogenetic analyses of sequence data generated limited support along the backbone of the phylogeny (Hall et al. 2002; Sanchez-Acebo 2005; Hall 2008; Inda et al. 2008; Feodorova et al. 2011).

Bayesian analysis of chloroplast sequence data has increased support for placement of these early diverging clades in Cleomaceae. The Western N.A. cleomoids are the most basal lineage in Cleomaceae, which is consistent with relationships proposed previously with low statistical support (Hall 2008; Feodorova et al. 2010). The *C. droserifolia* clade is sister to the remaining taxa in Cleomaceae, excluding the western North American cleomoids. This clade was also identified in Hall 2008, with limited support for a sister relationship with the western North American cleomoids.

The position of the type species for the genus *Cleome*, *C. ornithopodioides* is particularly important. If *Cleome* will be divided into smaller genera, which is the current trend with at least New World taxa (Iltis and Cochrane 2007; Tucker and Vanderpool 2010), the clade that houses *C. ornithopodioides* will retain the name *Cleome*. Although this clade was previously identified (Hall 2008), its relationship to the *C. droserifolia* and western North American cleomoids clade was ambiguous. Importantly, the combined chloroplast Bayesian data and Hall

2008 phylogenetic hypothesis specify a different *Cleome* s. s. than the Feodorova et al. 2010 topologies. Inclusion of additional accessions for this taxon is necessary to clarify designation of the *Cleome* s.s. clade.

As with previous studies, analyses presented here indicate that *Cleome* is not monophyletic. However, the monophyly of *Dactylaena* (two species sampled), *Podandrogyne* (6 species sampled), and *Polanisia* (two species sampled) are supported, again consistent with previously work (Hall 2008; Feodorova et al. 2010). Interestingly, the analyses presented here suggest that newly the described genera *Hemiscola, Peritoma,* and *Tarenaya* (Iltis and Cochrane 2007; Tucker and Vanderpool 2010) are not monophyletic (Figure 2.1).

Floral symmetry evolution in Cleomaceae

Although limited sampling of morphological characters across the Cleomaceae does not produce strong support for ancestral states of clades, the distribution of morphological character states across the phylogeny is informative regarding floral symmetry evolution. The most basal lineage in Cleomaceae, the Western North American cleomoids, is also the least monosymmetric. No adaxial/ abaxial differentiation between organs is observed in the sepal, petal, or stamen whorl (Table 2.1; Figures 2.10). In this regard, they are similar to a typical brassicaceous flower. Instead, floral monosymmetry is due to abaxial/ adaxial differentiation within individual petals that result in upward curvature of the petal bases. Although *C. droserifolia* also shows monosymmetry due to curvature rather than adaxial/abaxial differentiation of organs within whorls, it cannot be concluded that this clade does not include highly monosymmetric members with the available data.

Floral monosymmetry in more diverged lineages is variable, but includes differentiation of adaxial/ abaxial regions of the flower in at least one whorl, in addition to petal curvature. Strong differentiation of adaxial/ abaxial regions of the flower is first observed in the *Cleome* s. s. clade (Hall 2008) that includes the highly monosymmetric taxa *C. violacea*. Some taxa in this clade have differentiation of shape, size, and colour of adaxial and abaxial petals, size of the

abaxial sepal, and an enlarged adaxial nectar gland. Monosymmetry within whorls is labile in these derived clades, including at least one reversion to polysymmetry in *Dipterygium glaucum* (Hall et al. 2002).

The short internodes of the backbone between early diverging lineages in the combined chloroplast Bayesian topology is consistent with a periods of rapid speciation observed in other plant lineages (Hilu et al. 1999; Davis et al. 2005; Jian et al. 2008). Furthermore, these radiations are often associated with the origin of floral monosymmetry (Steele et al. 1994; Johnson and Soltis 1995; Moore et al. 2007; Wurdack and Davis 2009). Rapid speciation following divergence from the Brassicaceae would account for short branch lengths along the backbone of the phylogeny. Bayesian posterior probabilities for these relationships between clades are high. Although support for these relationships is lower based on corresponding MP bootstrap values, Bayesian posterior probabilities more accurately reflect relationships (Alfaro et al. 2003).

Conclusion

Phylogenetic relationships within Cleomaceae establish a framework with which to interpret floral evolution. The earliest diverging lineage, the Western North American cleomoids, exhibits the least monosymmetric flowers. In these flowers, monosymmetry is due to upward curvature of petal bases, but no abaxial/ adaxial differentiation within whorls. In more derived lineages, monosymmetry is due to differentiation of adaxial/ abaxial organs within whorls. Additional scoring of morphology based on herbarium specimens, descriptions in the literature (Iltis 1957; Iltis and Cochrane 2007), and existing data sets (Hall et al. 2002) will clarify the level of morphological variation within clades and facilitate less ambiguous ancestral state reconstructions.

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Tables

Table 2.1. Summary of Cleomaceae morphology and historical generic delimitations. Descriptions compiled from De Candolle 1824, Eichler 1865, Ducke 1930, Iltis 1957, Iltis 1958, Kers 2003.

Clade/ Genus	Distinctive Morphology	Floral Symmetry
***Cleomella* DC.**	Ovate-globose torus and stipitate silicula.	Not highly monosymmetric.
***Dactylaena* Schrad. ex Schult. f.**	Extreme stamen reduction - only a single fertile, adaxial; staminodes abaxial. Four linear petals and one fertile and four sterile stamens.	Monosymmetric; one sepal (abaxial?) larger than the others; petal pairs differentiated with smaller pair resembling staminodia; four sterile staminodes, one fertile stamen. Six species, New World. Supposed no close relationship to sect. *Dianthera* (Old World; two stamens), but rather to *Haptocarpum* in new world.
***Dipterygium* Deene.**	Cruciferous flowers, although without tetradynamous stamens. Fruit eseptate (without replum), but resembling some few seeded eseptate members of Brassicaceae. Not much evidence to suggest close relatives based on chemical or morphological data.	Not monosymmetric, flowers small and not beautiful.
***Gynandropsis* (L.) Briq.**	Has a torus, that of the latter "subhemisphaericus" and that of the former "elongates". Elongated torus base.	Monosymmetric, resembling dioecious *Podandrogyne*.
***Haptocarpum* Ule**	Only has abaxial petals; claw is wider than the blade (these are separated by a fold or ridge); four fertile stamens are adaxial, and opposed by two fused petaloid staminodes perched on a laminate protuberance; back of this double staminode is glandular projection of the disk. Plant is a vine, and uses two-pronged remains of replum as holdfasts in clambering over surrounding vegetation.	Highly monosymmetric; distinctive siliques and overlapping range and similar habitat indicate close relationship to *Dactylaena*.
***Oxystylis* Torr. & Frem.**	Globose racemose and ebracteate inflorescences, spinescent schizocarpic fruits with one-seeded mericarps (Iltis 1957; Kers 2003).	Monosymmetry within whorls not observed.
***Peritoma* DC.**	Calyx with circumscissile base and four dentate apex and monodelphus androecium.	Monosymmetry within whorls not observed.

***Physostemon* Mart. & Zucc.**	Open corolla aestivation, and 6-8 stamens that are usually inflated apically.	Monosymmetry within whorls not observed.
***Podandrogyne* Ducke**	Robust, sometimes woody herbs of American tropics, about 36 species, related to *Adinocleome* but segregated based on short to elongate androgynophore, unisexual flowers in monoecious racemes, arillate seeds, distinct fruit dehiscence (irregularly twisted) (Ducke in Archiv. Jard. Bot. Riode Janeiro 5: 115. pl. 7. 1930). Earliest flowers are pistillate with aborted stamens, and then alternating pistillate and staminate.	Monosymmetric. Conspicuous, brightly coloured adaxial gland. Petals fused laterally and curved upward adaxially in staminate flowers, free in pistillate flowers. Some small differentiation of petal pairs.
***Polanisia* Raf.**	Historically included any species with more than six stamens (De Candolle 1824), but more naturally lobed adaxial petals and brightly coloured adaxial gland.	Monosymmetric. Conspicuous adaxial gland. Differentiation in size/ shape (degree of lobbing). Also staggered maturation of stamens, open aestivation. Suspected affinity to Old World taxa based on morphology.
***Wislizenia* Engelm.**	Dense racemose ebracteate inflorescences and schizocarp fruits with 1-2(3) seeded mericarps.	Monosymmetry within whorls not observed.

Table 2.2. Accession table: taxa sampling, voucher identification (herbaria), geographic distribution, and GenBank accession numbers for samples included in this study. Species are listed alphabetically. Missing sequence data is indicated by "--". Sequences generated in this study are indicated by Genbank# in bold font. Herbarium vouchers are specified by citation to identify the original paper where sequences were published, the name of the collector and specimen number, followed by herbarium accession number. Herbarium acronyms are consistent with the Index Herbariorum (Thiers, continuously updated). Not all information is available for all taxa.

Taxon	**Herbarium Voucher**	**Geographic origin of specimen**	***ndhF***	***matK***	***YCF***	***rsp3***	***ITS***
Brassicaceae							
Aethionema arabica (L.) Rothm	[no voucher listed]	[no voucher listed]	--	--	--	--	AY254539
Aethionema saxatile R. Br.	Moore. s. n. (WIS)	[no country listed]	AY483250	EU371817	--	**Genbank#**	--
Arabidopsis thaliana L. Heynh	Inda et al. 2008; "Columbia" ecotipo: Hall s.n. (WIS)	[no country listed]	--	--	--	--	AJ232900
Arabidopsis thaliana (L.) Heynh.	Hall et al. 2002; Kock et al. 2001	[no country listed]	AY122394	AF144348	**Genbank#**	**Genbank#**	
Barbarea vulgaris R. Br.	Moore 9 (WIS)	[no country listed]	AY122395	EU371818	--	--	--
Brassica nigra (L.) W.D.J Koch	Inda et al. 2008; [no voucher listed]	[no voucher listed]	--	JN584951	--	--	AF128103
Brassica rapa L.	Inda et al. 2008; [no voucher listed]	[no voucher listed]	--	--	--	--	AF128098
Capsella bursa-pastoris (L.) Medik.	Moore 4; [no voucher listed]	[no voucher listed]	**Genbank#**	**Genbank#**	**Genbank#**	--	--
Iberis oppositifolia Pers.	Hall 2008; Cochrane 6 Apr. 2000	[no country listed]	AY122398	EU371819	--	**Genbank#**	--
Iberis spathulata Lag. ex Willk. & Lange	Feodorova et al. 2010; [no voucher listed]	[no voucher listed]	--	--	--	--	AJ440312
Nasturtium officinale R. Br.	Hall 2008; Stahmann 233 (WIS)	[no country listed]	AY122399	AY483225	**Genbank#**	--	--
Sisymbrium altissimum L.	Hall 2008; Leach et al. 1939	[no country listed]	--	JN585004	**Genbank#**	--	--
Stanleya pinnata (Pursh) Britton	Hall 2008; 1 (AZ)	[no country listed]	AY122401	AY483226	**Genbank#**	**Genbank#**	--

Taxon	Herbarium Voucher	Geographic origin of specimen	*ndhF*	*matK*	*YCF*	*rsp3*	*ITS*
Stanleya pinnata (Pursh) Britton	Feodorova et al. 2010; R. Price s. n. (GA)	[no country listed]	--	--	--	--	AF531620
Cleomaceae							
Arivela viscosa (L.) Raf. [=*Cleome viscosa* L.]	J.D. Sauer 3492 (WIS)	[no country listed]	EU373714	EU371806	**Genbank#**	--	**Genbank#**
Carsonia sparsifolia (S. Wats.) Greene [= *Cleome sparsifolia* S. Wats]	Feodorova et al. 2010	USA: Nevada	--	--	--	--	DQ455805
Cleome africana Botch.	Feodorova et al. 2010; E. Voznesenskaya 1 (WS)	Egypt (WSUG)	--	--	--	--	HM044222
Cleome africana Botsch.	Hall & Taggart (ALTA)	Canada	HQ452951	HQ452946	--	--	--
Cleome afrospina H. H. Iltis	Feodorova et al. 2010; F. J. Breteler 696 (MO)	Gabon	**Genbank#**	**Genbank#**	--	**Genbank#**	**Genbank#**
Cleome allamanii Chiov.	Feodorova et al. 2010; Agnew et al. 10879	Kenya	**Genbank#**	**Genbank#**	**Genbank#**	--	**Genbank#**
Cleome ambylocarpa Baratte & Murb.	Mankowski (ALTA)	[no country listed]	HQ452952	HQ452947	--	--	--
Cleome angustifolia Forssk.	Feodorova et al. 2010; O. Maurin s. n. (WS)	South Africa: National Park Kruger	--	--	--	--	HM044250
Cleome anomala Kunth	Inda et al. 2008; T. Ruiz y L. Hernandez 4980 (MY)	Venezuela: Tachira	--	--	--	--	DQ455782
Cleome arabica L.	J.C. Hall greenhouse (ALTA)	[no country listed]	EU373701	EU371791	**Genbank#**	--	**Genbank#**
Cleome arborea Kunth.	Feodorova et al. 2010; T. Ruiz y L. Hernandez 4981 (MY)	Venezuela: Las Chorreras de las Gonzalez, Merida	--	--	--	--	DQ455783
Cleome boliviensis boliviensis H. H. Iltis	Sanchez A. L., Aizama S. and Saravia C. 1122 (MO)	Bolivia	**Genbank#**	**Genbank#**	--	--	**Genbank#**
Cleome brachycarpa Vahl. ex DC.	J.C. Hall & Taggart (ALTA)		HQ452953	HQ452948	**Genbank#**	--	--

Taxon	Herbarium Voucher	Geographic origin of specimen	*ndhF*	*matK*	*YCF*	*rsp3*	*ITS*
Cleome breyeri B. Davy	Feodorova; A. O. D. Mogg et al. 19159 (MO)	South Africa: Norscot	**Genbank#**	**Genbank#**	**Genbank#**	--	**Genbank#**
Cleome briquetii Polhill	R. B. and A. J. Faden 74 (MO)	Kenya	**Genbank#**	Genbank#	**Genbank#**	**Genbank#**	**Genbank#**
Cleome burtii R. A. Graham	Sally Bidgood, L. Mwasumbi, and K. Vollesen	Tanzania	**Genbank#**	**Genbank#**	--	**Genbank#**	--
Cleome chalapensis H. H. Iltis	H. H. Iltis et al. 832 (USZ)	Mexico: Michoacan	--	--	--	--	DQ455800
Cleome chiliensis DC.	F. Billiet and B. Jadin (MO)	Chile	**Genbank#**	**Genbank#**	--	**Genbank#**	--
Cleome chrysantha Decne.	J. Leonard 4879 (MO)	Libya	**Genbank#**	**Genbank#**	--	--	--
Cleome cleomoides (F. Muell.) H. H. Iltis	Accession: 55989901 (MO)	[no country listed]	**Genbank#**	**Genbank#**	--	--	**Genbank#**
Cleome coluteoides Boiss	V. Btachanzev 136 (LE)	Turkmenistan	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**
Cleome cordobensis Eichler ex Grisebach	S. Victoria 1733 (MO)	Argentina	**Genbank#**	**Genbank#**	--	--	**Genbank#**
Cleome crenopetala DC	Inda et al. 2008; P. Dusen 7365 (MO)	Brasil: Parana	--	--	--	--	DQ455788
Cleome densifolia C. H. Wright	Accession: 3245723 (MO)	[no country listed]	--	--	--	--	**Genbank#**
Cleome diandra Burch.	J. J. F. E. De Wilde 5456 (MO)	Ethiopia	**Genbank#**	--	--	--	**Genbank#**
Cleome domingensis Iltis	DNA 2/17/89 [85-01-4]	[no country listed]	AY122383	EU371793	**Genbank#**	**Genbank#**	**Genbank#**
Cleome droserifolia (Forssk.) Del.	A.G. Miller 6387 (WIS)	[no country listed]	EU373703	EU371794	**Genbank#**	**Genbank#**	--
Cleome droserifolia (Forssk.) Delile	Feodorova et al. 2010; E. Voznesenskaya 41 (WS)	Egypt (WSUG)	--	--	--	--	HM044229
Cleome elegantissima Briq.	Lars Erik Kers 3651 (MO)	Angola	**Genbank#**	**Genbank#**	--	--	**Genbank#**
Cleome espinosa Jacq.	C. Grandez, G. Baquero, and G. Criollo 17060 (MO)	Peru	**Genbank#**	--	--	--	**Genbank#**

Taxon	Herbarium Voucher	Geographic origin of specimen	*ndhF*	*matK*	*YCF*	*rsp3*	*ITS*
Cleome fimbriata Vicary	Feodorova et al. 2010; V. Botchanzv 159a (LE)	Uzbekistan	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**
Cleome foliosa Hook. f.	Hall 2008; L. E. Kers 1750 (WIS)	[no country listed]	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**
Cleome gigantea L.	M. Smith s. n. (WS)	Prague Bot. Garden (WSUG)	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**
Cleome hemsleyana (Bullock) H. H. Iltis	R. L. Wilbur 36639 (MO)	Mexico	--	**Genbank#**	--	--	--
Cleome hirta (Klotzch) Oliv.	Hall 2008; Bayliss 10731	[no country listed]	HQ452949	HQ452954	--	--	--
Cleome hirta (Klotzch) Oliv.	Feodorova et al. 2010; N. A Mwangulango 791 (MO)	Tanzania	--	--	--	--	HM044264
Cleome iberidella Welw. ex Oliv.	Bidgood, I Darbyhire et al. (MO)	Tanzania	**Genbank#**	**Genbank#**			**Genbank#**
Cleome kalachariensis (Schinz) Gilg. and Ben	P. M. Burgoyne and N Snow	Namibia	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**
Cleome khorassanica Bunge and Bien. ex Boiss	D. Bukinich s. n. (LE)	Afganistan	**Genbank#**	**Genbank#**	--	**Genbank#**	**Genbank#**
Cleome lanceolata (Mart. & Zucc.) H. H. Iltis	R. W. Harley (MO)	Brazil	--	--	--	--	**Genbank**
Cleome lechleri Eichl.	Hall 2008; J. C. Solomaon & M. Morales 17236 (WIS)	[no country listed]	**Genbank#**	**Genbank#**	--	--	--
Cleome linearifolia (Stephens) Dinter	Feodorova et al. 2010; W. Giess et al. 5785	Namibia	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**
Cleome luderitziana Schinz	Feodorova et al. 2010; M. Bourele et al. 2827 (MO)	Namibia	**Genbank#**	**Genbank#**	**Genbank#**	--	**Genbank#**
Cleome macrophylla (Klotzsch) Briz. var. *macrophylla*	Feodorova et al. 2010; H. H. Schmidt et al. 2346 (MO)	Zambia	**Genbank#**	--	**Genbank#**	**Genbank#**	**Genbank#**

Taxon	Herbarium Voucher	Geographic origin of specimen	*ndhF*	*matK*	*YCF*	*rsp3*	*ITS*
Cleome maculata (Sond.) Szyszyl *556*	Feodorova et al. 2010; Balkwill et al. 5421 (MO)	South Africa: Transvaal	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	HM044263
Cleome microaustralica H. H. Iltis	Feodorova et al. 2010; A. V. Slee s. n. (CANB)	Northern Territory, Australia	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**
Cleome microcarpa Ule	Feodorova et al. 2010; R. M Harley 27228 (MO)	Brazil	--	--	--	--	DQ455793
Cleome microcarpa Ule.	Andre, M. A. & Amerin 1799 (WIS)	Brazil	**Genbank#**	**Genbank#**	--	--	--
Cleome monochroma J.F. Macbr.	Feodorova et al. 2010; P. Kuchar 23051	Tanzania	**Genbank#**	**Genbank#**	--	**Genbank#**	**Genbank#**
Cleome monophylla L.	Hall 2008; R. E. Gereau & C. J. Kayombo 3951 (MO)	[no country listed]	AY122384	EU371798	**Genbank#**	**Genbank#**	--
Cleome moritziana Klotzsch ex Eichler	Feodorova et al. 2010; T. Ruiz y L. Hernandez 4984 (MY)	Venezuela: Merida	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**
Cleome ornithopodioides L.	Hall 2008; WIS Botanical Garden	[no country listed]	EU373707	EU371799	**Genbank#**	**Genbank#**	**Genbank#**
Cleome oxalidea F. Muell	Feodorova et al. 2010; P. A. Fryxell 3958	Western Australia, Australia	**Genbank#**	**Genbank#**	--	**Genbank#**	**Genbank#**
Cleome oxyphylla Bursh.	Hall 2008; L.E. Kers 3003 (WIS)	[no country listed]	EU373708	EU371800	**Genbank#**	--	**Genbank#**
Cleome paradoxa R. Br. ex DC	Feodorova et al. 2010; E. Voznesenskaya 43 (WS)	Yemen (WSUG)	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**
Cleome parviflora Humboldt, Bonpland & Kunth subsp. *psoralaeifolia* (DC.) Iltis [=*Cleome psoralaeifolia* DC.]	Hall 2008; R. Seidel 321 (WIS)	[no country listed]	EU373709	EU371801	**Genbank#**	**Genbank#**	**Genbank#**
Cleome pernambucensis H. H. Iltis, Costa & Silva, ined.	Feodorova et al. 2010: Costa e Silva 1529 (MO)	Brazil: Pernambuco	--	--	--	--	DQ455798

Taxon	Herbarium Voucher	Geographic origin of specimen	*ndhF*	*matK*	*YCF*	*rsp3*	*ITS*
Cleome pilosa Benth.	Hall 2008; H.H. Iltis 30585 (WIS)	[no country listed]	AY122385	AY483231	**Genbank#**	**Genbank#**	--
Cleome quinquenervia DC.	Feodorova et al. 2010; E. Leontieva 127 (LE)	Turkmenistan	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**
Cleome rosea Vahl. ex DC. [= *Tarenaya rosea*, not sure if formalized yet]	Hall 2008; Ex Rio bot; JH greenhouse (WIS)	[no country listed]	EU373710	EU371802	**Genbank#**	**Genbank#**	**Genbank#**
Cleome rotundifolia Mart. & Zucc.	R. M. Harley 27032 (MO)	Brazil	**Genbank#**	**Genbank#**	--	--	**Genbank#**
Cleome rutidosperma DC	Hall 2008; A. A. Mitchell 6380 (WIS)	[no country listed]	**Genbank#**	**Genbank#**	**Genbank#**	--	--
Cleome rutidosperma DC	Feodorova et al 2010; T. Ruiz 4360 (MY)	Venezuela: Maracay	--	--	--	--	DQ455802
Cleome schimperi Pax	Feodorova et al. 2010; L. Festo &W. Bayona 1729 (MO)	Tanzania	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	HM044273
Cleome schweinforthii Gilg.	W. J. J. O de Wilde and B. E. E de Wilde-Duyfjes	Ethiopia	--	**Genbank#**	--	**Genbank#**	**Genbank#**
Cleome siliculifera Eichler	Feodorova; et al. 2010 R. M. Harley 26987 (NY)	Brazil: Bahia	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	HM044286
Cleome stenophylla Klotzsch ex urban	Inda 2008; T. Ruiz y R. Villafane 4987 (MY)	Venezuela: Guarico	--	--	--	--	DQ455814
Cleome strigosa (Boj.) Oliv.	F. R. Fosberg (MO)	U. S. A.: Colorado	--	--	--	--	**Genbank#**
Cleome stylosa Eichler	Feodorova et al. 21010; R. Ruiz y L. Hernandez 4977 (MY)	Venezuela: Tachira	--	--	--	--	DQ455812
Cleome sulfurea Bremek. & Oberm.	H. Wild 5131 (MO)	Zimbabwe	**Genbank#**	**Genbank#**	--	--	--
Cleome tenuifolia (Mart. & Zucc.) H. H. Iltis	R. M. Harley 163525 (NY)	Brazil: Bahia	--	--	--	--	HM044280

Taxon	Herbarium Voucher	Geographic origin of specimen	*ndhF*	*matK*	*YCF*	*rsp3*	*ITS*
Cleome tetranda f. *linophylla* (O. Schwarz) Iltis	Mitchell C. R. 3659 (MO)	Australia	**Genbank#**	**Genbank#**	--	--	--
Cleome titubans Spegaz	Feodorova et al. 2010; A. Krapovickas 2897 (MO)	Argentina: Buenos Aires	--	--	--	--	DQ455813
Cleome torticarpa H. H Iltis & T. Ruiz Zapata	Feodorova et al. 2010; T. Ruiz y R. Villafane 5011 (MO)	Venezuela: Falcon	--	--	--	--	DQ455810
Cleome trachycarpa Klotsch ex Eichler	Feodorova et al. 2010; A. Drapovickas & CL. Critobal 46421	Argentina	**Genbank#**	**Genbank#**	--	**Genbank#**	**Genbank#**
Cleome tucumanensis H. H. Iltis	R. Fortunato 6639 (MO)	Argentina	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	HM044291
Cleome turkmena Bobrov	Feodorova et al. 2010; D. Kurbanov 1055 (MO)	Turkmenistan	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**
Cleome uncifera Kers.	Feodorova et al. 2010; B. J. Pepschi and L. A. Craven 5624 (CANB)	Western Australia	**Genbank#**	**Genbank#**	**Genbank#**	--	**Genbank#**
Cleome usambarica Pax	M. A. Mwangoka 2967 (MO)	Tanzania	**Genbank#**	**Genbank#**	**Genbank#**	--	**Genbank#**
Cleome violacea L.	M. Bolton __ (ALTA)	[no country listed]	HQ452955	HQ452950	**Genbank#**	**Genbank#**	**Genbank#**
Cleome viridiflora Schreb	Feodorova et al. 2010; T. Ruiz y L. Herbandez 4987 (MY)	Venezuela: Barinitas, Barinas	--	--	--	--	DQ455820
Cleome viridiflora Schreb.	Solomon s.n. (MO)	[no country listed]	AY122386	AY483232	**Genbank#**	**Genbank#**	--
Cleome werdermannii Ernst	Feodorova; Sanchez 111a (MO)	Bolivia: Santa Cruz	**Genbank#**	**Genbank#**	--	--	DQ455809
Cleomella longipes Torr.	S. Vaderpool 1334 (OKL)	[no country listed]	AY122387	EU371807	**Genbank#**	**Genbank#**	**Genbank#**
Cleomella obtusifolia Torr. & Frem.	Hall 2008; S. Vanderpool1293 (OKL)	[no country listed]	EU373715	EU371808	--	--	--

Taxon	Herbarium Voucher	Geographic origin of specimen	*ndhF*	*matK*	*YCF*	*rsp3*	*ITS*
Cleoserrata melanosperma (S. wats.) H.H. Iltis [= *Cleome melanosperma* S. Wats.][1]	Feodorova et al. 2010; R. L. Reina G. 98-853 (NY)	Mexico: Sonora	--	--	--	--	HM044284
Cleoserrata paludosa (Willd. Ex Eichler) H.H. Iltis [= *Cleome paludosa* Willd. ex Eichler [C97][1]	Feodorova et al. 2010; R. H. Fortunato 2874 (MO)	Argentina	**Genbank#**	**Genbank#**	--	**Genbank#**	**Genbank#**
Cleoserrata speciosa (Raf.) H.H.Iltis [= *Cleome speciosa* Raf.]	Feodorova et al. 2010; R. Ruiz y L. Hernandez 4978 (MY)	Venezuela: Tachira	--	--	--	--	DQ455806
Dactylaena microphylla Eichler	Hall 2008; R. M. Harley 26503 B. Stannard &D. J. N. Hind (MO)	[no country listed]	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	--
Dactylaena microphylla Eichler	Feodorova et al. 2010; Callejas & A.M de Carvalho 1729 (NY)	Brazil: Bahia	--	--	--	--	HM044279
Dactylaena pauciflora Griseb.	J.C. Solomon & M. Nee 18108 (MO)	[no country listed]	EU373717	EU371810	**Genbank#**	--	--
Dipterygium glaucum Decne.	M.I. Bajwa 972-75 (MO)	[no country listed]	EU373718	EU371811	--	--	**Genbank#**
Gynandropsis gynandra (L.) Briq. [= *Cleome gynandra* L.]	Hall 2008; 238	[no country listed]	HQ452954	HQ452949 #	**Genbank#**	**Genbank#**	--
Gynandropsis gynandra (L.) Briq. [= *Cleome gynandra* L.]	Feodorova et al. 2010; I. D. Cowie s. n. (CANB)	Australia: Queensland	--	--	--	--	HM044253
Hemiscola aculeata (L.) Raf. [= *Cleome aculeata* L.]	H.H. Iltis 30563a (WIS)	[no country listed]	AY122382	EU371790	**Genbank#**	**Genbank#**	--

Taxon	Herbarium Voucher	Geographic origin of specimen	*ndhF*	*matK*	*YCF*	*rsp3*	*ITS*
Hemiscola aculeata (L.) Raf. [= *Cleome aculeata* L.]	Feodorova et al. 2010; F. Billeit & B. Jadin 7445 (MO)	French Guiana	--	--	--	--	HM044288
Hemiscola diffusa (Banks ex DC.) H.H. Iltis [= *Cleome diffusa* Banks ex DC.]	Follii 3782 (WIS)	[no country listed]	EU373702	EU371792	**Genbank#**	**Genbank#**	**Genbank#**
Oxystylis lutea Torr & Frem.	S. Vanderpool 1340 (WIS)	[no country listed]	AY122390	EU371814	**Genbank#**	**Genbank#**	**Genbank#**
Peritoma arborea (Nutt.) H.H. Iltis [=*Isomeris arborea* Nutt ex. Torr. & Gray]	M. Fishbein 4146 (WS)	[no country listed]	AY122389	EU371813	**Genbank#**	--	--
Peritoma arborea (Nutt.) H.H. Iltis [=*Isomeris arborea* Nutt ex. Torr. & Gray]	Feodorova et al. 2010; E. Voznesenskaya 6 (WS)	[no country listed]	--	--	--	--	HM044239
Peritoma lutea (Hook.) Raf. [=*Cleome lutea* Hook. subsp. *jonesii* (Macbr.) Iltis]	S. Vanderpool 1007 (OKL)	[no country listed]	EU373706	EU371797	--	**Genbank#**	**Genbank#**
Peritoma multicaulis (DC) H.H. Iltis [=*Cleome multicaulis* DC]	Inda 2008; H. Iltis 4359	U.S.A.: Colorado	--	--	--	--	DQ455795
Peritoma playtcarpa (Torr.) H.H. Iltis [= *Cleome platycarpa* Torr.]	Feodorova et al. 2010; A. Tiehm 8030 (WS)	U.S.A.: Nevada	--	--	--	--	HM044234

Taxon	Herbarium Voucher	Geographic origin of specimen	*ndhF*	*matK*	*YCF*	*rsp3*	*ITS*
Peritoma serrulata (Pursh) DC. [=*Cleome serrulata* Pursh]	M. Patchell (ALTA)	Canada: Alberta	**Genbank#**	**Genbank#**	**Genbank#**	**Genbank#**	
Podandrogyne chiriquensis (Standl.) Woodson	Feodorova et al. 2010; J. & K. Utley 4533 (MO)	Costa Rica	AY122393	AY483233	--	--	HM044281
Podandrogyne decipiens (Triana & Planch.) Woodson	Hall 2008; G. Mora 380	[no country listed]	EU373719	EU371815	--	--	--
Podandrogyne jamesonii (Briq.) T. S. Cochrane	Feodorova et al. 2010; G. P. Lewis et al. 3438 (MO)	Ecuador	--	--	--	--	HM044282
Podandrogyne jamesonii (Briq.) T. S. Cochrane	Hall 276	[no country listed]	--	--	**Genbank#**	**Genbank#**	--
Podandrogyne macrophylla (Turcs.) Woodson	Feodorova et al. 2010; T. Ruiz y L. Hernandez 4982	Venezuela: Merida	--	--	--	--	DQ455815
Podandrogyne mathewsii (Briq.) Cochrane	J.R.I. Wood 11536 (K)	[no country listed]	EU373720	EU371816	**Genbank#**	--	--
Podandrogyne pulcherrima (Standley) Woodson	Hall 2008; M.N. 45	[no country listed]	AY122393	AY483233	--	**Genbank#**	--
Podandrogyne pulcherrima (Standley) Woodson	Hall 2008; M. N. s. n.	[no country listed]	--	--	**Genbank#**	--	--
Polanisia dodecandra DC.	D.F. Grether 8603 (WIS)	[no country listed]	AY483251	AY483234	**Genbank#**	**Genbank#**	**Genbank#**
Polanisia uniglandulosa DC.	Feodorova et al. 2010; Stanford et al. 2098 (WS)	Mexico	--	--	--	--	HM044225
Tarenaya hassliarina (Chodat) H.H. Iltis [=*Cleome hassleriana* Chodat]	E. Voznesenskaya 6 (WS)	Harris Seeds #2285, Rochester, NY (WSUG)	**Genbank#**	**Genbank#**	**Genbank#**	--	HM044293

Taxon	Herbarium Voucher	Geographic origin of specimen	*ndhF*	*matK*	*YCF*	*rsp3*	*ITS*
Tarenaya spinosa (Jacq.) Raf. [= *Cleome spinosa* Jacq.]	Hall 2008; G. Ayala 91-11 (WIS)	[no country listed]	EU373713	EU371805	**Genbank#**	**Genbank#**	--
Tarenaya spinosa (Jacq.) Raf. [= *Cleome spinosa* Jacq.]	Feodorova et al. 2010: A. Grable 11178 (WS)	Puerto Rico	--	--	--	--	HM045529 6
Wislizenia refracta Engelm. subsp. *refracta*	Hall 2008; S. Vanderpool 1340 (OKL)	[no country listed]	AY122391	AY483235	**Genbank#**	**Genbank#**	**Genbank#**

[1] Listed in Iltis and Cochrane 2007, but not 100% certain formally moved from *Cleome*.

Table 2.3. Primer list used in PCR amplification and sequencing reactions.

Region	Primers name	Application	Primer Sequence (5'-3')
matK	trnK710F	amplification of whole region, or front half/ sequencing	CGCACTATGTGTCATTTCAGAACTC
matK	*matK495F*	amplification of back half/ sequencing	CTTGGTTCAAACCCTACGTTACCG
matK	*matK1010R*	amplification front half/ sequencing	CCACTAAAGGATTTAATCGCAAAC
matK	*trnK2R*	amplification of whole region, or back half/ sequencing	CCCGGAACTAGTCGGATGGAG
matK	*matK454R*	sequencing	CGGTAACGTAGGGTTTGAACCAAG
matK	*matK1010F*	sequencing	GTTTGCGATTAAATCCTTTAGTGG
ndhF	*ndhF972F*	amplification of whole region, or front half/ sequencing	GTCTCAATTGGGTTATATGATG
ndhF	*ndhF 1703R*	amplification of front half/ sequencing	GGCTCCAATAAAYAAAGT
ndhF	*ndhF1318F*	amplification of back half/ sequencing	GGATTAACTGCATTTTATATGTTTCG
ndhF	*ndhF2110R*	amplification of back half/ sequencing	CCCCCTA(C/T)ATATTTGATACCTTCTCC
ycf1	*rps15 rev*	amplification of whole region or front half/ sequencing	CAATTYCAAATGTGAAGTAAGTCTCC
ycf1	*YCF4497F*	amplification of whole region or front half/ sequencing	TKGATTGGATGGGRWTGAATG
ycf1	*YCF5778R*	amplification of front half/ sequencing	CAWAYGTATCCTTAASATACTGAAACG
ycf1	*YCF5710F*	amplification of back half/ sequencing	GCTTGTATGAATCGYTATTGGTTTG
ycf1	*YCF65F*	sequencing	AGAAACCGTGGGTGATAC
ycf1	*YCF950F*	sequencing	GTTCTTTCTTTGGCCCAATTTTCG

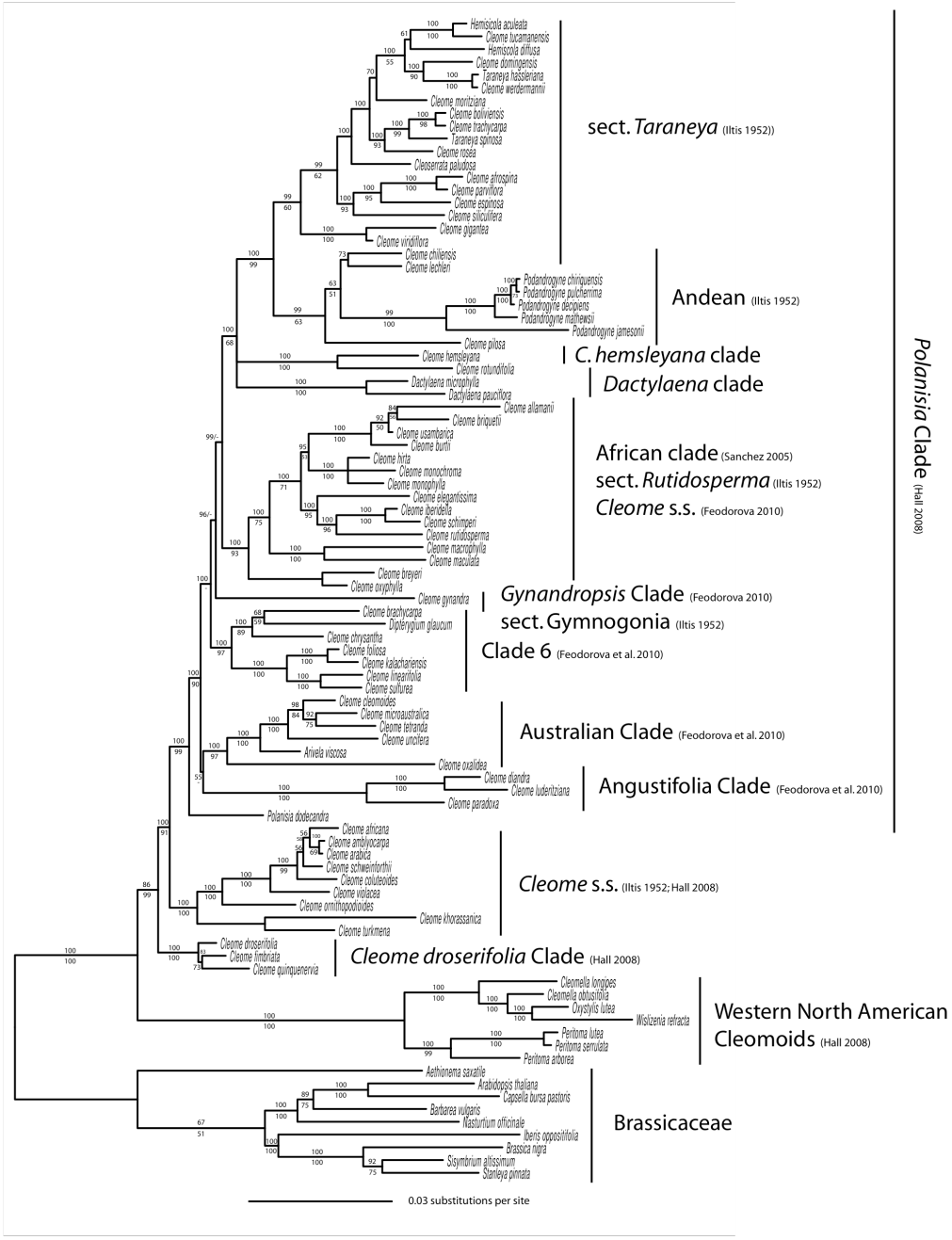
ycf1	*YCF785F*	sequencing	CTAAACGACGTAGAGAATTTCG
ycf1	*YCF1310R*	sequencing	GATTCGGATAGGTATCCAAAACGCA
rps3	*rps3F1*	amplification of whole regions or front half/ sequencing	GTTCGATACGTCCACCTAC
rps3	*rps3R1.5*	amplification of front half/ sequencing	CTATTCCCTTTATCAATTCTCCTAT
rps3	*rps3F2*	amplification of back half/ sequencing	CCCGTCGTAGTTCTCAATCATTTYG
rps3	*rps3R1*	amplification of whole region or back half/ sequencing	GTACGTTTCGGATATRGCA
rps3	*rps3F3*	sequencing	CGKGGCCTWCAAGCATCC
ITS1	*BMBCR*	amplification of whole region or front half/ sequencing	GTACACACCGCCCGTCG
ITS1	*ITS2*	amplification of front half/ sequencing	GCTGCGTTCTTCATCGATGC
ITS1	*ITS3*	amplification of back half/ sequencing	GCATCGATGAAGAACGCAGC
ITS1	*ITS4*	amplification of whole region or back half/ sequencing	TCCTCCGCTTATTGATATGC

Table 2.4. Summary of phylogenetic datasets, including sequence data for Cleomaceae and outgroup Brassicaceae. The chloroplast data set includes *matK*, *ndhF*, and *ycf1*. The total evidence data set includes sequence data from all five genes.

	ITS1	*matK*	*ndhF*	*ycf1*	*rps3*	Chloroplast	Total evidence
Number of accessions sampled	95	86	85	60	49	86	95
Number of characters	1230	1589	1109	2050	1558	4748	8271
Variable characters	657	879	420	1024	410	2154	3554
Parsimony-informative characters	500	710	274	668	198	1404	2301
Percent missing data	0.40	0.091	0.067	0.32	0.18	0.16	0.51
Bayesian							
Model of molecular evolution	*GTR + I + Γ*	GTR + Γ	GTR + I + Γ	GTR + I + Γ (coding) GTR + Γ (noncoding)	GTR + I + Γ	NA	NA

Figures

Figure 2.1. Phylogenetic relationships within Cleomaceae. Bayesian 50% majority rule consensus tree inferred from chloroplast (*matK*, *ndhF*, and *ycf1*) sequence data. Posterior probabilities greater than 70% are indicated above branches; bootstrap values greater than 50% are indicated below branches.


Hemiscola aculeata
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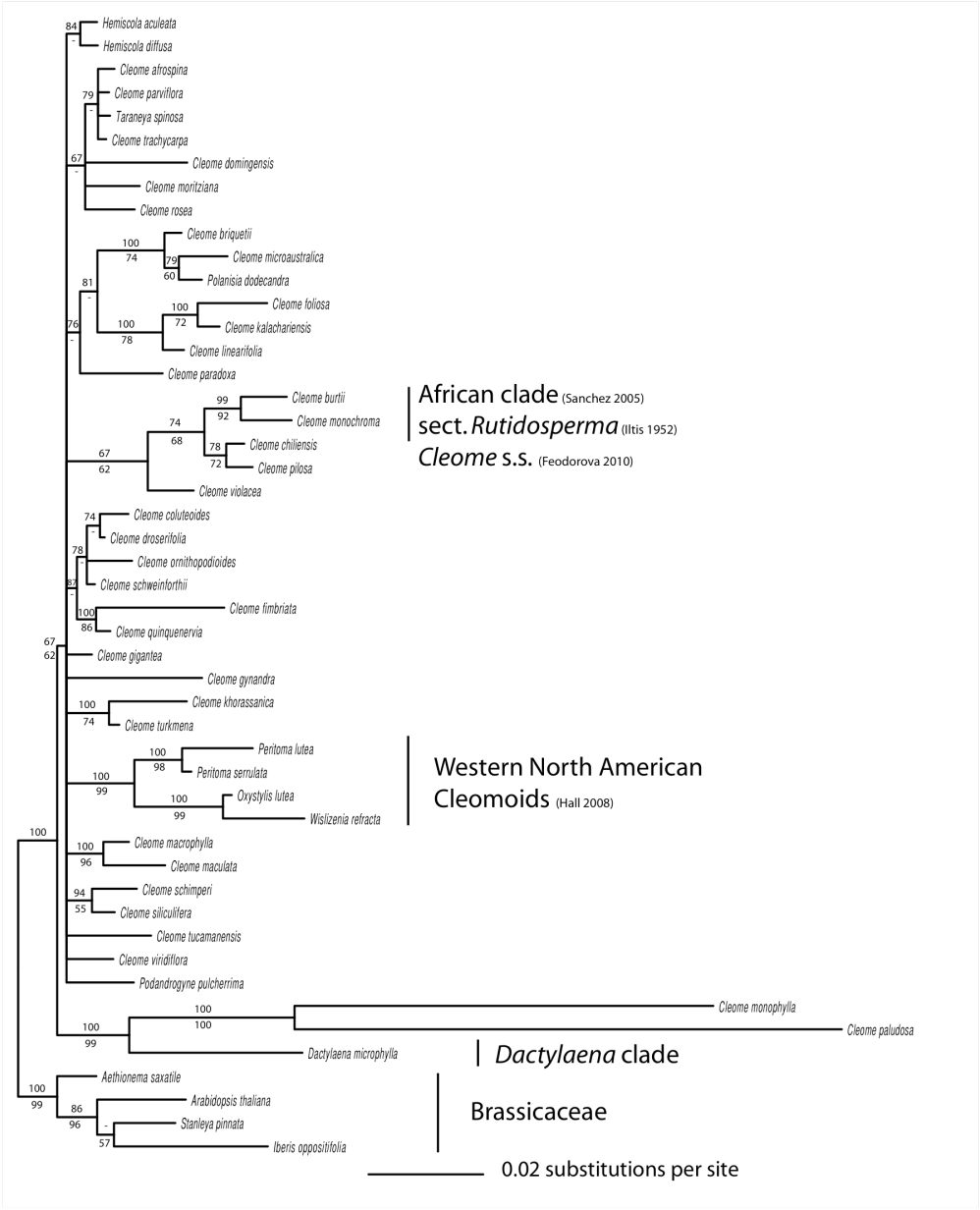
Hemiscola diffusa
Cleome dominguensis
Tarenaya hassleriana
Cleome wurdemannii
Cleome moritziana
Cleome boliviensis
Cleome trachycarpa
Tarenaya spinosa
Cleome rosea
Cleoserrata paludosa
Cleome afrospina
Cleome parviflora
Cleome espinosa
Cleome siliculifera
Cleome gigantea
Cleome viridiflora
Cleome chilensis
Cleome lechleri
Podandrogyne chiriquensis
Podandrogyne pulcherrima
Podandrogyne decipiens
Podandrogyne mathewsii
Podandrogyne jamesonii
sect. Taraneya (Iltis 1952))
Andean (Iltis 1952)
Cleome pilosa
Cleome hemsleyana
Cleome rotundifolia
C. hemsleyana clade
Dactylaena microphylla
Dactylaena pauciflora
Dactylaena clade
Cleome allamanii
Cleome briquetii
Cleome usambarica
Cleome burtii
Cleome hirta
Cleome monochroma
Cleome monophylla
Cleome elegantissima
Cleome iberidella
Cleome schimperi
Cleome rutidosperma
Cleome macrophylla
Cleome maculata
Cleome breyeri
Cleome oxyphylla
African clade (Sanchez 2005)
sect. Rutidosperma (Iltis 1952)
Cleome s.s. (Feodorova 2010)
Cleome gynandra
Gynandropsis Clade (Feodorova 2010)
Cleome brachycarpa
Dipterygium glaucum
Cleome chrysantha
Cleome tolosa
Cleome kalachariensis
Cleome linearifolia
Cleome sulfurea
sect. Gymnogonia (Iltis 1952)
Clade 6 (Feodorova et al. 2010)
Cleome cleomoides
Cleome microaustralica
Cleome tetrandra
Cleome uniflora
Arivela viscosa
Australian Clade (Feodorova et al. 2010)
Cleome oxalidea
Cleome diandra
Cleome luderitziana
Cleome paradoxa
Angustifolia Clade (Feodorova et al. 2010)
Polanisia dodecandra
Cleome africana
Cleome amblyocarpa
Cleome arabica
Cleome schweinfurthii
Cleome colutioides
Cleome violacea
Cleome ornithopodioides
Cleome khorassanica
Cleome turkmena
Cleome s.s. (Iltis 1952; Hall 2008)
Cleome droserifolia
Cleome fimbriata
Cleome quinquenervia
Cleome droserifolia Clade (Hall 2008)
Cleomella longipes
Cleomella obtusifolia
Oxystylis lutea
Wislizenia refracta
Peritoma lutea
Peritoma serrulata
Peritoma arborea
Western North American Cleomoids (Hall 2008)
Aethionema saxatile
Arabidopsis thaliana
Capsella bursa pastoris
Barbarea vulgaris
Nasturtium officinale
Iberis oppositifolia
Brassica nigra
Sisymbrium altissimum
Stanleya pinnata
Brassicaceae
Polanisia Clade (Hall 2008)
0.03 substitutions per site

Figure 2.2. Bayesian 50% majority rule consensus tree inferred from mitochondrial (*rps3*) sequence data for Cleomaceae. Posterior probabilities greater than 70% are indicated above branches; bootstrap values greater than 50 % are indicated below branches.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Hemiscola diffusa
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Cleome parviflora
Taraneya spinosa
Cleome trachycarpa
Cleome domingensis
Cleome moritziana
Cleome rosea
Cleome briquetii
Cleome microaustralica
Polanisia dodecandra
Cleome foliosa
Cleome kalachariensis
Cleome linearifolia
Cleome paradoxa
Cleome burtii
Cleome monochroma
Cleome chiliensis
Cleome pilosa
Cleome violacea
African clade (Sanchez 2005)
sect. Rutidosperma (Iltis 1952)
Cleome s.s. (Feodorova 2010)
Cleome coluteoides
Cleome droserifolia
Cleome ornithopodioides
Cleome schweinforthii
Cleome fimbriata
Cleome quinquenervia
Cleome gigantea
Cleome gynandra
Cleome khorassanica
Cleome turkmena
Peritoma lutea
Peritoma serrulata
Oxystylis lutea
Wislizenia refracta
Western North American
Cleomoids (Hall 2008)
Cleome macrophylla
Cleome maculata
Cleome schimperi
Cleome siliculifera
Cleome tucamanensis
Cleome viridiflora
Podandrogyne pulcherrima
Cleome monophylla
Cleome paludosa
Dactylaena microphylla
Dactylaena clade
Aethionema saxatile
Arabidopsis thaliana
Stanleya pinnata
Iberis oppositifolia
Brassicaceae
0.02 substitutions per site

Figure 2.3. Bayesian 50% majority rule consensus tree inferred from nuclear ribosomal (*ITS1*) sequence data for Cleomaceae. Posterior probabilities greater than 70% are indicated above branches; bootstrap values greater than 50% are indicated below branches.

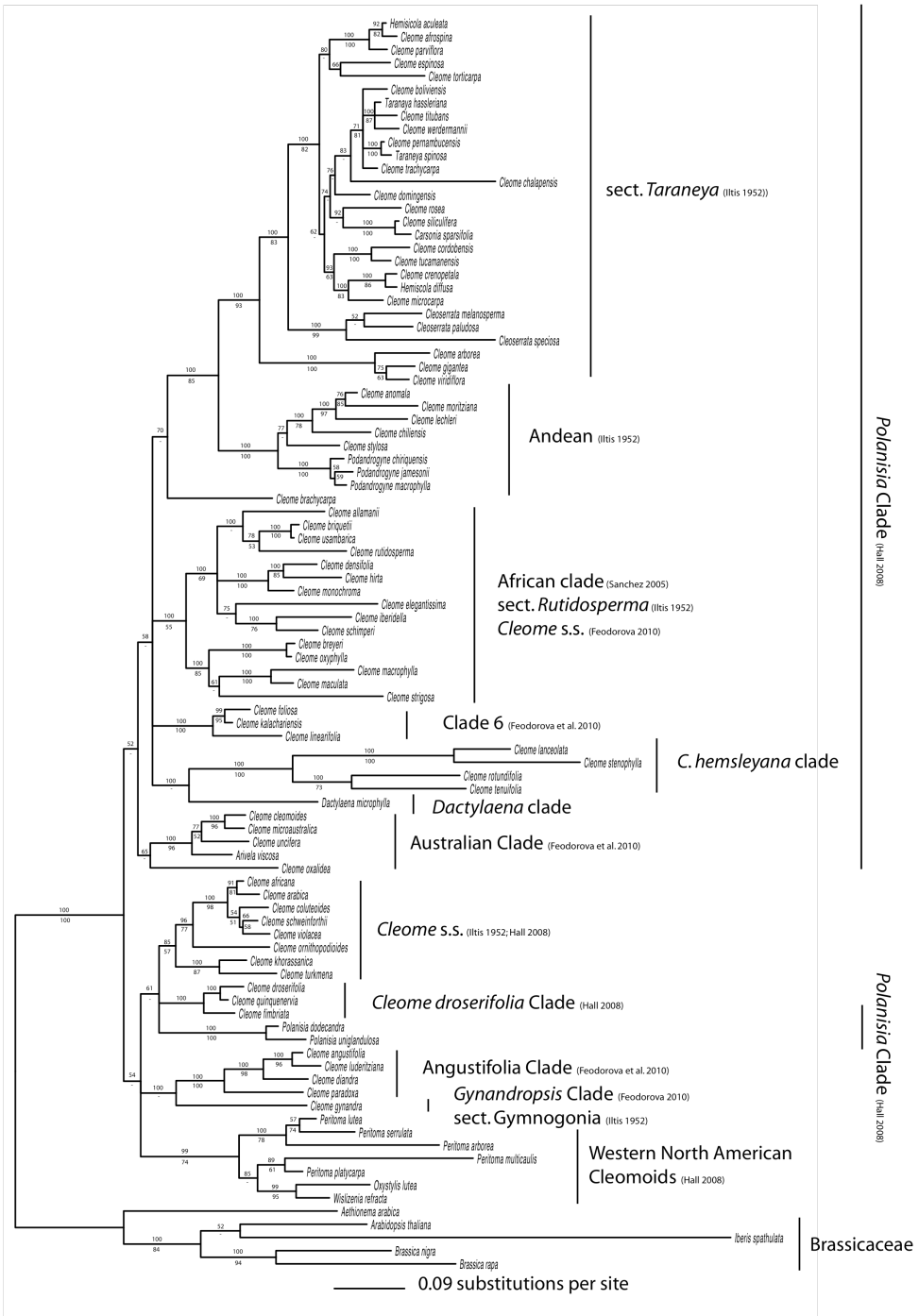

Hemiscola aculeata
Cleome afrospina
Cleome parviflora
Cleome espinosa
Cleome torticarpa
Cleome boliviensis
Taraniaya hassleriana
Cleome titubans
Cleome werdermannii
Cleome pernambucensis
Taraneya spinosa
Cleome trachycarpa
Cleome chalapensis
Cleome domingensis
Cleome rosea
Cleome siliculifera
Carsonia sparsifolia
Cleome cordobensis
Cleome tucamanensis
Cleome crenopetala
Hemiscola diffusa
Cleome microcarpa
Cleoserrata melanosperma
Cleoserrata paludosa
Cleoserrata speciosa
Cleome arborea
Cleome gigantea
Cleome viridiflora
sect. Taraneya (Iltis 1952))
Cleome anomala
Cleome moritziana
Cleome lechleri
Cleome chiliensis
Cleome stylosa
Podandrogyne chiriquensis
Podandrogyne jamesonii
Podandrogyne macrophylla
Andean (Iltis 1952)
Cleome brachycarpa
Cleome allamanii
Cleome briquetii
Cleome usambarica
Cleome rutidosperma
Cleome densifolia
Cleome hirta
Cleome monochroma
Cleome elegantissima
Cleome iberidella
Cleome schimperi
Cleome breyeri
Cleome oxyphylla
Cleome macrophylla
Cleome maculata
Cleome strigosa
African clade (Sanchez 2005)
sect. Rutidosperma (Iltis 1952)
Cleome s.s. (Feodorova 2010)
Cleome foliosa
Cleome kalachariensis
Cleome linearifolia
Clade 6 (Feodorova et al. 2010)
Cleome lanceolata
Cleome stenophylla
Cleome rotundifolia
Cleome tenuifolia
C. hemsleyana clade
Dactylaena microphylla
Dactylaena clade
Cleome cleomoides
Cleome microaustralica
Cleome uncifera
Arivela viscosa
Cleome oxalidea
Australian Clade (Feodorova et al. 2010)
Cleome africana
Cleome arabica
Cleome coluteoides
Cleome schweinfurthii
Cleome violacea
Cleome ornithopodioides
Cleome khorassanica
Cleome turkmena
Cleome s.s. (Iltis 1952; Hall 2008)
Cleome droserifolia
Cleome quinquenervia
Cleome fimbriata
Cleome droserifolia Clade (Hall 2008)
Polanisia dodecandra
Polanisia uniglandulosa
Cleome angustifolia
Cleome ludentziana
Cleome diandra
Cleome paradoxa
Cleome gynandra
Angustifolia Clade (Feodorova et al. 2010)
Gynandropsis Clade (Feodorova 2010)
sect. Gymnogonia (Iltis 1952)
Peritoma lutea
Peritoma serrulata
Peritoma arborea
Peritoma muticaulis
Peritoma platycarpa
Oxystylis lutea
Wislizenia refracta
Western North American Cleomoids (Hall 2008)
Aethionema arabica
Arabidopsis thaliana
Iberis spathulata
Brassica nigra
Brassica rapa
Brassicaceae
0.09 substitutions per site
Polanisia Clade (Hall 2008)
Polanisia Clade (Hall 2008)

Figure 2.4. Bayesian 50% majority rule consensus tree inferred from total evidence: chloroplast (*matK*, *ndhF*, and *ycf1*), mitochondrial (*rps3*), and nuclear ribosomal (*ITS1*). Posterior probabilities greater than 70% are indicated above branches; bootstrap values greater than 50% are indicated below branches.

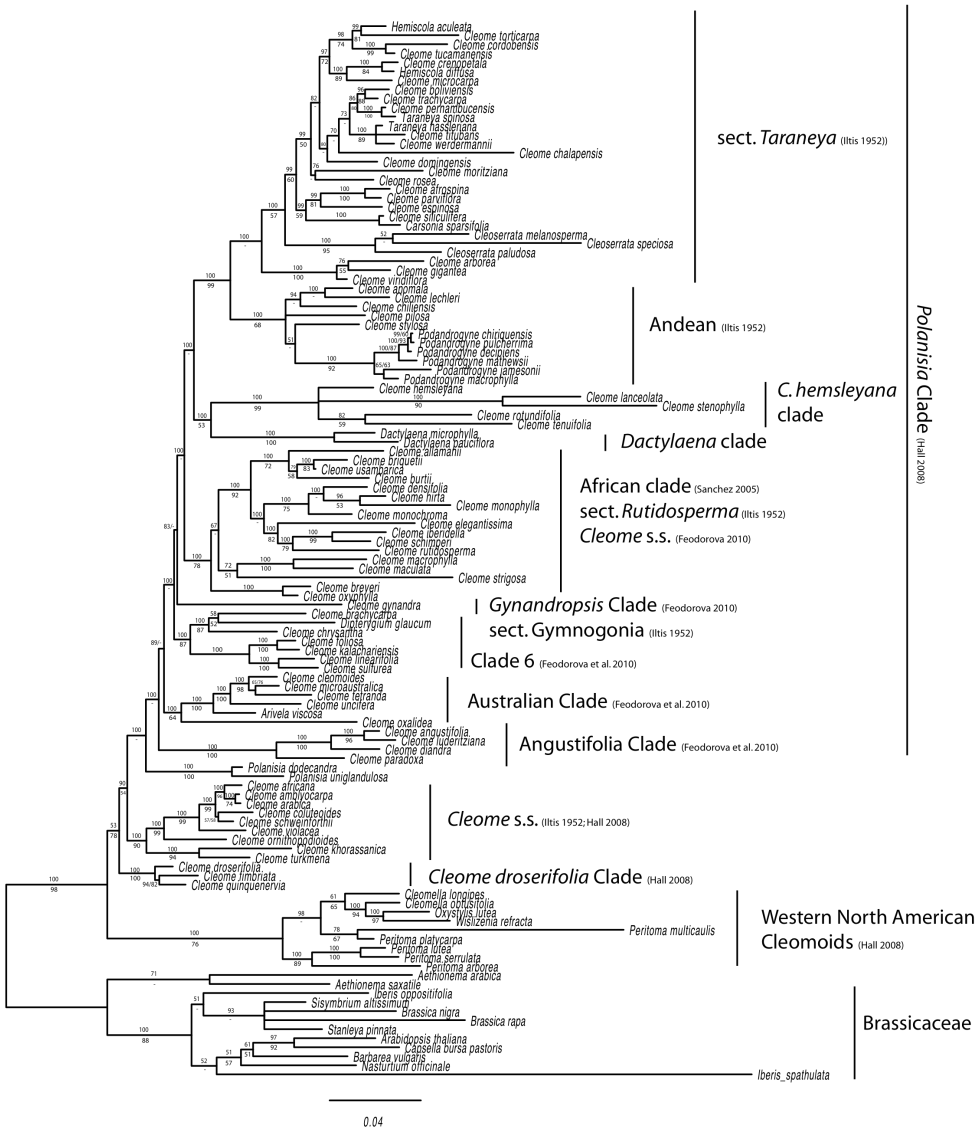

Hemiscola aculeata
Cleome torticarpa
Cleome cordobensis
Cleome tucamanensis
Cleome crenopetala
Hemiscola diffusa
Cleome microcarpa
Cleome boliviensis
Cleome trachycarpa
Cleome pernambucensis
Taraneya spinosa
Taraneya hassleriana
Cleome titubans
Cleome werdermannii
Cleome chalapensis
Cleome dodringensis
Cleome moritziana
Cleome rosea
Cleome afrospina
Cleome parvilobra
Cleome espinosa
Cleome silicuifera
Carsonia sparsifolia
Cleoserrata melanosperma
Cleoserrata speciosa
Cleoserrata paludosa
Cleome arborea
Cleome gigantea
Cleome viridiflora
Cleome eremophila
Cleome lechleri
Cleome chilensis
Cleome pilosa
Cleome stylosa
Podandrogyne chiriquensis
Podandrogyne pulcherrima
Podandrogyne decipiens
Podandrogyne mathewsii
Podandrogyne jamesonii
Podandrogyne macrophylla
Cleome hemsleyana
Cleome lanceolata
Cleome stenophylla
Cleome rotundifolia
Cleome tenuifolia
Dactylaena microphylla
Dactylaena patuliflora
Cleome allamanii
Cleome briquetii
Cleome usambarica
Cleome burtii
Cleome densifolia
Cleome hirta
Cleome monophylla
Cleome monocroma
Cleome elegantissima
Cleome iberidella
Cleome schimperi
Cleome rutidosperma
Cleome macrophylla
Cleome maculata
Cleome strigosa
Cleome breveri
Cleome oxyphylla
Cleome gynandra
Cleome brachycarpa
Dipterygium glaucum
Cleome chrysantha
Cleome foliosa
Cleome kalahariensis
Cleome linearifolia
Cleome sulfurea
Cleome cleomoides
Cleome microaustralica
Cleome tetranda
Cleome uncifera
Arivela viscosa
Cleome oxalidea
Cleome angustifolia
Cleome luderitziana
Cleome diandra
Cleome paradoxa
Polanisia dodecandra
Polanisia uniglandulosa
Cleome africana
Cleome amblyocarpa
Cleome arabica
Cleome colluteoides
Cleome schweinfurthii
Cleome violacea
Cleome ornithopodioides
Cleome khorassanica
Cleome turkmena
Cleome droserifolia
Cleome himbriata
Cleome quinquenervia
Cleomella longipes
Cleomella obtusifolia
Oxystylis lutea
Wislizenia refracta
Peritoma multicaulis
Peritoma platycarpa
Peritoma lutea
Peritoma serrulata
Peritoma arborea
Aethionema arabica
Aethionema saxatile
Iberis oppositifolia
Sisymbrium altissimum
Brassica nigra
Brassica rapa
Stanleya pinnata
Arabidopsis thaliana
Capsella bursa pastoris
Barbarea vulgaris
Nasturtium officinale
Iberis_spathulata
0.04
sect. Taraneya (Iltis 1952))
Andean (Iltis 1952)
C. hemsleyana clade
Dactylaena clade
African clade (Sanchez 2005)
sect. Rutidosperma (Iltis 1952)
Cleome s.s. (Feodorova 2010)
Gynandropsis Clade (Feodorova 2010)
sect. Gymnogonia (Iltis 1952)
Clade 6 (Feodorova et al. 2010)
Australian Clade (Feodorova et al. 2010)
Angustifolia Clade (Feodorova et al. 2010)
Cleome s.s. (Iltis 1952; Hall 2008)
Cleome droserifolia Clade (Hall 2008)
Western North American Cleomoids (Hall 2008)
Brassicaceae
Polanisia Clade (Hall 2008)

Figure 2.5. Evolution of monosymmetry in the sepal whorl across the majority rule consensus tree based on chloroplast sequence data (*matK*, *ndhF*, and *ycf1*). Pie charts represent maximum likelihood ancestral state reconstructions across the majority rule consensus tree of the last 500 topologies recovered from Bayesian analysis and show the proportion of reconstructions in which each state is significant. The monosymmetric state is characterized by having an enlarged abaxial sepal. The polysymmetric state is characterized by having equally sized sepals. Designation of "node absent" indicates the percentage of trees (of the 500) in which that node is not present. Equivocal states at nodes were not assigned a statistically significant anscestral state.

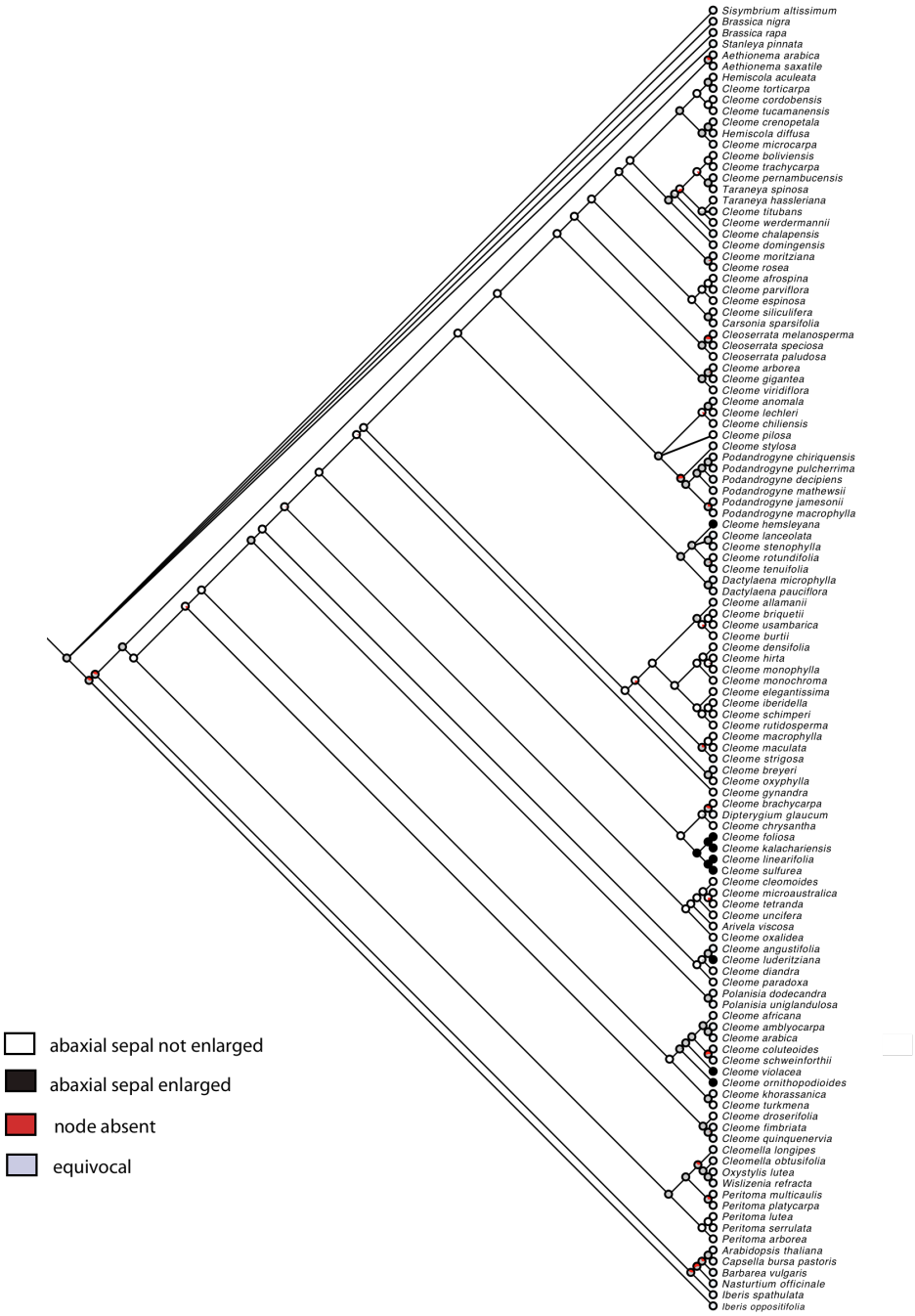

Sisymbrium altissimum
Brassica nigra
Brassica rapa
Stanleya pinnata
Aethionema arabica
Aethionema saxatile
Hemiscola aculeata
Cleome torticarpa
Cleome cordobensis
Cleome tucamanensis
Cleome crenopetala
Hemiscola diffusa
Cleome microcarpa
Cleome boliviensis
Cleome trachycarpa
Cleome pernambucensis
Taraneya spinosa
Taraneya hassleriana
Cleome titubans
Cleome werdermannii
Cleome chalapensis
Cleome domingensis
Cleome moritziana
Cleome rosea
Cleome afrospina
Cleome parviflora
Cleome espinosa
Cleome siliculifera
Carsonia sparsifolia
Cleoserrata melanosperma
Cleoserrata speciosa
Cleoserrata paludosa
Cleome arborea
Cleome gigantea
Cleome viridiflora
Cleome anomala
Cleome lechleri
Cleome chiliensis
Cleome pilosa
Cleome stylosa
Podandrogyne chiriquensis
Podandrogyne pulcherrima
Podandrogyne decipiens
Podandrogyne mathewsii
Podandrogyne jamesonii
Podandrogyne macrophylla
Cleome hemsleyana
Cleome lanceolata
Cleome stenophylla
Cleome rotundifolia
Cleome tenuifolia
Dactylaena microphylla
Dactylaena pauciflora
Cleome allamanii
Cleome briquetii
Cleome usambarica
Cleome burtii
Cleome densifolia
Cleome hirta
Cleome monophylla
Cleome monochroma
Cleome elegantissima
Cleome iberidella
Cleome schimperi
Cleome rutidosperma
Cleome macrophylla
Cleome maculata
Cleome strigosa
Cleome breyeri
Cleome oxyphylla
Cleome gynandra
Cleome brachycarpa
Dipterygium glaucum
Cleome chrysantha
Cleome foliosa
Cleome kalachariensis
Cleome linearifolia
Cleome sulfurea
Cleome cleomoides
Cleome microaustralica
Cleome tetranda
Cleome uncifera
Arivela viscosa
Cleome oxalidea
Cleome angustifolia
Cleome luderitziana
Cleome diandra
Cleome paradoxa
Polanisia dodecandra
Polanisia uniglandulosa
Cleome africana
Cleome amblyocarpa
Cleome arabica
Cleome coluteoides
Cleome schweinforthii
Cleome violacea
Cleome ornithopodioides
Cleome khorassanica
Cleome turkmena
Cleome droserifolia
Cleome fimbriata
Cleome quinquenervia
Cleomella longipes
Cleomella obtusifolia
Oxystylis lutea
Wislizenia refracta
Peritoma multicaulis
Peritoma platycarpa
Peritoma lutea
Peritoma serrulata
Peritoma arborea
Arabidopsis thaliana
Capsella bursa pastoris
Barbarea vulgaris
Nasturtium officinale
Iberis spathulata
Iberis oppositifolia
abaxial sepal not enlarged
abaxial sepal enlarged
node absent
equivocal

Figure 2.6. Evolution of monosymmetry due to differentiation of colour pattern in the petal whorl across the majority rule consensus tree based on chloroplast sequence data (*matK*, *ndhF*, and *ycf1*). Pie charts represent maximum likelihood ancestral state reconstructions across the majority rule consensus tree of the last 500 trees recovered from the Bayesian analysis and show the proportion of reconstructions in which each state is significant. The monosymmetric state is characterized by having different colour patterns between the adaxial and abaxial petal pairs. The polysymmetric state is characterized by having similar colour patterns on all petals. Designation of "node absent" indicates the percentage of trees (of the 500) in which that node is not present. Equivocal states at nodes were not assigned a statistically significant anscestral state.

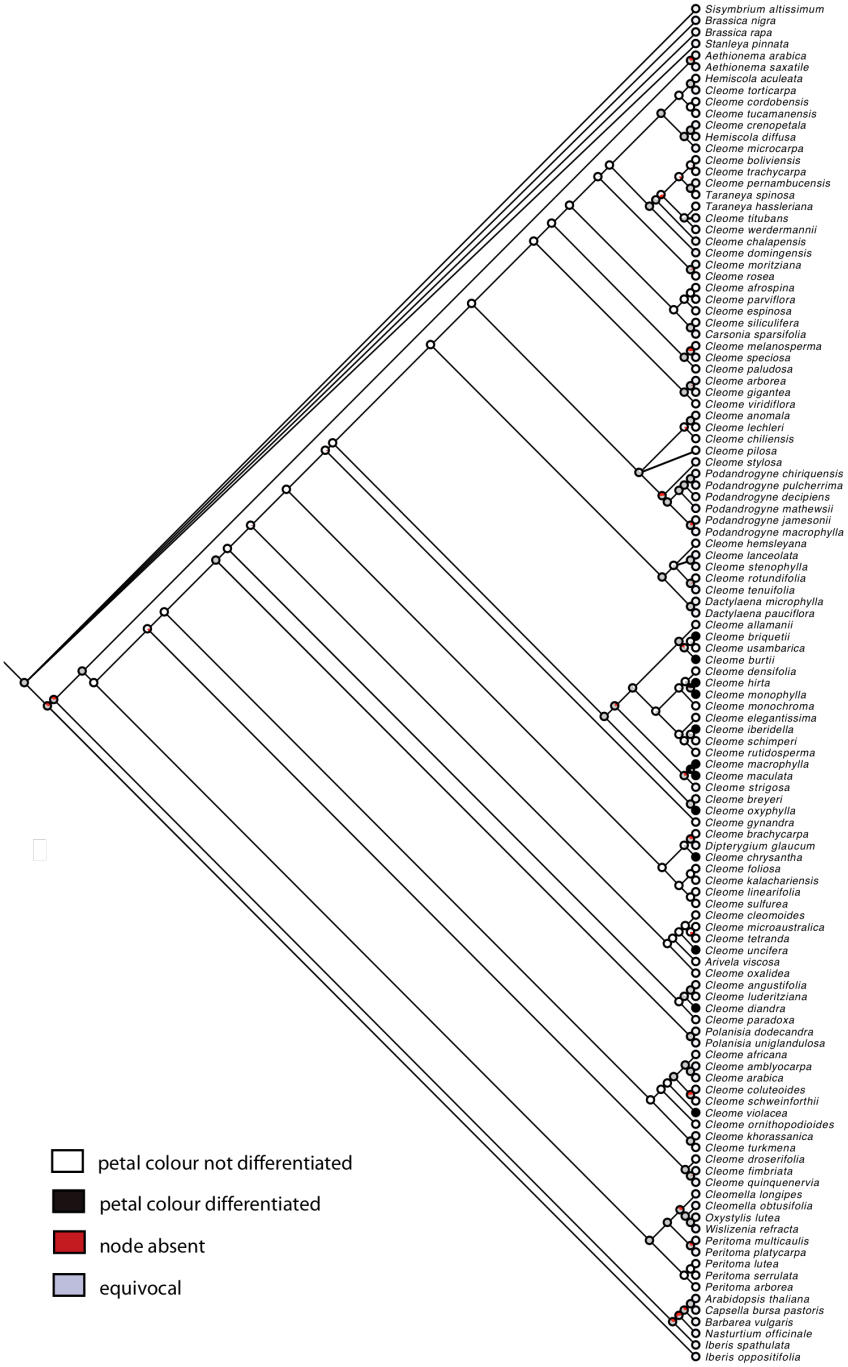

Sisymbrium altissimum
Brassica nigra
Brassica rapa
Stanleya pinnata
Aethionema arabica
Aethionema saxatile
Hemiscola aculeata
Cleome torticarpa
Cleome cordobensis
Cleome tucamanensis
Cleome crenopetala
Hemiscola diffusa
Cleome microcarpa
Cleome boliviensis
Cleome trachycarpa
Cleome pernambucensis
Taraneya spinosa
Taraneya hassleriana
Cleome titubans
Cleome werdermannii
Cleome chalapensis
Cleome domingensis
Cleome moritziana
Cleome rosea
Cleome afrospina
Cleome parviflora
Cleome espinosa
Cleome siliculifera
Carsonia sparsifolia
Cleome melanosperma
Cleome speciosa
Cleome paludosa
Cleome arborea
Cleome gigantea
Cleome viridiflora
Cleome anomala
Cleome lechleri
Cleome chiliensis
Cleome pilosa
Cleome stylosa
Podandrogyne chiriquensis
Podandrogyne pulcherrima
Podandrogyne decipiens
Podandrogyne mathewsii
Podandrogyne jamesonii
Podandrogyne macrophylla
Cleome hemsleyana
Cleome lanceolata
Cleome stenophylla
Cleome rotundifolia
Cleome tenuifolia
Dactylaena microphylla
Dactylaena paucilflora
Cleome allamanii
Cleome briquetii
Cleome usambarica
Cleome burtii
Cleome densifolia
Cleome hirta
Cleome monophylla
Cleome monochroma
Cleome elegantissima
Cleome iberidella
Cleome schimperi
Cleome rutidosperma
Cleome macrophylla
Cleome maculata
Cleome strigosa
Cleome breyeri
Cleome oxyphylla
Cleome gynandra
Cleome brachycarpa
Dipteryglium glaucum
Cleome chrysantha
Cleome foliosa
Cleome kalachariensis
Cleome linearifolia
Cleome sulfurea
Cleome cleomoides
Cleome microaustralica
Cleome tetranda
Cleome uncifera
Arivela viscosa
Cleome oxalidea
Cleome angustifolia
Cleome luderitziana
Cleome diandra
Cleome paradoxa
Polanisia dodecandra
Polanisia uniglandulosa
Cleome africana
Cleome amblyocarpa
Cleome arabica
Cleome coluteoides
Cleome schweinforthii
Cleome violacea
Cleome ornithopodioides
Cleome khorassanica
Cleome turkmena
Cleome droserifolia
Cleome fimbriata
Cleome quinquenervia
Cleomella longipes
Cleomella obtusifolia
Oxystylis lutea
Wislizenia refracta
Peritoma multicaulis
Peritoma platycarpa
Peritoma lutea
Peritoma serrulata
Peritoma arborea
Arabidopsis thaliana
Capsella bursa pastoris
Barbarea vulgaris
Nasturtium officinale
Iberis spathulata
Iberis oppositifolia
petal colour not differentiated
petal colour differentiated
node absent
equivocal

Figure 2.7. Evolution of monosymmetry differentiation in size of adaxial and abaxial petal pairs across the majority rule consensus tree based on chloroplast sequence data (*matK*, *ndhF*, and *ycf1*). Pie charts represent maximum likelihood ancestral state reconstructions across the majority rule consensus tree of the last 500 Bayesian topologies and show the proportion of reconstructions in which each state is significant. The monosymmetric state is characterized by having different sizes between adaxial and abaxial petal pairs. The polysymmetric state is characterized by having equally sized petals. Designation of "node absent" indicates the percentage of trees (of the 500) in which that node is not present. Equivocal states at nodes were not assigned a statistically significant anscestral state.

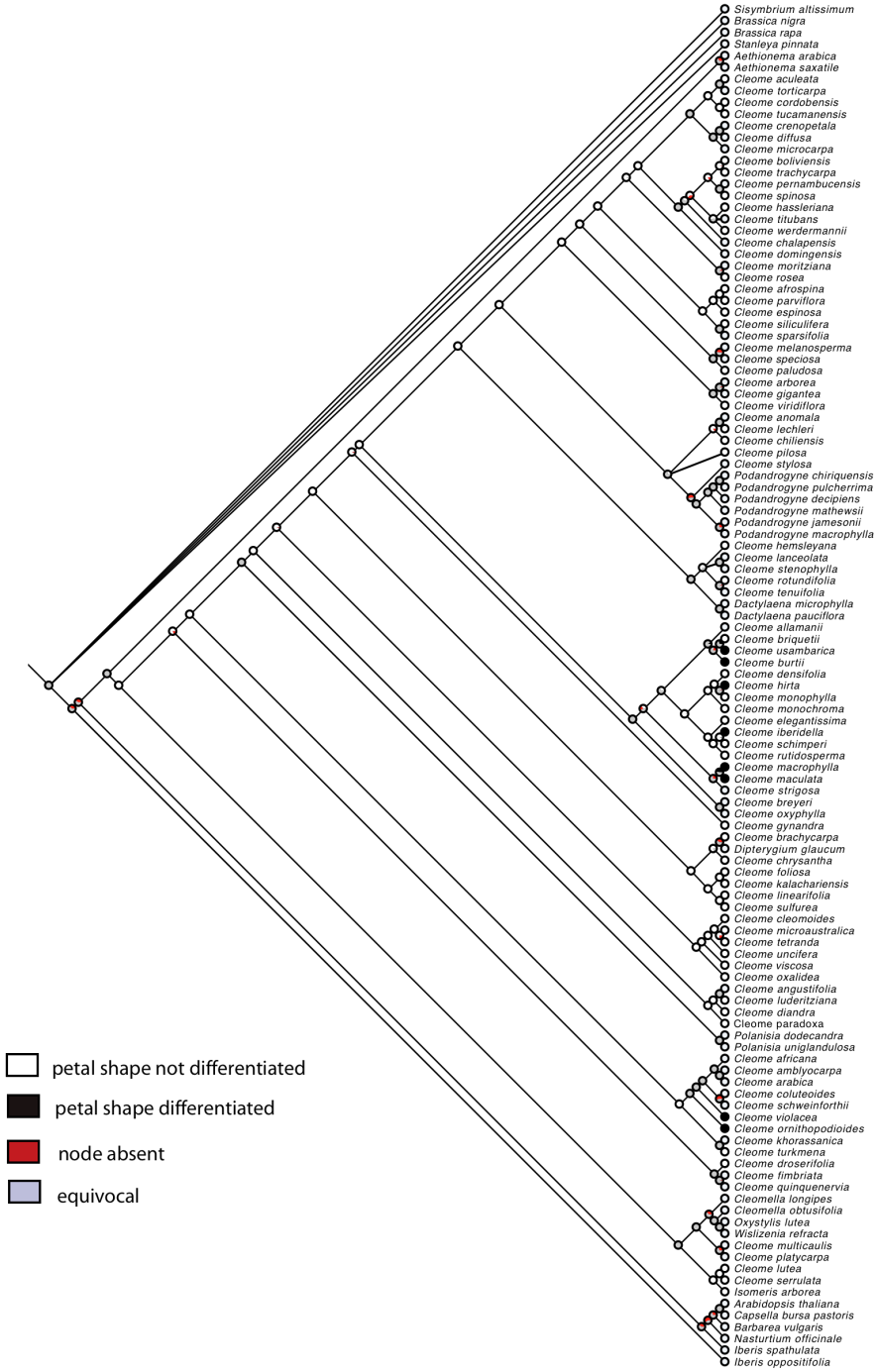

Sisymbrium altissimum
Brassica nigra
Brassica rapa
Stanleya pinnata
Aethionema arabica
Aethionema saxatile
Cleome aculeata
Cleome torticarpa
Cleome cordobensis
Cleome tucamanensis
Cleome crenopetala
Cleome diffusa
Cleome microcarpa
Cleome boliviensis
Cleome trachycarpa
Cleome pernambucensis
Cleome spinosa
Cleome hassleriana
Cleome titubans
Cleome werdermannii
Cleome chalapensis
Cleome domingensis
Cleome moritziana
Cleome rosea
Cleome afrospina
Cleome parviflora
Cleome espinosa
Cleome siliculifera
Cleome sparsifolia
Cleome melanosperma
Cleome speciosa
Cleome paludosa
Cleome arborea
Cleome gigantea
Cleome viridiflora
Cleome anomala
Cleome lechleri
Cleome chiliensis
Cleome pilosa
Cleome stylosa
Podandrogyne chiriquensis
Podandrogyne pulcherrima
Podandrogyne decipiens
Podandrogyne mathewsii
Podandrogyne jamesonii
Podandrogyne macrophylla
Cleome hemsleyana
Cleome lanceolata
Cleome stenophylla
Cleome rotundifolia
Cleome tenuifolia
Dactylaena microphylla
Dactylaena pauciflora
Cleome allamanii
Cleome briquetii
Cleome usambarica
Cleome burtii
Cleome densifolia
Cleome hirta
Cleome monophylla
Cleome monochroma
Cleome elegantissima
Cleome iberidella
Cleome schimperi
Cleome rutidosperma
Cleome macrophylla
Cleome maculata
Cleome strigosa
Cleome breyeri
Cleome oxyphylla
Cleome gynandra
Cleome brachycarpa
Dipterygium glaucum
Cleome chrysantha
Cleome foliosa
Cleome kalachariensis
Cleome linearifolia
Cleome sulfurea
Cleome cleomoides
Cleome microaustralica
Cleome tetranda
Cleome uncifera
Cleome viscosa
Cleome oxalidea
Cleome angustifolia
Cleome luderitziana
Cleome diandra
Cleome paradoxa
Polanisia dodecandra
Polanisia uniglandulosa
Cleome africana
Cleome amblyocarpa
Cleome arabica
Cleome coluteoides
Cleome schweinforthii
Cleome violacea
Cleome ornithopodioides
Cleome khorassanica
Cleome turkmena
Cleome droserifolia
Cleome fimbriata
Cleome quinquenervia
Cleomella longipes
Cleomella obtusifolia
Oxystylis lutea
Wislizenia refracta
Cleome multicaulis
Cleome platycarpa
Cleome lutea
Cleome serrulata
Isomeris arborea
Arabidopsis thaliana
Capsella bursa pastoris
Barbarea vulgaris
Nasturtium officinale
Iberis spathulata
Iberis oppositifolia
petal shape not differentiated
petal shape differentiated
node absent
equivocal

Figure 2.8. Evolution of monosymmetry in the stamen whorl across the majority rule consensus tree based on chloroplast sequence data (*matK*, *ndhF*, and *ycf1*). Pie charts represent maximum likelihood ancestral state reconstructions across the majority rule consensus tree of the last 500 Bayesian topologies and show the proportion of reconstructions in which each state is significant. The monosymmetric state is characterized by deviation from six adaxial stamens, either by production of more or less stamens. The polysymmetric state is characterized by having six adaxial stamens. Designation of "node absent" indicates the percentage of trees (of the 500) in which that node is not present. Equivocal states at nodes were not assigned a statistically significant anscestral state.

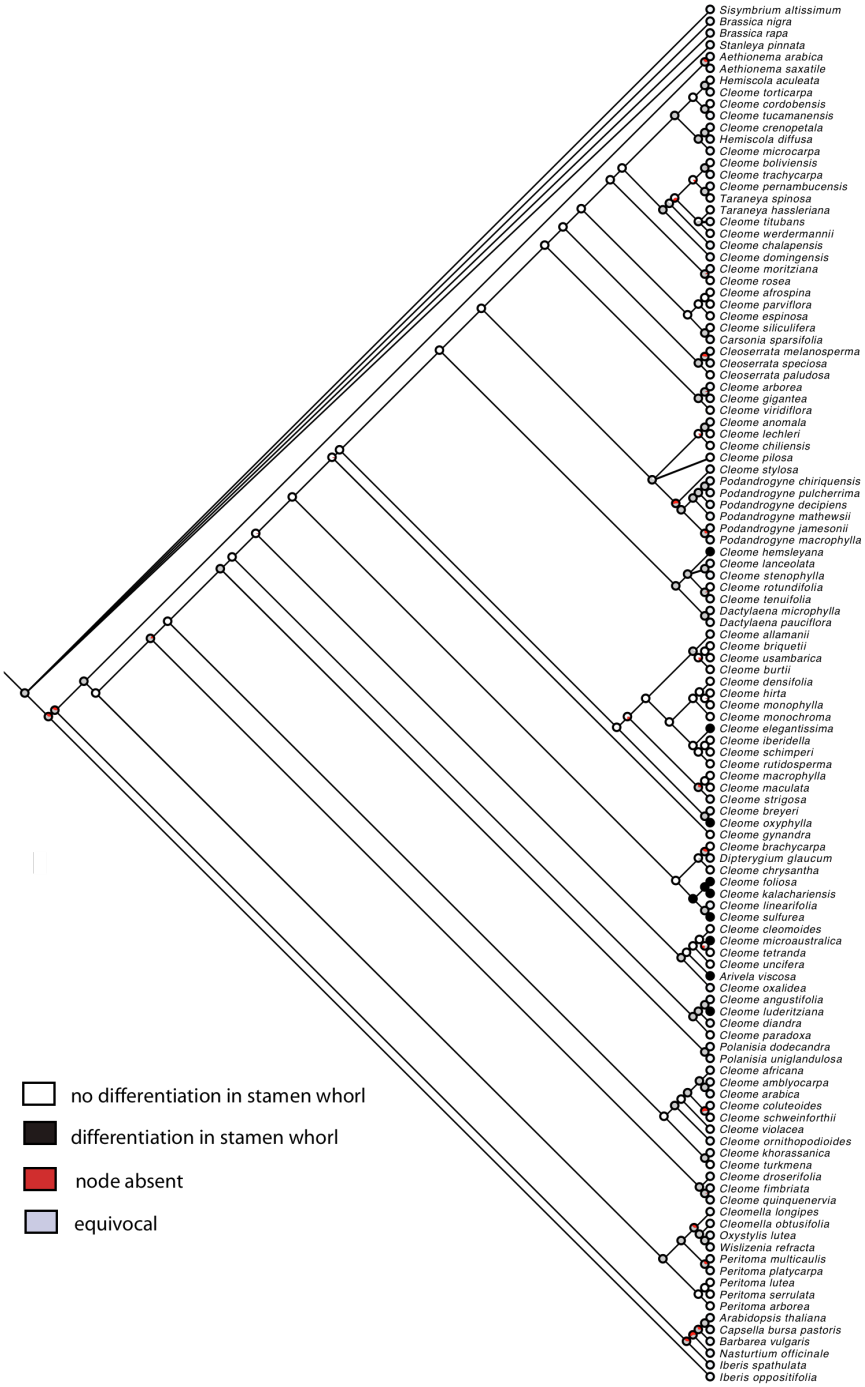

Sisymbrium altissimum
Brassica nigra
Brassica rapa
Stanleya pinnata
Aethionema arabica
Aethionema saxatile
Hemiscola aculeata
Cleome torticarpa
Cleome cordobensis
Cleome tucamanensis
Cleome crenopetala
Hemiscola diffusa
Cleome microcarpa
Cleome boliviensis
Cleome trachycarpa
Cleome pernambucensis
Taraneya spinosa
Taraneya hassleriana
Cleome titubans
Cleome werdermannii
Cleome chalapensis
Cleome domingensis
Cleome moritziana
Cleome rosea
Cleome afrospina
Cleome parviflora
Cleome espinosa
Cleome siliculifera
Carsonia sparsifolia
Cleoserrata melanosperma
Cleoserrata speciosa
Cleoserrata paludosa
Cleome arborea
Cleome gigantea
Cleome viridiflora
Cleome anomala
Cleome lechleri
Cleome chiliensis
Cleome pilosa
Cleome stylosa
Podandrogyne chiriquensis
Podandrogyne pulcherrima
Podandrogyne decipiens
Podandrogyne mathewsii
Podandrogyne jamesonii
Podandrogyne macrophylla
Cleome hemsleyana
Cleome lanceolata
Cleome stenophylla
Cleome rotundifolia
Cleome tenuifolia
Dactylaena microphylla
Dactylaena pauciflora
Cleome allamanii
Cleome briquetii
Cleome usambarica
Cleome burtii
Cleome densifolia
Cleome hirta
Cleome monophylla
Cleome monochroma
Cleome elegantissima
Cleome iberidella
Cleome schimperi
Cleome rutidosperma
Cleome macrophylla
Cleome maculata
Cleome strigosa
Cleome breyeri
Cleome oxyphylla
Cleome gynandra
Cleome brachycarpa
Dipterygium glaucum
Cleome chrysantha
Cleome foliosa
Cleome kalachariensis
Cleome linearifolia
Cleome sulfurea
Cleome cleomoides
Cleome microaustralica
Cleome tetranda
Cleome uncifera
Arivela viscosa
Cleome oxalidea
Cleome angustifolia
Cleome luderitziana
Cleome diandra
Cleome paradoxa
Polanisia dodecandra
Polanisia uniglandulosa
Cleome africana
Cleome amblyocarpa
Cleome arabica
Cleome coluteoides
Cleome schweinforthii
Cleome violacea
Cleome ornithopodioides
Cleome khorassanica
Cleome turkmena
Cleome droserifolia
Cleome fimbriata
Cleome quinquenervia
Cleomella longipes
Cleomella obtusifolia
Oxystylis lutea
Wislizenia refracta
Peritoma multicaulis
Peritoma platycarpa
Peritoma lutea
Peritoma serrulata
Peritoma arborea
Arabidopsis thaliana
Capsella bursa pastoris
Barbarea vulgaris
Nasturtium officinale
Iberis spathulata
Iberis oppositifolia
no differentiation in stamen whorl
differentiation in stamen whorl
node absent
equivocal

Figure 2.9. Evolution of monosymmetry due to presence of an enlarged adaxial gland across the majority rule consensus tree based on chloroplast sequence data (*matK*, *ndhF*, and *ycf1*). Pie charts represent maximum likelihood ancestral state reconstructions across the majority rule consensus tree of the last 500 Bayesian topologies and show the proportion of reconstructions in which each state is significant. The monosymmetric state is characterized by having an enlarged adaxial nectar gland. The polysymmetric state is characterized by not having an adaxial nectar gland. Designation of "node absent" indicates the percentage of trees (of the 500) in which that node is not present. Equivocal states at nodes were not assigned a statistically significant anscestral state.

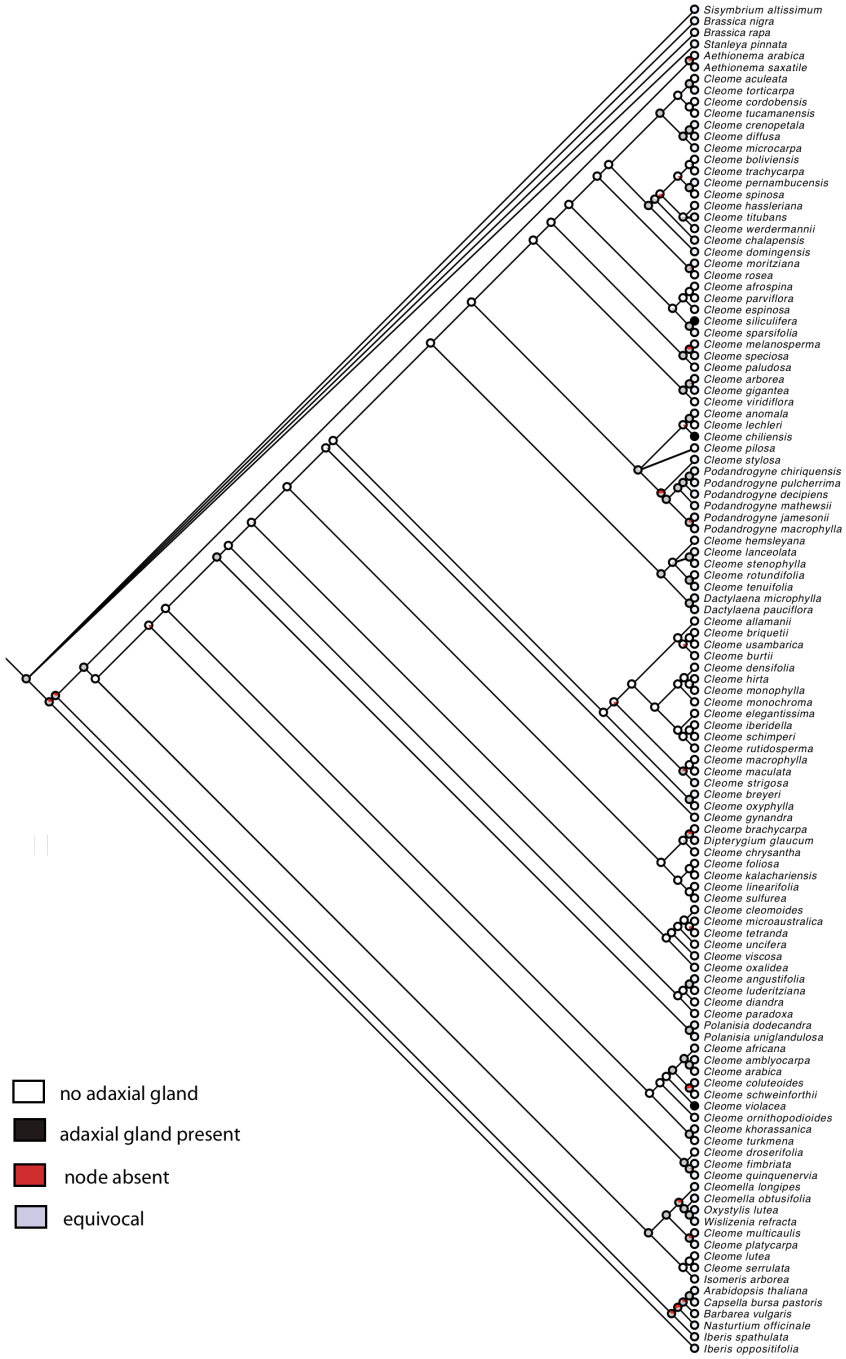
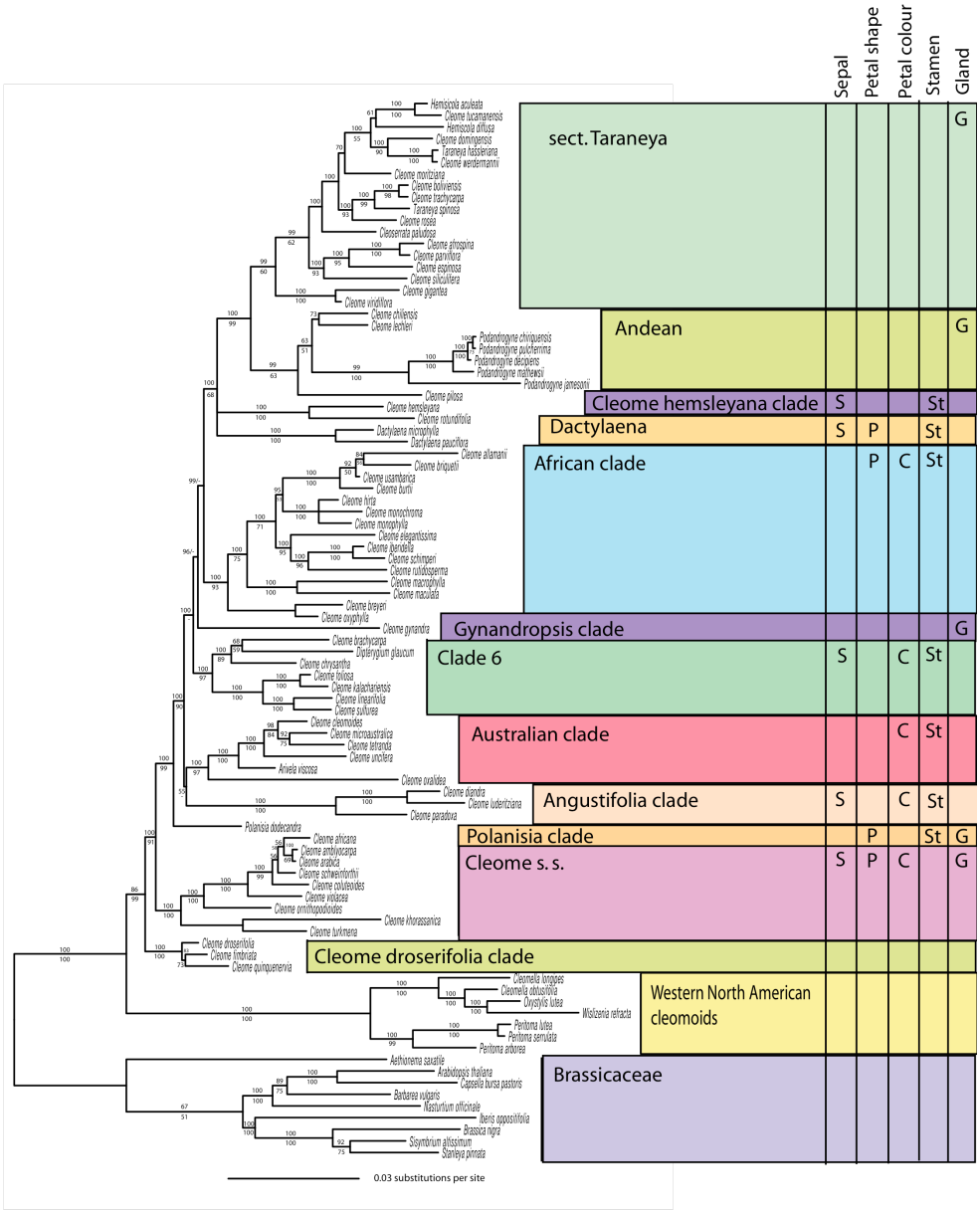

Sisymbrium altissimum
Brassica nigra
Brassica rapa
Stanleya pinnata
Aethionema arabica
Aethionema saxatile
Cleome aculeata
Cleome torticarpa
Cleome cordobensis
Cleome tucamanensis
Cleome crenopetala
Cleome diffusa
Cleome microcarpa
Cleome boliviensis
Cleome trachycarpa
Cleome pernambucensis
Cleome spinosa
Cleome hassleriana
Cleome titubans
Cleome werdermannii
Cleome chalapensis
Cleome domingensis
Cleome moritziana
Cleome rosea
Cleome afrospina
Cleome parviflora
Cleome espinosa
Cleome siliculifera
Cleome sparsifolia
Cleome melanosperma
Cleome speciosa
Cleome paludosa
Cleome arborea
Cleome gigantea
Cleome viridiflora
Cleome anomala
Cleome lechleri
Cleome chiliensis
Cleome pilosa
Cleome stylosa
Podandrogyne chiriquensis
Podandrogyne pulcherrima
Podandrogyne decipiens
Podandrogyne mathewsii
Podandrogyne jamesonii
Podandrogyne macrophylla
Cleome hemsleyana
Cleome lanceolata
Cleome stenophylla
Cleome rotundifolia
Cleome tenuifolia
Dactylaena microphylla
Dactylaena pauciflora
Cleome allamanii
Cleome briquetii
Cleome usambarica
Cleome burtii
Cleome densifolia
Cleome hirta
Cleome monophylla
Cleome monochroma
Cleome elegantissima
Cleome iberidella
Cleome schimperi
Cleome rutidosperma
Cleome macrophylla
Cleome maculata
Cleome strigosa
Cleome breyeri
Cleome oxyphylla
Cleome gynandra
Cleome brachycarpa
Dipterygium glaucum
Cleome chrysantha
Cleome foliosa
Cleome kalachariensis
Cleome linearifolia
Cleome sulfurea
Cleome cleomoides
Cleome microaustralica
Cleome tetranda
Cleome uncifera
Cleome viscosa
Cleome oxalidea
Cleome angustifolia
Cleome luderitziana
Cleome diandra
Cleome paradoxa
Polanisia dodecandra
Polanisia uniglandulosa
Cleome africana
Cleome amblyocarpa
Cleome arabica
Cleome coluteoides
Cleome schweinforthii
Cleome violacea
Cleome ornithopodioides
Cleome khorassanica
Cleome turkmena
Cleome droserifolia
Cleome fimbriata
Cleome quinquenervia
Cleomella longipes
Cleomella obtusifolia
Oxystylis lutea
Wislizenia refracta
Cleome multicaulis
Cleome platycarpa
Cleome lutea
Cleome serrulata
Isomeris arborea
Arabidopsis thaliana
Capsella bursa pastoris
Barbarea vulgaris
Nasturtium officinale
Iberis spathulata
Iberis oppositifolia
no adaxial gland
adaxial gland present
node absent
equivocal

Figure 2.10. Summary of floral monosymmetry across the sepal, petal, and stamen whorls, and including presence or absence of a nectar gland, in Cleomaceae. Bayesian 50% majority rule consensus tree is inferred from chloroplast (*matK*, *ndhF*, and *ycf1*) sequence data. Posterior probabilities greater than 70 % are indicated above branches; bootstrap values greater than 50 % are indicated below branches. The position of strongly supported clades is identified by name and colour in the right margin of the phylogenetic tree. States that increase monosymmetry of the flower are listed vertically at the top of the phylogeny. States that increase monosymmetry in at least one representative of a clade are indicated in the corresponding column. Abbreviations: C = colour; P = petal; G = gland; S = sepal; St = stamen.


Sepal
Petal shape
Petal colour
Stamen
Gland
sect. Taraneya
G
Andean
G
Cleome hemsleyana clade
S
St
Dactylaena
S
P
St
African clade
P
C
St
Gynandropsis clade
G
Clade 6
S
C
St
Australian clade
C
St
Angustifolia clade
S
C
St
Polanisia clade
P
St
G
Cleome s. s.
S
P
C
G
Cleome droserifolia clade
Western North American cleomoids
Brassicaceae
0.03 substitutions per site

Appendices

Appendix 2.1. Morphological character matrix. Taxa are listed in the left column. Characters 1-8 are listed in the right columns. Characters: character 1 = sepal size; character 2 = petal shape; character 3 = petal colour; character 4 = petal aestivation; character 5 = gland; character 6 = stamen number; character 7 = curvature. Character states: 1 = monosymmetric state; 0 = polysymmetric state; ? = state unknown.

Taxon \ Character	1	2	3	4	5	6	7
Aethionema arabica	?	?	?	?	?	?	?
Aethionema saxatile	?	?	?	?	?	?	?
Arabidopsis thaliana	?	?	?	?	?	?	?
Barbarea vulgaris	?	?	?	?	?	?	?
Brassica nigra	?	?	?	?	?	?	?
Brassica rapa	?	?	?	?	?	?	?
Capsella bursa pastoris	?	?	?	?	?	?	?
Cleome aculeata	0	0	0	0	0	0	1
Cleome africana	?	?	?	?	?	?	?
Cleome afrospina	0	0	0	1	0	0	1
Cleome allamanii	?	?	?	?	0	?	1
Cleome amblyocarpa	?	?	?	?	?	?	?
Cleome angustifolia	?	?	?	?	?	?	?
Cleome anomala	?	?	?	?	?	?	?
Cleome arabica	?	?	?	?	?	?	?
Cleome arborea	?	?	?	?	?	?	?
Cleome boliviensis	0	0	0	0	0	0	1
Cleome brachycarpa	0	0	0	0	0	0	1
Cleome breyeri	?	?	?	?	?	?	?
Cleome briquetii	0	0	1	0	0	0	1
Cleome burtii	0	1	1	0	0	0	1
Cleome chalapensis	?	?	?	?	?	?	?
Cleome chiliensis	0	0	0	0	1	0	1
Cleome crenopetala	?	?	?	?	?	?	?
Cleome chrysantha	0	0	1	1	0	0	1
Cleome cleomoides	0	0	0	0	0	0	1
Cleome coluteoides	?	?	?	?	?	?	?
Cleome cordobensis	0	0	0	0	0	0	1
Cleome densifolia	0	0	0	0	0	0	1
Cleome diandra	0	0	1	1	0	?	1
Cleome diffusa	?	?	?	?	?	?	?
Cleome domingensis	?	?	?	?	?	?	?
Cleome droserifolia	0	0	0	0	0	?	1
Cleome elegantissima	0	0	0	0	0	1	1
Cleome espinosa	0	0	0	0	0	0	1
Cleome fimbriata	?	?	?	?	?	?	?

Cleome foliosa	1	0	0	1	0	1	0
Cleome gigantea	?	?	?	?	?	?	?
Cleome gynandra	0	0	0	0	0	0	1
Cleome hassleriana	0	0	0	0	0	0	1
Cleome hemsleyana	1	0	0	1	0	1	?
Cleome hirta	0	1	1	0	0	0	1
Cleome iberidella	0	1	1	0	0	0	1
Cleome kalachariensis	1	0	0	0	0	1	1
Cleome khorassanica	?	?	?	?	?	?	?
Cleome lanceolata	?	?	?	?	?	?	?
Cleome lechleri	0	0	0	0	0	0	1
Cleome linearifolia	1	0	0	1	0	?	1
Cleomella longipes	?	?	?	?	?	?	?
Cleomella obtusifolia	?	?	?	?	?	?	?
Cleome luderitziana	1	0	0	1	0	1	1
Cleome lutea	0	0	0	0	0	0	1
Cleome macrophylla	0	1	1	0	0	0	1
Cleome maculata	0	1	1	0	0	0	1
Cleome melanosperma	?	?	?	?	?	?	?
Cleome microaustralica	0	0	0	0	0	1	1
Cleome microcarpa	?	?	?	?	?	?	?
Cleome monochroma	0	0	0	0	0	0	1
Cleome monophylla	0	0	1	0	0	0	1
Cleome moritziana	?	?	?	?	?	?	?
Cleome multicaulis	?	?	?	?	?	?	?
Cleome ornithopodioides	1	1	0	1	0	?	1
Cleome oxalidea	0	0	0	0	0	?	1
Cleome oxyphylla	0	?	1	0	0	1	1
Cleome paludosa	0	0	0	0	0	0	1
Cleome paradoxa	0	0	0	0	0	0	1
Cleome parviflora	0	0	0	0	0	0	1
Cleome pernambucensis	?	?	?	?	?	?	?
Cleome pilosa	0	0	0	0	0	0	1
Cleome platycarpa	?	?	?	?	?	?	?
Cleome quinquenervia	?	?	?	?	?	?	?
Cleome rosea	0	0	0	0	0	0	1
Cleome rotundifolia	0	0	0	0	0	0	1
Cleome rutidosperma	0	0	0	0	0	0	1
Cleome schimperi	0	0	0	0	0	0	1
Cleome schweinforthii	?	?	0	1	0	0	1
Cleome serrulata	0	0	0	0	0	0	1

Cleome siliculifera	0	0	0	0	1	0	1
Cleome sparsifolia	?	?	?	?	?	?	?
Cleome speciosa	?	?	?	?	?	?	?
Cleome spinosa	0	0	0	0	0	0	1
Cleome stenophylla	?	?	?	?	?	?	?
Cleome strigosa	?	?	?	?	?	?	?
Cleome stylosa	?	?	?	?	?	?	?
Cleome sulfurea	1	0	0	1	0	1	1
Cleome tetranda	0	0	0	0	0	0	1
Cleome titubans	?	?	?	?	?	?	?
Cleome torticarpa	?	?	?	?	?	?	?
Cleome trachycarpa	0	0	0	0	0	?	1
Cleome tucamanensis	0	0	0	0	0	?	1
Cleome turkmena	0	0	0	0	0	0	1
Cleome uncifera	0	0	1	0	0	0	1
Cleome usambarica	0	1	0	0	0	0	1
Cleome violacea	1	1	1	1	1	0	1
Cleome viridiflora	0	0	0	0	0	0	1
Cleome viscosa	0	0	0	0	0	1	1
Cleome werdermannii	0	0	0	0	0	0	1
Dactylaena microphylla	?	?	?	?	?	?	?
Dactylaena pauciflora	?	?	?	?	?	?	?
Dipterygium glaucum	?	?	?	?	?	?	?
Iberis oppositifolia	?	?	?	?	?	?	?
Iberis spathulata	?	?	?	?	?	?	?
Isomeris arborea	0	0	0	0	0	0	1
Nasturtium officinale	?	?	?	?	?	?	?
Oxystylis lutea	?	?	?	?	?	?	?
Cleome tenuifolia	?	?	?	?	?	?	?
Podandrogyne chiriquensis	?	?	?	?	?	?	?
Podandrogyne decipiens	?	?	?	?	?	?	?
Podandrogyne jamesonii	?	?	?	?	?	?	?
Podandrogyne macrophylla	?	?	?	?	?	?	?
Podandrogyne mathewsii	?	?	?	?	?	?	?
Podandrogyne pulcherrima	?	?	?	?	?	?	?
Polanisia dodecandra	?	?	?	?	?	?	?
Polanisia uniglandulosa	?	?	?	?	?	?	?
Sisymbrium altissimum	?	?	?	?	?	?	?
Stanleya pinnata	?	?	?	?	?	?	?
Wislizenia refracta	?	?	?	?	?	?	?

Chapter 3: Correlation of Early Floral Development to Expression of *TCP1* Homologues in *Cleome violacea*

Introduction

Floral types can be broadly categorized as either monosymmetric (zygomorphic, irregular) or polysymmetric (actinomorphic, regular) based on the number of planes of symmetry exhibited around the floral axis. While both of these floral types generally have four concentric whorls of leaf-like organs arranged around the apical meristem (specifically sepals, petals, stamens, and carpels) monosymmetric flowers also have distinct identities according to their adaxial-abaxial position on the floral meristem (Coen and Meyerowitz 1991; Endress 1999). This complicated morphology evolved numerous times from polysymmetric ancestors in response to interaction with insect pollinators (Endress 1999; Sargent 2004; Gomez et al. 2006). Transitions to monosymmetry thus play an important role in angiosperm diversification, to the extent that the most diverse lineages are dominated by monosymmetric taxa (e.g. Lamiaceae, Orchidaceae, Fabaceae; Endress 1999). Transitions from monosymmetry to polysymmetry are rare (Citerne et al. 2006; Wang et al. 2008; Zhang et al. 2010; Howarth et al. 2011; Preston et al. 2011).

At the molecular level, members of the large TCP gene family of transcription factors are consistently implicated in shifts from polysymmetry to monosymmetry (Cubas et al. 1999; Endress 2001; Howarth and Donoghue 2006; Busch and Zachgo 2007; Rosin and Kramer 2009). The name of this gene family is based on the first four proteins in which the TCP domain was identified: *teosinte branched1* (*TB1*) from maize (*Zea mays*; Poaceae; Doebley et al. 1997), *CYCLOIDEA* (*CYC*) from snapdragon (*Antirrhinum majus;* Plantaginaceae; Luo et al. 1996), and *PROLIFERATING CELL FACTORS 1* and *2 (PCF1* and *PCF2*) from rice (*Oryza sativa,* Poaceae; Kosugi and Ohashi 1997). Collectively, these transcription factors are associated with cell proliferation, either suppression or enhancement depending on the organ (Martin-Trillo and Cubas 2009). Structurally, members of this large and diverse gene family have a conserved TCP domain that adopts a helix-loop-helix conformation known to bind DNA (Martin-Trillo and Cubas 2009). A subgroup of closely related TCP genes forms the ECE

clade (Howarth and Donoghue 2006); members have a conserved arginine rich R domain in addition to a TCP domain (Martin-Trillo 2009). Asymmetric expression of TCP genes in the ECE clade are implicated in adaxial-abaxial differentiation during floral development in taxonomically diverse angiosperms: Papaveraceae (Damerval et al. 2007), Plantaginaceae (Hileman et al. 2003); Fabaceae (Feng et al. 2006); Malpighiaceae (Zhang et al. 2010); Caprifoliaceae (Howarth et al. 2011); Commelinaceae (Preston and Hileman 2012). No exceptions have been found within the core eudicots (Busch et al. 2012).

The molecular framework of TCP genes underlying development of monosymmetric flowers is characterized in the most detail for snapdragon (*Antirrhinum majus* L.). Wildtype flowers of snapdragon have five petals, four stamens, and an adaxial staminode. Monosymmetry is due to fusion of two adaxial petals independently of the fused two lateral and single abaxial petals. Prolonged expression in adaxial regions of the flower of the *AtTCP1* homologue *CYCLOIDEA* (*CYC*) underlies retarded growth rate and reduced number of petal and stamen primordia initiated in adaxial regions of the flower, as well as size and cell types of organs in the stamen and petal whorls (Luo et al. 1996). A paralogue of *CYC*, *DICHOTOMA* (*DICH*), contributes to monosymmetry of mature flowers in *Antirrhinum*, specifically internal asymmetry of adaxial petals (Luo et al. 1996). Expression of *DICH* in later stages of development is restricted to the adaxial petal lobes (Luo et al. 1996). In *cyc*/*dich* double mutants, flowers are strongly polysymmetric and all petals exhibit abaxial morphology (Luo et al. 1996). Downstream targets of *CYC* include MYB-like transcription factors *RADIALIS* (*RAD*) and *DIVARICATA* (*DIV*) (Corley et al 2005; Almeida et al. 1997; Galego and Almeida 2002). *RAD* inhibits *DIV*, which otherwise confers adaxial petal identity (Corley et al. 2005). Relatively little is known about upstream regulation of *TCP1* (Martin-Trillo and Cubas 2009).

Recent studies on TCP and floral monosymmetry have extended to the Brassicaceae (Busch and Zachgo 2007, Zachgo et al. 2012), a family with predominantly polysymmetric flowers that includes the genetic model *Arabidopsis thaliana*. Sequencing of the *Arabidopsis* genome reveals a total of 24

TCP genes (Damerval and Manuel 2003; Riechmann et al. 2000; Xiong et al. 2005). Only one of these genes is orthologous to *CYC*-like genes: *AtTCP1* (Cubas et al. 2001). Interestingly, *AtTCP1* is expressed adaxially in developing floral buds, even though mature flowers in *Arabidopsis* are polysymmetric (Cubas et al. 2001). In addition, flowers of *tcp1* mutants exhibit no discernable phenotype (Cubas 2004). Monosymmetric flowers in Brassicaceae are limited to a single clade, expanded lineage II, in which flowers of some species exhibit a simple monosymmetry type characterized by enlarged abaxial petals (Busch et al. 2012). In these species, *AtTCP1* homologues are expressed in adaxial regions of the flower during developmental stages that correspond to differential growth rates between adaxial and abaxial petals with no associated interaction with a paralogue (Busch et al. 2012). Thus, investigation of interactions between *AtTCP1* homologues is limited in Brassicaceae by available variation at the morphological and molecular level.

There is evidence that further investigation of mechanisms underlying floral monosymmetry in Cleomaceae, the sister family to Brassicaceae, will reveal additional levels of molecular interactions. Specifically, the close relationship of this family to Brassicaceae, and corresponding similarity at the molecular level, facilitates application of molecular techniques developed for *Arabidopsis* to a family that produces morphologically diverse, monosymmetric flowers (Iltis et al. 2011). Furthermore, floral monosymmetry in Cleomaceae appears to be evolutionarily labile relative to the cruciform flowers of Brassicaceae (Hall 2002; Hall 2008; Iltis 1957). Finally, Cleomaceae has also undergone a whole genome duplication independent of Brassicaceae (Schranz and Mitchell-Olds 2006), suggesting multiple copies of *TCP1* may be involved in the unique floral monosymmetry of cleomoid flowers.

Monosymmetry in Cleomaceae is most pronounced in the corolla due to curvature of the petal bases, and differences in size, shape, and colour of petals (Endress 2001; Hall et al. 2002; Patchell et al. 2011). Upward curvature of the androecial and gynoecial whorls also contributes to overall monosymmetry of the anthetic flower (Endress 2001; Hall et al. 2002; Patchell et al. 2011).

Furthermore, a minimum of two distinct patterns of early development leads to morphologically similar monosymmetric flowers in Cleomaceae (Patchell et al. 2011), which indicate potentially diverse underlying molecular interactions and/or different molecular basis of monosymmetry. Species with flowers that exhibit early monosymmetry produce broad abaxial sepals early in development of the bud, while those with early disymmetry have equally sized sepals (Patchell et al. 2011). Considering Cleomaceae plus Brassicaceae are sister to Capparaceae, which is also dominated by polysymmetric taxa (Hall et al. 2002), Cleomaceae represents both an opportunity to investigate a radiation of a monosymmetric clade from polysymmetric ancestors and a new system in which to investigate molecular interactions involved in the development of complex monosymmetric flowers. Moreover, this system offers the opportunity to compare different developmental trajectories underlying a seemingly similar mature morphology.

One of the most markedly monosymmetric taxa, *Cleome violacea* L, exhibits characteristics that make it an ideal focal taxon for examining monosymmetry (Figure 3.1). Flowers in this species exhibit clear differences in adaxial and abaxial organs in the sepal and petal whorls including internal asymmetry and abaxial/adaxial differences in petal colour and size (Patchell et al. 2011). Although this species has been included in previous developmental studies (Patchell et al. 2011; Karrer 1991; Erbar and Leins 1997), critical developmental stages have not been described, which currently limits use of this species as a model for floral development. However, investigation of floral symmetry in *C. violacea* is very timely because rapid generation time, small genome size (Schranz, personal communication), ease of propagation from seed (personal observation), rapidly increasing molecular tools including development of a transformation protocol (Mankowski unpublished), a transcriptome library (Pires, unpublished data), and recent identification by the Brassicaceae community as high priority for full genome sequencing (Pires, personal communication) continue to increase research utility and interest in this species.

Two *TCP1* paralogues are involved in establishing monosymmetry in snapdragon flowers (Luo et al. 1996; Almeida et al. 1997; Galego and Almeida

2002; Corley et al. 2005), whereas a single copy is implicated in monosymmetric Brassicaceae (Busch and Zachgo 2007, Busch et al. 2012). In *Antirrhinum*, expression of both *TCP1* homologues is asymmetric across the developing flowers, with higher levels of expression observed in adaxial regions of the flower. Furthermore, high levels of expression in stem tissue observed in Brassicaceous species indicate the importance of asymmetrical expression, independent of total expression level, within whorls to establishment of monosymmetry. Consistent with expression patterns documented in these and other taxa (Damerval et al. 2007; Hileman et al. 2003; Feng et al. 2006; Zhang et al. 2010; Howarth et al. 2011; Preston and Hileman 2012) expression of TCP1 homologues in Cleomaceae are expected to be expressed asymmetrically across the developing floral whorls and at higher levels in adaxial regions of the buds. Furthermore, timing of asymmetrical expression should correspond to periods of asymmetrical growth within floral whorls.

The purpose of this study is to provide a foundation in understanding the molecular basis of floral monosymmetry in Cleomaceae with emphasis on *Cleome violacea*. First, I identified *TCP1* homologues from three species of Cleomaceae, which represent the two developmental pathways to achieve monosymmetry. These data permit comparison of TCP sequences among *Cleome spinosa, C. violacea,* and *C. viridiflora* as well as *A. thaliana*. Second, I examined expression patterns of one TCP1 gene in *C. violacea* to test the hypothesis that this gene exhibits asymmetrical expression patterns that can be correlated with its monosymmetric flowers. Finally, I build on previous developmental work of *C. violacea* in order to provide detailed context for TCP1 gene expression patterns as well as future floral studies. Hypotheses regarding timing of expression during development and potential interaction of *TCP1* homologues in Cleomaceae are discussed.

Methods

Plant Material and growth conditions

TCP1-like genes were isolated for three *Cleome* species, including one representative that exhibits early monosymmetry (*Cleome violacea*) and two representatives of the early disymmetric pattern (*C. spinosa* and *C. viridiflora*). Wild type plants of each species were grown from seed in the University of Alberta growth chambers. Seeds were germinated in water agar, transplanted to individual four-inch plastic pots, and grown at 24 °C under long day conditions (16 hours of light, 8 hours of dark). Genetic isolation was maintained using perforated plastic bags to enclose each plant. Voucher specimens were deposited in the University of Alberta Vascular Plant Herbarium (ALTA): *Cleome spinosa* Jacq. (Hall and Mankowski 25 July 2008; 403258 from B&T World Seeds), *Cleome violacea* L. (Hall and Bolton Feb. 2008; 813 from Hortus Botanicus), and *Cleome viridiflora* Shreb. (Mankowski & Bolton 23 June 2008; 814 Hortus Botanicus).

Developmental and floral morphology of Cleome violacea

In preparation for examination by scanning electron microscopy (SEM), buds and inflorescence tips from *Cleome violacea* fixed in FAA (50% ethanol, 10% formalin, and 5% acetic acid) dehydrated in an ethanol series, and critical point dried in liquid CO2 using a Baltec Critical Point Dryer model 030. Dehydrated tissue was dissected onto carbon conductive tabs mounted on SEM stubs. Floral organs were selectively removed from older buds to expose inner whorls. Stubs were sputter coated with gold in Anatech Hummer Sputtering System and micrographs were prepared using a Philips/ FEI laB6 Environmental Scanning Electron Microscope (ESEM). Size measurements correspond to the size range of at least three flowers at each developmental stage described. Fresh buds and anthetic flowers were dissected on filter paper and photographed using a Nikon SMZ 1500 dissecting microscope. Photographs were processed in Photoshop (Adobe Photoshop CS2 version 9.0.2) to adjust contrast and remove textured background.

Identification of TCP1 homologues

Putative orthologues of *AtTCP1* were identified from both genomic DNA and cDNA pools of *Cleome violacea* and genomic DNA of *C. spinosa, C. violacea*, and *C. viridiflora*. Total RNA was extracted from fresh *C. violacea* inflorescences, including multiple stages of floral development, using Concert Plant RNA Reagent kit (Invitrogen, Carlsbad, CA, USA). From the total RNA extract, mRNA was purified using a Dynabeads mRNA Direct Kit (Invitrogen). cDNA was then synthesized using Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). Total DNA was extracted and purified from dried *C. spinosa, C. violacea*, and *C. viridiflora* vegetative tissue stored in silica using DNeasy Plant mini kits (Qiagen inc. Mississauga, Ont.).

Isolation of TCP1 homologues required experimentation to determine suitable reaction conditions prior to cloning: suitable primers were identified from previously published primer sets, proofreading polymerases that do not leave A' overhangs necessary for efficient cloning were identified as an important factor in successful PCR reactions, and pGem cloning kits (Promega) were concluded to be more successful at cloning than TOPO TA kits (Invitrogen). The 3' untranslated region (UTR) was easily amplified, but remains resistant to cloning. A complete primer list is included in Table 3.1, and the range of primers combinations, enzymes, and cloning kits used in experiments are summarized in Table 3.2.

Here, I describe the experimental procedure used to identify TCP1 homologues in *Cleome*. The primers TCP 5' fwd and TCP 3' rev used to amplify TCP1 homologues in *Iberis amara* L. (Table 3.1; Busch and Zachgo 2007) consistently amplified a 1000-1100 bp long fragment from both cDNA and genomic templates. Polymerase chain reaction (PCR) conditions included an initial denaturation of 10 min. at 94 °C, followed by 36 cycles of denaturation at 94 °C for 0.5 min, annealing at 55°C for 1 min, and extension at 72 °C for 2 min, followed by a final extension of 72°C for 10 min. The amplified fragment was excised from agarose gel, purified using the QIA quick gel extraction kit (Qiagen), and cloned using pGem T-easy plasmids (Promega) propagated in JM109 cells (Promega). Forty-six to 135 colonies were screened using the M13 forward and M13 reverse primers to visualize variation in the size of cloned

fragments. From these, plasmids were isolated from 20-66 colonies per species using Qiagen miniprep kits (Qiagen; Table 3.3). Sequences were obtained by cycle-sequencing reactions using M13 forward and M13 reverse primers (ABI Big Dye v. 3.1, Applied Biosystems, Foster City, CA). Sequence reactions were then purified using Performa DTR V3 96-well short plates (Edge Biosystems, Gaithersburg, MD) and run on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

To determine sequence identity, sequences were edited using Sequencher v. 4.10.1 (Gene Codes Corporation, Ann Arbor, MI), putatively identified as TCP1 homologues using BLAST (NCBI Blast), and then aligned with ClustalW v. 2.1 (Larkin et al. 2007) implemented in MacVector v. 12.0.2. Sequences were then codon aligned using visual inspection in Mesquite v. 2.74 (Maddison and Maddison 2010) to the known *Arabidopsis* sequence (Genbank # NM 001160982). Orthology of the sequences with the *Arabidopsis* homologue, *AtTCP1*, was confirmed by phylogenetic analysis (Figure 3.4 A). When preliminary phylogenetic analysis using neighbour-joining methods, in addition to visual inspection of the alignment, showed that sequences with high sequence similarity constitute the same clade only one representative sequence from each species was selected to include in a more rigorous phylogenetic analysis (data not shown). The *Arabidopsis* sequences for *TCP-P* (*AtTCP16*) was used as the outgroup, and representatives of major clades in the TCP gene family were used to determine orthology: *CYC3* (*AtTCP12*) and *CIN* (*AtTCP4*) (Figure 3.3 A). Maximum parsimony analyses were conducted on aligned nucleotides in PAUP* v. (Swofford 2000) with the following search parameters: simple addition sequence and tree-bisection-reconnection (TBR) branch swapping. Bootstrap support (Felsenstein 1985) was assessed using 1000 replicates of the same search parameters.

In Situ *Hybridization*

Buds and inflorescence tips prepared for use in *in situ* experiments were dissected directly from *Cleome violacea* plants, fixed in chilled, freshly prepared

FAA (50% ethanol, 10% formalin, and 5% acetic acid), dehydrated in an ethanol series, cleared with Citrisolv* (Fisherbrand, Fisher Scientific), and infiltrated with Paraplast Xtra (McCormick Scientific) using a microwave procedure (Table 3.4; modified from Kramer unpublished). Tissue was subsequently embedded in plastic moulds and stored at 4°C prior to sectioning. Blocks were sectioned to 8 micrometers using a Microm HM 325 (Walldorf, Germany) microtome. Sections were adhered to Probe on Plus slides (Thermo Fisher Scientific Inc.) by floating ribbons of attached sections on pools of water, removing water with a pipette, and allowing drying for four hours at 35°C. Slides were used immediately for *in situ* hybridization.

Probe preparation and *in situ* hybridization was performed as described (Kramer 2005). The 1100 bp long *CvTCP1.1* cDNA fragment isolated from *Cleome violacea* was used as a template for RNA probe preparation. A 615 bp long probe sequence from the downstream of the conserved TCP was amplified from minipreps (Qiagen) using *ClevioTCP1.1* sequence specific primers: PVF1 (forward) and PVR1 (reverse). Fragments were cloned into pCR 2.1-TOPO plasmids (Invitrogen) propagated in One Shot Mach1-T1 competent cells (Invitrogen). Plasmids were purified using midiprep kit Nucleobond Xtra Midi, (Machery-Nagel). Amplicon identity was confirmed by sequencing. Plasmids were linearized by digestion with either SPE1 (sense) or Not1 (antisense). Sense and antisense digoxygenin-labelled RNA probes were generated by reverse transcription using a 35% mix of digoxygenin labeled nucleotides (Roche) and catalyzed by either T7 (sense) or T3 (antisense) RNA polymerases. The 615 bp long probe was hydrolyzed to a final length of 150 base pairs.

In situ hybridization was performed on recently sectioned tissue mounted on slides. Sectioned tissue was rehydrated in an ethanol series, cleared of surrounding wax matrix, and then digested with protease for 10 minutes at a concentration 800 µL/300 mL to make cell walls permeable to probes. Sections were hybridized with digoxygenin-labelled *CvTCP1.1* antisense hydrolyzed RNA probe. A subset of slides was treated with hydrolyzed probe identical in sequence to the endogenous *CvTCP1.1* mRNA (sense) rather than complementary

(antisense) to control for nonspecific hybridization. Slides were then treated with RNase A to leave only the double stranded products. Anti-digoxigenin antibodies conjugated to an alkaline phosphatase (Roche 1 093 274) produced a bluish-coloured precipitate when allowed to develop in a substrate solution of NBT (Roche 1 383 213) and BCIP (Roche 1 383 221). Sections were then visualized and imaged using a combination of white and fluorescent light after counterstaining with calcofluor. Sections were digitally photographed using a NIKON H550L fluorescence microscope equipped with a Nikon DS-Ri1 imaging system.

Results

Anthetic Cleome violacea *flowers*

Cleome violacea flowers are monosymmetric at anthesis. Monosymmetry in the corolla is due to upward orientation of petal bases and position of the adaxial nectiferous gland (Figure 3.1 A). Adaxial petals have yellow eyespots on a maroon background, while abaxial petals are uniform maroon in colour (Figure 3.1 A). Adaxial and abaxial petal pairs are also different in shape and size. Adaxial petals are narrower at 1.5-2.0 mm relative to abaxial petals that are 2.5-3.5 mm wide. Adaxial and abaxial petals are similar in length, measuring 3-4 mm. The enlarged abaxial sepal also contributes to monosymmetry of the mature flower. Abaxial sepals are 0.8-1.0 mm wide compared to lateral or adaxial sepals, which are 0.6-0.8 mm and 0.3-0.5 mm respectively. Although cells at the base of the petals are somewhat elongate (Figure 3.1 C), the shape of cells in the abaxial petals (Figure 3.1 D) is similar to the shape of cells in the adaxial petals (Figure 3.1 E). Furthermore, cells of the abaxial sepals (Figure 3.1 F) are similar to cell of the adaxial sepals (Figure 3.1 G). Flowers are not noticeably scented (data not shown).

Development of Cleome violacea *flowers*

Cleome violacea inflorescences are indeterminate racemes. Flowers arise from the apical meristem in spiral succession such that a convenient range of developmental stages is represented in each inflorescence. The following description ascribes developmental events to a series of 12 stages (Table 3.5) that parallel descriptions for the closely related species *Arabidopsis thaliana* (Smyth et al. 1990). Use of similar developmental markers to track development will be used for description of gene expression patterns.

Stages 1 to 5

Flower primordia are first visible as lateral growths 15-20 μm across on the outer margin of the dome shaped apical meristem, and are already subtended by a developing bract (Figure 3.2 A). Stage 1 begins with the initial appearance and lateral growth of the floral buttress (Figure 3.2 A). Formation of a transverse groove delimiting the floral buttress from the apical meristem marks the transition to stage 2 (Figure 3.2 A). The size of the buttress increases during stage 2 to a size of 30-76 μm (Figure 3.2 A, B). Stage 3 begins when a transverse groove delimits abaxial sepal primordia from the floral buttress (Figure 3.2 A). The appearance of the abaxial sepal primordia is followed by lateral sepal primordia later in stage 3 (Figure 3.2 A). The abaxial sepal is broad relative to the other sepals, and remains so throughout development. Buds at this stage become stalked, showing a short pedicel (Figure 3.2 A). Flower primordia are 95-100 μm in size. The abaxial sepal elongates and overarches the adaxial region of the bud at stage 4 (Figure 3.2 A, C). Stage 5 is marked by the appearance of petal primordia at the corners of the trapezoid shaped floral apex and the adaxial sepal (Figure 3.2 D). The distance between the adaxial petal primordia, delimiting the adaxial margin of the trapezoid shaped floral apex, is 40-53 μm. The distance between the abaxial petal primordia, delimiting the abaxial margin of the floral apex, is 100-130 μm. Near the end of stage 5, the six stamen primordia appear around the central dome of the floral apex (Figure 3.2 E). The size of the stamens decreases from the abaxial to the adaxial region of the bud. The larger adaxial stamens appear before the smaller adaxial stamens. Size asymmetry in the stamen

whorl persists throughout development. Sepal growth continues through stages 3-5 to overarch the inner whorls (Figure 3.2 A, C - F).

Stages 6 to 12

The larger abaxial sepal completely envelops the inner whorls at the beginning of stage 6 (Figure 3.3 A). Stage 7 begins when the larger abaxial stamens become stalked, delimiting the young anther and filament (Figure 3.3 B). At this stage, the floral primordia are 200-260 μm across. Petal primordia are hemispherical to lens shaped and remain small (approximately 35-50 μm). Buds transition rapidly to stage 8 begins when anther locules can be observed on the inner surface of the larger abaxial stamens (Figure 3.3 B). Stage 9 is marked by elongation of petal primordia, which at this point become stalked and differentiated between adaxial and abaxial petal pairs (Figure 3.3 C). Abaxial petals are longer, and wider relative to adaxial petals. Petals increase in length, eventually surpassing the stamens but never closely enveloping the inner whorls. Stage 10 begins when petals reach the shorter adaxial stamens (Figure 3.3 D). Stigmatic papillae were not observed to mark the transition to stage 11. During this stage, the nectiferous gland appears and increases rapidly in size in during subsequent development (Figure 3.3 E-F). Flowers transition rapidly to stage 12 at which point the elongating petals reach the tips of the larger abaxial stamens (Figure 3.3 F). Stage 12 is the mature bud prior to anthesis (Figure 3.3 F). During this stage the gynoecium, stamens, and petals continue to elongate. Petals are uncurved in the bud. Curvature of petal bases occurs just prior to anthesis (Figure 3.4 A-D).

Isolation of TCP1-like genes in Cleome

All *Cleome* TCP sequences form a well-supported clade that includes *AtTCP1*, indicating that all copies recovered in these experiments belong to the *CYC2* clade (Figure 3.5 A). Importantly, TCP sequences from *Cleome* are found in two clades, indicating at least two divergent *TCP1*-like sequences in *C. spinosa, C. violacea,* and *C. viridiflora* (Figure 3.5 A). Current taxon sampling

precludes assessing whether this duplication occurred within Cleomaceae or before Brassicaceae and Cleomaceae diverged. The same length of *ClevioTCP1.1* sequences isolated from both cDNA and genomic DNA indicates the lack of introns in this region of the gene (data not shown), which is consistent with structure of *TCP1* in *Arabidopsis* (TAIR: http://www.arabidopsis.org/index.jsp; gene model for accession AT1G67260.2).

The *ClevioTCP1.1* cDNA sequence is distinct from *ClevioTCP1.2* at the nucleotide level. These copies share 48% percent identity at the amino acid level. In the region of probe overlap, the probe shares 100 percent identity with the *ClevioTCP1.1* sequence, but only 52% percent identity with *ClevioTCP1.2*. An alignment of nucleotide sequences of these TCP1 homologues in *Cleome* is shown in Figure 3.5 B. Furthermore, the probe sequence used for *in situ* hybridization excludes the conserved TCP domain. A protein alignment showing position of the *in situ* probe relative to the conserved *Arabidopsi*s TCP domain (Cubas et al. 1999) is shown in Figure 3.5 C. The R domain (Cubas et al. 1999) was not recovered.

Expression pattern ClevioTCP1.1 *in developing flowers of* Cleome violacea

In situ hybridization was used to assess the temporal and spatial expression patterns of *ClevioTCP1.1.* I was unable to observe expression patterns in the earliest stages of development, including the formation of the floral meristem, stages 1-2, and earliest stages of sepal development when size asymmetries are first established (stages 3-4). *ClevioTCP1.1* expression was first observed in the primordia of the abaxial and lateral sepals, stamens, and petals at stage 5 (Figure 3.6 A). Expression was observed in petal and stamen primordia of stage 6 buds (Figure 3.6 B), but no longer in the sepals. In stage 7 buds, expression was observed in petals and stamens (Figure 3.6 C). In stage 8 buds, the expression domain encompassed petals, stamens, and the gynoecium (Figure 3.6 D). In stage 9 buds, expression is observed in adaxial and abaxial petals and gynoecium (Figure 3.6 E). Similarly, expression is observed in adaxial and abaxial petals in stage 10 buds (Figure 3.6 F). Prolonged expression could be

detected during stamen development, from appearance of stamen primordia at stage 5 (Figure 3.6 A), through to differentiation of anther locules at stage 8 (Figure 3.6 1 D). Prolonged expression was also observed in developing petals, from their appearance at stage 5 through to early stages of elongation and differentiation between petal pairs at stage 9 (Figure 3.6 A-F), and in the gynoecium at stage 7 (Figure 3.6 D). No asymmetric expression between adaxial and abaxial regions of the bud was detected at any developmental stage (Figure 3.6 A-F). Hybridization with sense probes generated limited background signal in the cell walls of developing stamens, but no clear expression in the inner whorls (Figure 3.6 G-H).

Discussion

A clade of TCP transcription factors, including *TCP1*, has been shown to have strong effects on floral symmetry across distantly related angiosperms (Damerval et al. 2007; Hileman et al. 2003; Feng et al. 2006; Zhang et al. 2010; Howarth et al. 2011; Preston and Hileman 2012). However, data from the Rosid order Brassicales, which contains *Arabidopsis*, is limited to the Brassicaceae, a family dominated by disymmetric flowers (Busch and Zachgo 2007; Zachgo et al. 2012). This study represents the first examination of *TCP1* homologue expression in the Cleomaceae, which includes taxa that are highly amenable to investigation of floral evolution. Two divergent *TCP1*-like sequences were recovered from three species: *Cleome violacea, C. spinosa*, and *C. viridiflora*. The highly monosymmetric taxa *C. violacea* was chosen as a representative of the early monosymmetric pattern of development, characterized by the early appearance of an enlarged abaxial sepal (Patchell et al. 2011). *In situ* experiments reveal that expression of one *TCP1* homologue, *ClevioTCP1.1,* occurs early in development in the petals, stamens, and gynoecium. Developmental stages documented by scanning electron microscopy show that this expression occurs during developmental events that impart monosymmetry to the bud: appearance of petals define the corners of a trapezoidal shaped floral apex, a size gradient of

stamens from larger in the abaxial region to smaller in the adaxial region, and the differentiation in shape and size of abaxial and adaxial petal pairs.

Development of monosymmetry in Cleome violacea*: characterization in stages and candidate stages for* TCP1 *expression.*

Two patterns of early development are documented in Cleomaceae: early monosymmetry and early disymmetry (Patchell et al. 2011). *Cleome violacea* exhibits early monosymmetry, in which the abaxial sepal is larger than the lateral and adaxial sepals and envelopes the inner whorls, the floral apex is trapezoid in shape, and petals remain small until later stages closer to anthesis (Patchell et al. 2011). Development in *C. violacea* can be further described in stages that parallel those assigned to *Arabidopsis* development (Smyth et al. 1990). Early stages of *C. violacea* development correspond well with those documented in *Arabidopsis*, while intrinsic differences in mature flowers, including gland proliferation and differentiation of petal pairs, manifest in later stages of development and make direct comparisons difficult. Furthermore, developmental trajectories of *Cleome* species exhibiting early monosymmetry can be described using the stages described for *C. violacea*. However, species that exhibit early disymmetry, such as *C. spinosa*, will require separate consideration in assigning developmental events to comparable stages. In these taxa, sepals are equal in size and petals form at the corners of a square shaped floral apex; monosymmetry is first observed at anthesis following petal curvature (Patchell et al. 2011).

Early *TCP1* expression in adaxial regions of the floral meristems is likely ancestral in Brassicaceae (Busch et al. 2012). However, the timing of expression in the few monosymmetric taxa undergoes a heterochronic shift so that expression corresponds to periods of differential growth between adaxial and abaxial petal pairs (Busch et al. 2012). Similarly, candidate stages for asymmetrical TCP expression in *Cleome violacea* are those associated with differential proliferation of adaxial and abaxial organs that increase monosymmetry of the developing bud. Monosymmetry is first imparted to developing *C. violacea* flowers at stage 3 when asymmetric sepal sizes are established (Figure 3.2). The abaxial sepal

continues to increase in size relative to the lateral and adaxial sepals through to stage 6. These relative sepal sizes are maintained throughout subsequent development. Monosymmetry in the corolla originates with the monosymmetric orientation of primordia at the corners of the trapezoid shaped floral apex (stage 5). Differentiation in size and shape of petal pairs is apparent at stage 9. The size of stamen primordia decreases towards the adaxial region of the developing bud, thereby imparting monosymmetry to the stamen whorl. The gland proliferates rapidly during stage 11. Adaxial curvature of the petal bases occurs just prior to anthesis. The gynoecium also exhibits adaxial curvature in mature flowers. Thus, monosymmetry is documented in all floral whorls and differentiation of adaxial and abaxial regions within whorls occurs at different developmental stages, rather than only in the petal whorl as in Brassicaceae (Busch et al. 2012).

Minimum of two copies of TCP1 in Cleome

Two TCP-like genes were identified in *Cleome spinosa*, *C. violacea*, and *C. viridiflora* (Figure 3.5). This sequence diversity was not necessarily expected because only a single *TCP1* homologues has been isolated in all Brassicaceae examined to date (Busch and Zachgo 2007; Busch et al. 2012) including a whole genome sequence of *A. thaliana* (Reichmann et al. 2000). Additional sampling, including members of Capparaceae, is necessary to determine if the duplication observed here occurred before Cleomaceae and Brassicaceae diverged or if the duplication occurred within Cleomaceae. Alternatively, there may be currently unidentified copies in Brassicaceae considering an exhaustive effort to determine copy number in monosymmetric members of Brassicaceae, such as *Iberis*, *Calepina*, *Teesdalia*, *Ionopsidium*, *Streptanthus*, and *Notoceras* using degenerate primers or Southern blots has not been conducted (Busch et al. 2012; Busch and Zachgo 2007). Interestingly, representatives of both types of developmental pattern, early monosymmetry and early disymmetry, contain the same complement of putative paralogues. No paralogue is unique to one trajectory or the other. Although additional copies may be identified, current evidence

indicates that it is unlikely that sequence diversity alone underlies observed differences in development.

However, multiple *TCP1* genes increase the potential for variation in expression patterns associated with more pronounced floral monosymmetry, including differentiation between adaxial and abaxial regions in more than one whorl, and monosymmetry of organs within whorls. For example, floral monosymmetry in Brassicaceae is limited to differences in a single whorl (the petals), while independent duplication of *TCP1* members is the rule rather than the exception in a range of core eudicot clades that exhibit more pronounced monosymmetry: Plantaginaceae (Hileman and Baum 2003), Gesneraceae (Gao et al. 2008), Asteraceae (Donoghue et al. 1998; Chapman et al. 2008), Fabaceae (Citerne 2003; Fukuda et al. 2003), Malpighiaceae (Zhang et al. 2010, 2012), and Dispsacales (Howarth and Donoghue 2005). Furthermore, these duplications have been correlated with variation in expression patterns (i.e. orthologues have different expression domains) that correspond to changes in degree of monosymmetry. For example, the high degree of monosymmetry in snapdragon flowers is due not only to differentiation between adaxial and abaxial regions of the flower, but also differentiation between left and right halves of each dorsal petal caused by differences in expression domain of the two *TCP1* paralogues, *CYC* and *DICH*. Thus, it is perhaps not surprising that multiple copies have been identified in Cleomaceae, which have monosymmetry exhibited in all floral whorls and petal curvature (differentiation between adaxial and abaxial regions of the petal).

ClevioTCP1.1 *expression correlates with development of stamen, petal, and gynoecial whorls in* Cleome violacea

Stages of development that increase monosymmetry of the bud are expected to correspond to asymmetrical accumulation of *TCP1* transcripts within whorls undergoing differentiation between adaxial and abaxial regions of the developing flower. In *Cleome violacea*, early developmental events that both increase monosymmetry of the bud and correspond with observed expression

include decreasing size of stamens from abaxial to adaxial region of the bud, monosymmetry of the floral apex due to position of petal primordia, and differentiation of shape of petal pairs (summarized in Table 3.5). However, no differences in the strength of expression are observed between adaxial and abaxial regions of the flower in these whorls. There are several explanations for this broad expression pattern.

First, *ClevioTCP1.1* may not be sufficient for adaxial/abaxial differentiation as in Brassicaceae. Increasing evidence suggests involvement of numerous transcriptional regulators functioning as multimeric regulatory modules (reviewed in Martin-Trillo and Cubas 2009). In this case, *ClevioTCP1.1* could participate as a component of such a regulatory complex, the activity of which is determined by interaction with a currently unidentified, adaxially expressed gene. Since homo-dimerization or hetero-dimerization between members of the same class is required for DNA binding of TCP genes, and heterodimers bind more efficiently (Kosugi and Ohashi 2002), the participating gene is likely a *TCP1* paralogue. Clearly, expression patterns of *ClevioTCP1.2* are needed to determine if this gene is involved. In addition, a *TCP1* orthologue may not actually be involved in establishing floral monosymmetry in Cleomaceae, although this hypothesis is hard to reconcile with the maintenance of multiple copies. Evidence is accumulating that *CYC3* genes may also be implicated in floral monosymmetry in some taxa (E. Kramer, pers. communication). Moreover, B-class MADS-Box genes have been shown to be important in establishing monosymmetry in monocots (Preston and Hileman 2012). Finally, I cannot completely rule-out that my probe may be non-specific and perhaps picking up more than one copy. However, considering probes used in Brassicaceae included the conserved TCP domain (Busch and Zachgo 2007; Busch et al. 2012), while the *ClevioTCP1.1* probe targeted regions with high sequence divergence between the two copies, cross hybridization is expected to be minimal.

Second, differences in expression between adaxial and abaxial regions of the flower might still occur but fail to be detected by *in situ* hybridization at the relevant stage of development. *TCP1* genes have stronger expression in the

adaxial domain in a wide range of rosids including *Arabidopsis* (Brassicaceae; Cubas et al. 2001), *Cardamine* (Brassicaceae; Busch and Zachgo 2012), Malpighiaceae (Zhang et al. 2010, 2012), and *Lupinus* (Fabaceae, Citerne et al., 2006). However, expression patterns of *ClevioTCP1.1* do not show an accumulation of transcripts in adaxial regions of the developing flowers (Figure 3.6), but rather a strong accumulation in stamen, petal and gynoecial whorls during early development. This pattern is consistent with observations of expression patterns in some monosymmetric Brassicaceae (Busch et al. 2012). However, in these monosymmetric Brassicaceae adaxial transcript accumulation is observed in later stages of development following differentiation in shape of abaxial and adaxial petal pairs that is only detected using semi-quantitative reverse transcriptase PCR (RT-PCR) and quantitative reverse transcriptase PCR (qPCR) (Busch et al. 2012). Importantly, limited differentiation between adaxial regions of the bud occurs during earlier stages of development in monosymmetric Brassicaceae.

Finally, it is possible that *ClevioTCP1.1* is not involved in adaxial/abaxial differences across the developing bud, but rather differentiation between left and right halves of individual petals that result in curvature. This possibility might not be detected using in situ hybridization because regions of the petals are difficult to observe in sectioned flowers. Although adaxial transcript accumulation of a single copy of *TCP1* across the developing flower imparts monosymmetry to some taxa in Brassicaceae (Busch and Zachgo 2007; Busch 2012), multiple homologues underlie more complex monosymmetric morphologies in other eudicot lineages. For example, multiple *TCP1* genes in Papilionoideae and *Antirrhinum* underlie two observed types of monosymmetry in the petal whorl: dorsoventral asymmetry (DV), identified as differentiation between adaxial and abaxial regions of the flower, and organ internal asymmetry (IN), characterized by differentiation between the two halves of an individual organ within a whorl (Luo et al. 2006; Wang et al. 2008). Interestingly, IN asymmetry is unique to lineages with multiple TCP homologues (Wang et al. 2008; Rosin and Kramer 2009). Peas in subfamily Papilionoideae have one adaxial (standard), two lateral (wings) and

two fused abaxial petals (keel). The standard is internally symmetric while two wings and keel are internally asymmetric (Wang et al. 2008). In *Antirrhinum*, the ventral petal is internally symmetric, while two dorsal and two lateral petals are internally asymmetric.

In cleomoid flowers, IN asymmetry is due to upward curvature of abaxial and adaxial petal bases and is the only character that imparts monosymmetry to all cleomoid flowers. Involvement of *CleovioTCP1.1* in IN asymmetry of *Cleome* flowers would not be detected in these *in situ* hybridization experiments because expression patterns within individual petals are difficult to observe using either transverse or longitudinal sections. Furthermore, the role of *TCP1* paralogues in IN asymmetry is known from analysis of mutant lines (Luo et al. 2006; Wang et al. 2008). Stable mutant lines that exhibit a loss of IN asymmetry are unknown in Cleomaceae. However, it is possible to transiently abolish gene function using virus-induced gene silencing (VIGS; Mankowski personal communication). In this case, a putative role of *ClevioTCP1.1* in IN asymmetry and *ClevioTCP1.2* in DV asymmetry would require abolition of gene function in both copies to produce a completely polysymmetric flower.

Conclusion

Floral monosymmetry in Cleomaceae is achieved via two different developmental pathways, although mature flowers are similar (Patchell et al., 2011). Representatives from both species have multiple copies of *TCP1* homologues, which is consistent with other monosymmetric taxa as well as with the testable hypothesis that both copies are necessary in establishing monosymmetry. Developmental data for the focal taxon *C. violacea* indicate that monosymmetry of the different whorls is imparted at different stages and provide a valuable framework for future developmental genetic inquires. Expression data of *ClevioTCP1.1* provides information in that the gene is expressed throughout all the whorls that exhibit monosymmetry. However, the broad expression pattern prohibits a strong statement on how the gene may have a role in establishing monosymmetry. The expression pattern of *ClevioTCP1.1* is consistent with a role

in differentiation of adaxial and abaxial petal pairs based on similarities of the expression pattern to observations of monosymmetric Brassicaceae in which stronger transcript accumulation was only detected using RT-PCR and qPCR. It is also possible that *ClevioTCP1.1* is not directly involved in abaxial/ adaxial differentiation within the flower. Another gene may be responsible, or *ClevioTCP1.1* could potentially interact with an adaxially expressed gene, such as *ClevioTCP1.2*. Expression studies of *ClevioTCP1.2* could test this hypothesis. Alternatively, *ClevioTCP1.1* could be involved in petal curvature. VIGS experiments of *ClevioTCP1.1* and *ClevioTCP1.2* function would test this hypothesis.

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Tables

Table 3.1. Primer list

Primers and primer combinations assessed for amplification of *TCP1*-like gene fragments in *Cleome*:

Primer name	**Citation**	**Derived from**	**Primer Sequence** 5'>3'
LEGCYC-F1 ***LEGCYC-R1***	Citerne et al. 2003	*Lotus japonicus* and *Glycine max*	fwd: TCAGGGSYTGAGGGACCG rev: TCCCTTGCTCTTGCTCTTGC
TCP-fwd 5' ***TCP-rev 5'***	Busch and Zachgo 2007	*Iberis amara*	fwd: ACAATGGAGTGTACCCTCTCTCTCTTTACC rev: TTATAGTTGCTGCTAGAACTCTGSTCTAC
TCPf1-malpig polyT	Zhang et al. 2010	Malpighiaceae	fwd: AARGAYMGICAYAGYAARAT rev: CCGGATCCTCTAGAGCGGCCGCTTTTTTTT TTTTTTTT
TCPf2-malpig polyT	Zhang et al. 2010	Malpighiaceae	fwd: GCIAGRAARTTYTTYGAYYTICARGAYATG rev: CCGGATCCTCTAGAGCGGCCGCTTTTTTTT TTTTTTTT
CYCF1 ***CYCF2***	Howarth and Donoghue 2006	diverse eudicot sequences	fwd: AAAGAYCGYCACAGC rev: CTCGCYTTCGCCCTCCWCTC
PV1 fwd ***PV1 rev***	Chapter 3, this thesis	*Cleome violacea* ClevioTCP1.1 cDNA	fwd: GAG TCT GGT GAA CGG TGG AT rev: AGT CCT CAT CAA AGG GTG CA

Table 3.2. Summary of *TCP1* cloning experiments.

Experiments and reaction conditions used for amplification of *TCP1*-like gene fragments in *Cleome*: See table 3.1 for primer sequences and information.

Species	Template	Forward primer	Reverse primer	Polymerase	Cell competency	Cloning kit	Insert preparation	Notes
Cleome violacea	*cDNA/ gDNA*	TCP fwd 5'	TCP rev 3'	Platinum taq.	TOP10 chemically competent	TOPO TA	None.	Very few colonies, screening does not amplify insert.
Cleome violacea	*cDNA/ gDNA*	TCP fwd 5'	TCP rev 3'	Platinum taq Qiagen taq. Top taq.	NA	NA	NA	Failed at PCR. A selection of polymerases that leave 3' A overhangs amplify poorly.
Cleome violacea	*cDNA/ gDNA*	TCP fwd 5'	TCP rev 3'	E2 taq	TOP10 chemically competent	TOPO TA	Gel extracted and retailed.	Very few colonies, screening does not amplify insert.
Cleome violacea	*cDNA*	TCP f2 malpig	Poly T	rTaq	TOP10 electrocompetent	TOPO TA	Gel extracted.	Failed. No colonies; suspect issues with electroporator.
Cleome violacea	*cDNA*	TCP F2 malpig	Poly T	rTaq	TOP10 electrocompetent	TOPO TA blunt	Gel extracted; half reaction.	Failed. No colonies.
Cleome violacea	cDNA	TCP F2 malpig	Poly T	rTaq	TOP 10 chemically competent.	TOPO TA blunt	Gel extracted; full reaction.	Failed. No colonies.

Species	Template	Forward primer	Reverse primer	Polymerase	Cell competency	Cloning kit	Insert preparation	Notes
Cleome violacea	*cDNA/ gDNA*	TCP fwd 5'	TCP rev 3'	Platinum taq.	TOP10 chemically competent	TOPO TA	None.	Very few colonies, screening does not amplify insert.
Cleome violacea	*cDNA/ gDNA*	TCP fwd 5'	TCP rev 3'	Platinum taq Qiagen taq. Top taq.	NA	NA	NA	Failed at PCR. A selection of polymerases that leave 3' A overhangs amplify poorly.
Cleome violacea	*cDNA/ gDNA*	TCP fwd 5'	TCP rev 3'	E2 taq	TOP10 chemically competent	TOPO TA	Gel extracted and retailed.	Very few colonies, screening does not amplify insert.
Cleome violacea	*cDNA*	TCP f2 malpig	Poly T	rTaq	TOP10 electrocompetent	TOPO TA	Gel extracted.	Failed. No colonies; suspect issues with electroporator.
Cleome violacea	*cDNA*	TCP F2 malpig	Poly T	rTaq	TOP10 electrocompetent	TOPO TA blunt	Gel extracted; half reaction.	Failed. No colonies.
Cleome violacea	cDNA	TCP F2 malpig	Poly T	rTaq	TOP 10 chemically competent.	TOPO TA blunt	Gel extracted; full reaction.	Failed. No colonies.

Cleome violacea/ Cleome spinosa/ Cleome viridiflora	gDNA	TCP fwd 5'	TCP rev 3'	rTaq	Chemically competent.	pGem	Gel extracted.	Worked well, *TCP* from all three taxa.

Table 3.3. Summary of colony screening and identification of *TCP1* homologues.

Taxa	**Number of colonies screened**	**Number of colonies miniprepped**	**Number of TCP sequences recovered**
Cleome violacea	135	66	22
Cleome spinosa	132	50	5
Cleome viridiflora	46	20	5

Table 3.4. Microwave fixation protocol modified from Kramer (http://www.oeb.harvard.edu/faculty/kramer/Site/Home.html). The same solutions are used for samples and probes.

Solution	**Sample temperature** (°C)	**Water bath**[1]	**Time (minutes)**
Fixative	37	Ice	15
Fixative	37	Ice	15
Fixative	37	Ice	15
50% EtOH	67	room temperature	1.2
70% EtOH	67	room temperature	1.2
95% EtOH	67	room temperature	1.2
100% EtOH	67	room temperature	1.2
100% EtOH	67	room temperature	1.2
100% EtOH: Citrisolv[2] (50:50)	67	room temperature	1.5
100% Citrisolv	67	room temperature	1.5
citrisolv: Paraplast X-tra[3] (50:50)	67	hot water	10
Paraplast X-tra	67	hot water	10
Paraplast X-tra[4]	67	hot water	30

[1] Immerse sample vials in appropriate water bath.

[2] CitriSolv (Fisherbrand, catalogue # 22143795)

[3] Paraplast X-tra (McCormick Scientific, catalogue # 503002)

[4] Vacuum infiltrate for 2-3 minutes and repeat five times. Clean the temperature probe in CitriSolv for 5 min at 67 °C after the last Paraplast X-tra step.

Table 3.5. Comparison of *ClevioTCP1.1* expression data and floral development.

Stage	**Morphological changes that occur at this stage**	**Hypothesized expression (is this stage associated with increased monosymmetry of the bud?)**	**Expression observed with *ClevioTCP1.1* probe**
1	Flower primordia differentiate from apical meristem.	None.	Not observed.
2	Transverse groove isolates floral buttress from apical meristem.	None.	Not observed.
3	Transverse groove marks differentiation of abaxial sepal from floral buttress, followed by appearance of adaxial and lateral sepals.	Asymmetric sepals sizes are established.	Not observed.
4	Adaxial sepal begins to overarch inner whorls.	None.	Not observed.
5	Petal primordia appear at the corners of trapezoid shaped floral apex, followed by appearance of 6 stamens.	Petals define corners of trapezoidal floral apex; abaxial stamens are larger than adaxial stamens.	Expression in petals and stamens.
6	Abaxial sepal completely envelopes inner whorls.	None.	Expression in petals and stamens.
7	Abaxial stamens become stalked and gynoecium invaginates.	None.	Expression in petals, stamens, and gynoecium.
8	Anther locules appear on abaxial stamens.	None.	Expression in petals and stamens.
9	Petals elongate and differentiate between adaxial and abaxial pairs.	Differentiation in shape and size of petal pairs.	Expression in petals.

10	Petals are the same length as the shorter adaxial stamens.	None.	None.
11	Nectiferous gland rapidly increases in size.	Nectiferous gland increases rapidly in size; colour patterns become visible on petals.	Not observed.
12	Mature bud: petals are the same length as the longer abaxial stamens.	None.	Not observed.

Figures

Figure 3.1. Mature morphology of *Cleome violacea* flowers. A, Front view. B, Side view. C, Surface detail of cells at the base of petals on abaxial surface. D, Cells on abaxial surface of petals. E, Cells on adaxial surface of petals. F, Cells on abaxial surface of sepals. G, Cells on adaxial surface of sepals. Scale bars = 1 mm (A, B), 50 µm (D, E, and F, G), 25 µm (C). Abbreviations: G = gynoecium; Gl = gland; Pab = abaxial petal; Pad = adaxial petal; Sab = abaxial sepal; S = stamen.

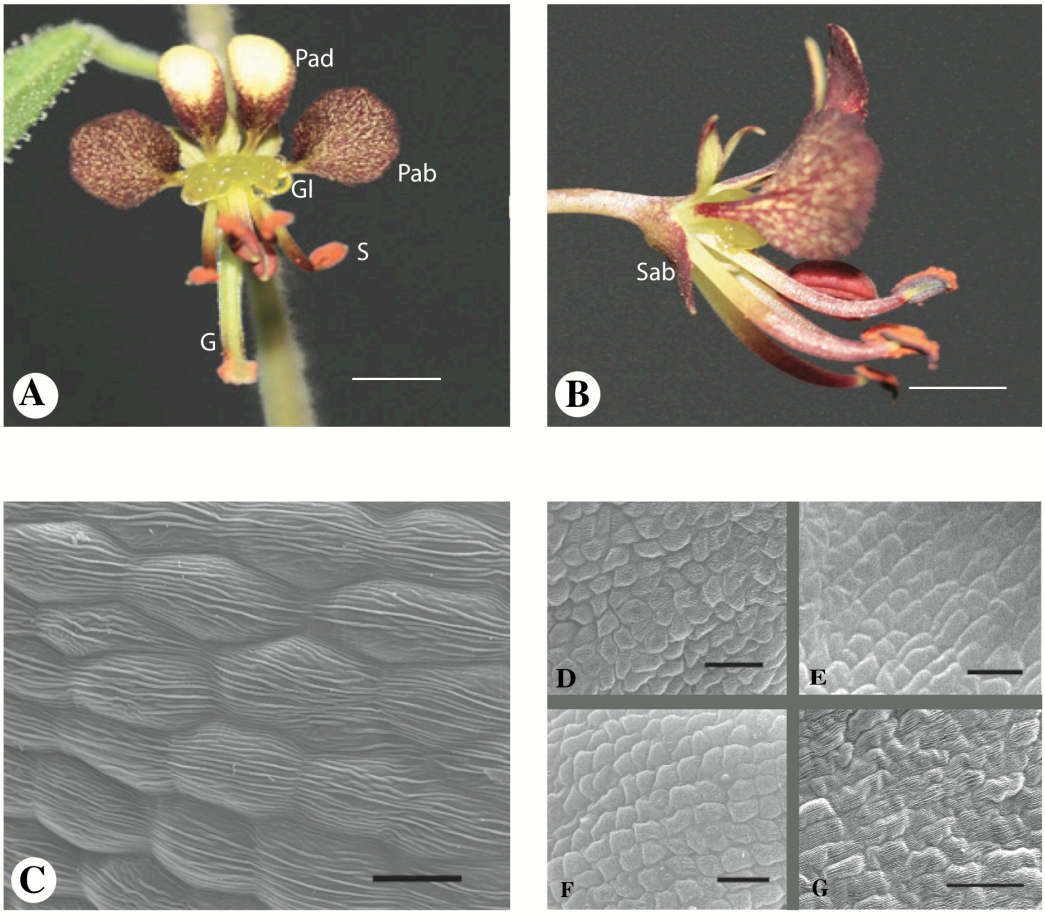

Pad
Pab
Gl
S
G
A
Sab
B
C
D
E
F
G

Figure 3.2. Stages 1-5 of early floral development in *Cleome violacea*. Scanning electron micrographs with selected organs removed, abaxial sepal towards the bottom when appropriate. A, Floral meristem showing stages 1-4. B, Late stage 2. C, Stage 4 showing enlarged abaxial sepal beginning to overarch bud. D, Early stage 5 showing appearance of petal primordia. E, Late stage 5 showing first appearance of stamen primordia. F, Undissected late stage 5 bud showing overarching abaxial sepal. Scale bars = 200 μm (A), 50 μm (D, E, and F), 20 μm (B, C). Abbreviations: B = bract; P = petal; Sab = abaxial sepal; Sad = adaxial sepal; Sl = lateral sepal; S = stamen.

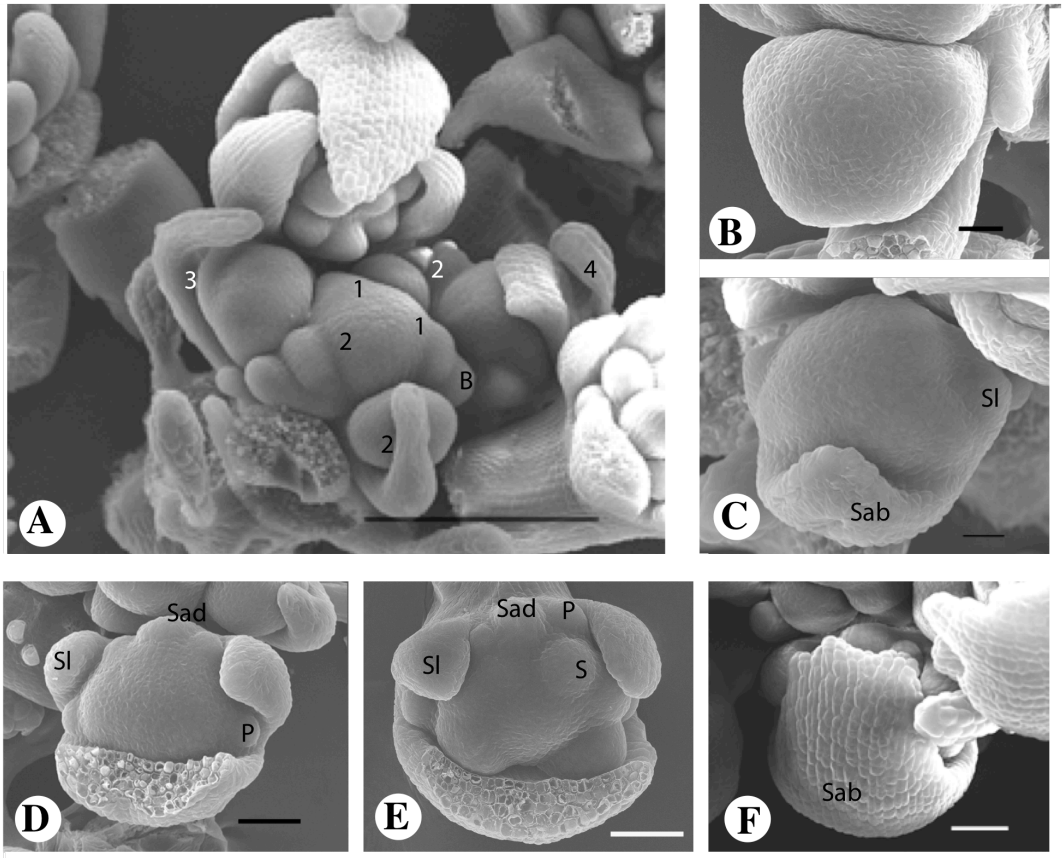

A
3
1
2
2
1
2
B
4
B
C
Sl
Sab
D
Sad
Sl
P
E
Sad
P
Sl
S
F
Sab

Figure 3.3. Stages 6-12 of middle to late development in *Cleome violacea*. Scanning electron micrographs or colour photos with selected organs removed, abaxial sepal towards the bottom when appropriate. A, Stage 6 showing abaxial sepal completely enveloping inner whorls. B, Stage 8 showing anther locules on abaxial stamens and stalked adaxial stamen. C, Stage 9 showing differentiation of adaxial and abaxial petals. D, Stage 10 begins when petals reach the shorter adaxial stamens. E, Nectiferous gland appears at stage 11. F, Stage 12 is the mature bud prior to anthesis. Scale bars = 1 mm (D, E, F); 200 µm (C); 100 µm (A, B). Abbreviations: G = gynoecium; Gl = gland; Pab = abaxial petal; Pad = adaxial petal; Sab = abaxial sepal; Sad = adaxial sepal; Sl = lateral sepal; S = stamen.

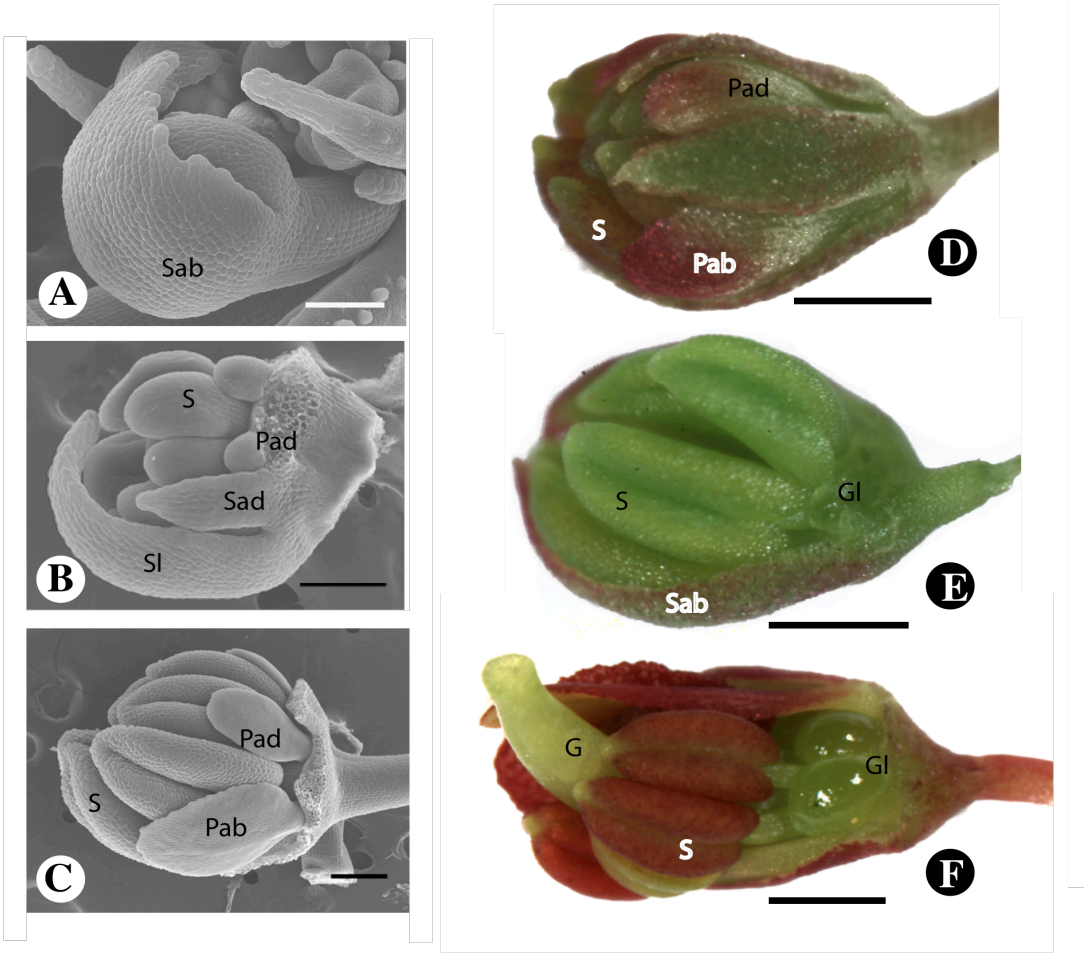

Sab
A
S
Pad
Sad
Sl
B
Pad
S
Pab
C
Pad
S
Pab
D
S
Gl
Sab
E
G
Gl
S
F

Figure 3.4. Petal curvature in *Cleome violacea*. A. Adaxial petals before anthesis. B. Abaxial petals before anthesis. C. Adaxial petals at anthesis showing curvature of petal bases. D, Adaxial petal at anthesis. Scale bar = 1 mm.

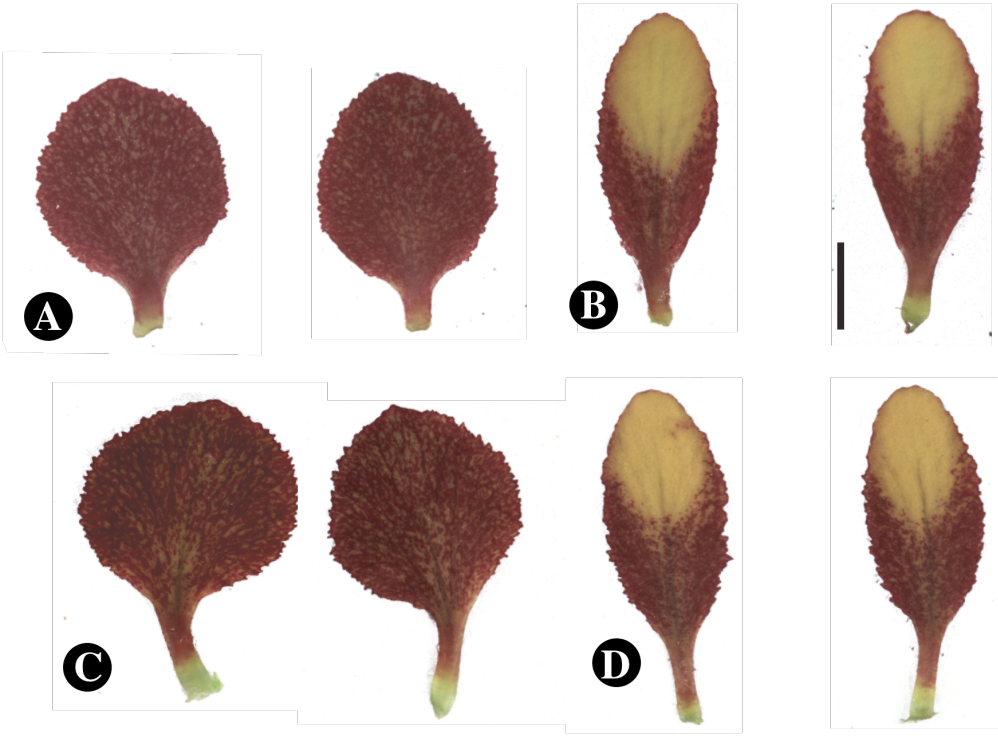

A
B
C
D

Figure 3.5. *TCP1* homologues as pertaining to *in situ* hybridization experiments. A, Summary of phylogenetic relationships between TCP-like sequences isolated from *Cleome viridiflora*, *C. spinosa*, and *C. violacea*. *Arabidopsis* sequences from the *TCP1*, *CYC3*, *CIN*, and *TCP-P* clades are included to demonstrate orthology. Topology derived from one of three trees identified by maximum parsimony (MP) analysis with simple addition sequence (length = 2377; CI = 0.890; RI = 0.703). Bootstrap values based on 1000 replicates of the MP search are indicated above branches. B, Alignment of TCP-like sequences isolated from *C. viridiflora*, *C. spinosa*, and *C. violacea* showing position and sequence of *ClevioTCP1.1* probe used in in-situ experiments. C, Protein sequence of *ClevioTCP1.1* showing position of conserved TCP domain and probe used for *in situ* hybridization experiments.

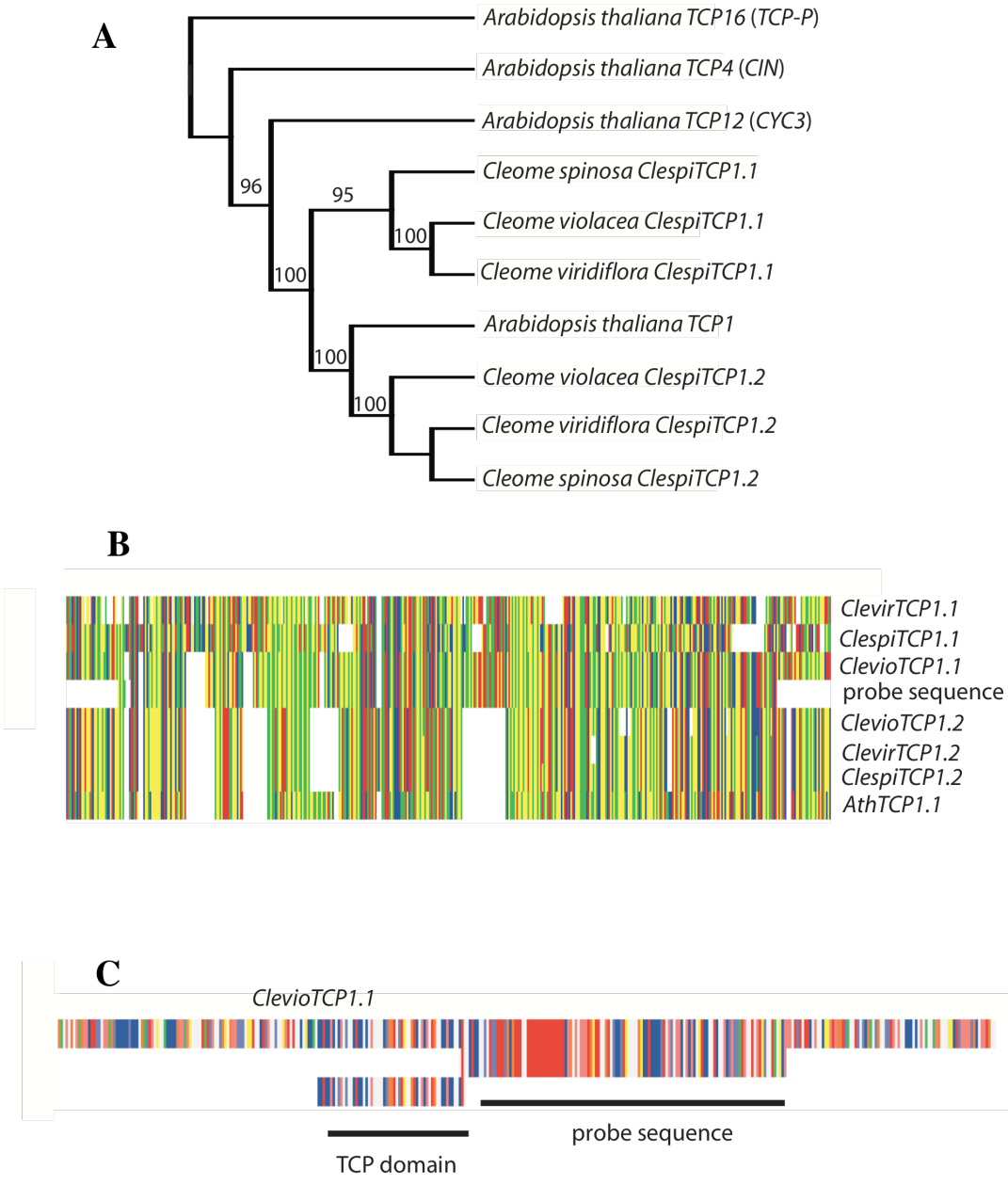
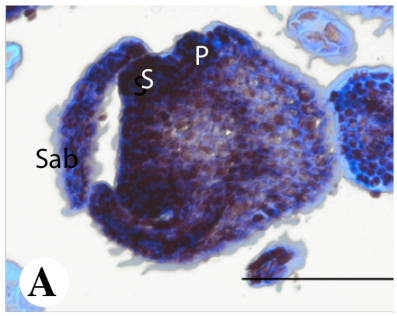
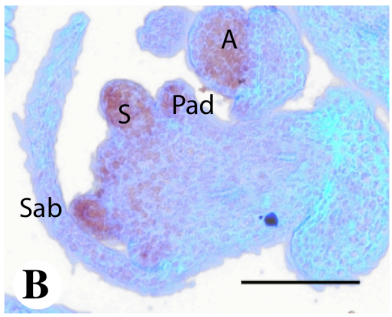
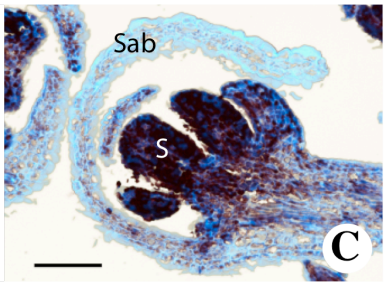
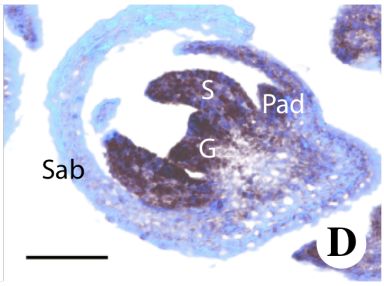

A
Arabidopsis thaliana TCP16 (TCP-P)
Arabidopsis thaliana TCP4 (CIN)
Arabidopsis thaliana TCP12 (CYC3)
Cleome spinosa ClespiTCP1.1
Cleome violacea ClespiTCP1.1
Cleome viridiflora ClespiTCP1.1
Arabidopsis thaliana TCP1
Cleome violacea ClespiTCP1.2
Cleome viridiflora ClespiTCP1.2
Cleome spinosa ClespiTCP1.2
96
95
100
100
100
100
B
ClevirTCP1.1
ClespiTCP1.1
ClevioTCP1.1
probe sequence
ClevioTCP1.2
ClevirTCP1.2
ClespiTCP1.2
AthTCP1.1
C
ClevioTCP1.1
probe sequence
TCP domain

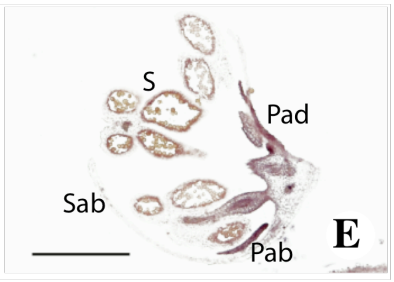
Figure 3.6. Tissue specific expression pattern of *ClevioTCP1-1* in *Cleome violacea* visualized with *in situ* hybridization. Digoxigenin-labelled antisense *ClevioTCP1.1* was hybridized to longitudinal sections through buds and inflorescences tips. Abaxial sepal is oriented to the bottom when appropriate. A, Stage 5 bud showing expression in developing sepal, stamen, and petal primordia. B, Stage 6 bud showing expression in petals and stamen primordia. C, Stage 7 bud showing expression in petals and stamens. D, Stage 8 bud showing expression in petals, stamens, and gynoecium. E, Stage 11 bud showing expression in petals. F, Stage 10 bud showing expression in petals. G - I, Hybridization with sense *ClevioTCP1.1* in early (Figure 3.6 G) and middle (Figure 3.6 H) stages of development. Scale bars = 50 μm (C, D, H); 100 μm (A, B); 100 μm (I); 200 μm (E, F). Abbreviations: A = apical meristem; G = gynoecium; P = petal; Pab = abaxial petal; Pad = adaxial petal; Sab = abaxial sepal; S = stamen.

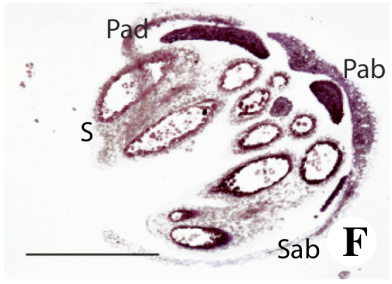

P
S
Sab
A


A
S
Pad
Sab
B


Sab
S
C


S
Pad
G
Sab
D


S
Pad
Sab
Pab
E


Pad
Pab
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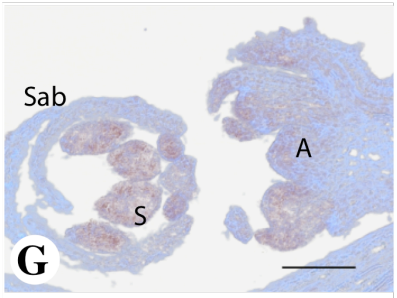
F


Sab
A
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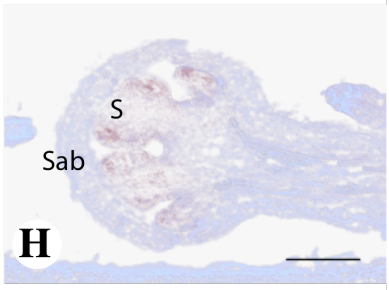
S
Sab
H

Chapter 4: Conclusions

Chapter 4: Conclusions

This thesis addresses important milestones in evolutionary developmental genetic investigations of Cleomaceae. First, resolution of the Cleomaceae phylogeny provides an important framework for understanding direction and frequencies of morphological transitions. Importantly, a strong phylogenetic hypothesis will guide selection of focal taxa, as closely related taxa with few morphological differences are ideal to investigate the genetic basis of that variation. Finally, resolution of the Cleomaceae phylogeny permits the generation of hypotheses regarding evolutionary transitions. Development and phylogenetic relationships of a highly monosymmetric species, *Cleome violacea*, were characterized to facilitate interpretation of expression patterns of *ClevioTCP1.1*, one of two homologues of the candidate gene *TCP1* isolated from three species of *Cleome*. These data provide foundation for further investigations of whether this gene family is responsible for the monosymmetric flowers observed in the family. In this concluding chapter, I explore evolutionary consequences for floral evolution in Cleomaceae based on these observations and propose directions for future investigation.

TCP1 sequence diversity in a phylogenetic context

The diversity of TCP sequences is an important consideration regarding interpretation of floral symmetry evolution at the morphological and molecular level within Cleomaceae. Rather than a single copy expressed adaxially in the petal whorl, as in Brassicaceae (Busch and Zachgo 2007; Busch et al. 2012), two TCP paralogues were isolated in the focal taxon, *Cleome violacea*. However, early stages of development show a broad expression domain across multiple whorls that is similar to comparable stages of development in monosymmetric Brassicaceae (Busch et al. 2012). A single copy would be expected to show expression patterns similar to those associated with monosymmetry in Brassicaceae, where there is a broad expression domain early in development, which is later restricted to adaxial petals. Isolation of two copies in Cleomaceae

generates three testable hypotheses: 1) differences in relative expression between adaxial and abaxial parts of the flower occur but are not detected with *in situ* hybridization and will be detected with qPCR experiments; 2) *ClevioTCP1.2*, rather than *ClevioTCP1.1*, is responsible for adaxial/abaxial differentiation between whorls and may have an expression domain restricted to the adaxial region of developing flowers; 3) *ClevioTCP1.1* is involved in differentiation of left and right halves of organs within whorls, such as petal curvature. Sequence similarity between paralogues is also a consideration for designing molecular tools based on specificity of sequence interactions, such as *in situ* hybridization and virus-induced gene silencing (VIGS) experiments. VIGS experiments represent one avenue to obtain functional data (Baulcombe 1999) and have been shown to be promising in *C. violacea* (Mankowski, unpublised).

Currently, little is known about *TCP1* sequence diversity in Cleomaceae and how potential diversity may relate to differences in floral monosymmetry. Two *TCP1*-like sequences were recovered from the three species surveyed: *Cleome spinosa*, *C. violacea*, and *C. viridiflora*. However, *C. violacea* is in a basal clade that predates a genome duplication in Cleomaceae (Schranz, personal communication; Schranz and Mitchell-Olds 2006), although the placement of the Cleomaceae-specific duplication needs to be re-evaluated in light of the stronger phylogenetic hypothesis presented here. As a result of this genome duplication, additional copies of *TCP1*-like genes may be recovered from *C. spinosa* or *C. viridiflora*, both from the more derived *Tarenaya* clade (Chapter 2). Continued cloning effort from genomic DNA using degenerate primers has the potential to recover additional copies within Cleomaceae. Interestingly, lineages that diverge after the genome duplication include taxa that produce flowers with a range in degree of monosymmetry, from minimal differentiation between whorls as in *C. spinosa*, to highly monosymmetric taxa such as *Haptocarpum* and *Dactylaena*. If *TCP1*-like sequences isolated from these taxa that have differentiation of adaxial and abaxial petal pairs in addition to curvature form a unique clade within the family, differences at the sequence level may indicate functional domains or consensus sequences involved in gene function/regulation. Thus, phylogenetic

analysis of *TCP1*-like sequences across Cleomaceae is necessary to correlate sequence diversity with floral morphology.

The expression patterns of *TCP1* paralogues in other lineages of Cleomaceae are likely to be different than those documented in the *Cleome* s.s. clade, which includes *C. violacea*. For example, *C. spinosa* has a developmental trajectory that exhibits early disymmetry rather than early monosymmetry (Patchell et al. 2011), imparted to *C. violacea* largely by the appearance of an enlarged abaxial sepal in early stages of development (Patchell et al. 2011; Chapter 3). The effects of *TCP1* expression and expression domain on cell growth and proliferation (Martin-Trillo and Cubas 2009) would account for these developmental differences. Comparative expression data for *C. spinosa* is required to determine if differences in expression pattern can be correlated with the two major developmental trajectories. Alternatively, considering the potential that adaxial expression is not detected in adaxial regions of the flower with *in situ* hybridization, RT-PCR or qPCR could be used to determine differential expression between adaxial and abaxial organs within whorls at candidate stages of development. Importantly, species from both developmental pathways have at least two copies of TCP homologues. Expression patterns of both copies need to be thoroughly characterized to test for possible examples of sub- and/or neofunctionalization. The very large-flowered species, *C. viridiflora*, exhibits the early disymmetric pattern of expression (Patchell et al. 2011). Due to its large size (unopened buds are approximately 8 cm long) this species is an ideal candidate for these expression studies. I have prepared dissected tissue at different developmental stages for this investigation.

The structure of *TCP1*-like genes in Cleomaceae needs to be characterized for a complete understanding of floral symmetry regulation in Cleomaceae. For example, the R domain and 3' untranslated region (UTR) were not recovered from *TCP1*-like sequences in the three taxa sampled. The 3' UTR has poorly understood roles in mRNA stability, localization, and translation efficiency (Chung et al. 2006). All of these roles affect expression levels visualized using *in situ* hybridization. Furthermore, gene structure determines function (Cubas 1999;

Howarth and Donoghue 2006). Alternative splicing that results in proteins lacking the R domain is documented in some TCP1-like sequences (Poza and Cubas unpublished). It is possible that a difference in a particular component of the *TCP1* gene, such as the consensus sequence that determines interaction with other molecules (reviewed in Martin-Trillo and Cubas 2009), rather than expression domain, lead to changes in molecular interactions that affect floral development. Experiments based on heterologous gene transformation would account for function of *TCP1* in Cleomaceae based on gene structure rather than expression domain.

Little is known about upstream regulation that specifies differences in the expression domain of TCP1 transcription factors (reviewed in Martin-Trillo and Cubas 2009). However, there may be an unexpected link between floral symmetry and gravity. For example, Vochting (1886) used a clinostat to show that monosymmetry in some flowers is induced by gravity. Termed positional monosymmetry, these flowers tend to exhibit monosymmetry in the latest stages of development and are held laterally in inflorescences. Furthermore, these species occasionally produce polysymmetric flowers at the apical position within racemes. These polysymmetric flowers are held vertically, rather than laterally, in the inflorescence and experience limited gravity in the plane of normal monosymmetric development (Endress 1999). Flowers that are monosymmetric regardless of the direction of gravity exhibit constitutional monosymmetry (Endress 1999). Although Endress has recently described the role of gravity in establishing monosymmetry in developing flowers (Endress 1999), the possibility of a connection between expression of *TCP1-like* homologues and gravity has not been explored.

Interestingly, it is unknown whether *Cleome violacea* flowers exhibit constitutional or positional monosymmetry. However, the type of monosymmetry can be determined experimentally. Plants of *C. violacea* grown from seed will flower in approximately 4 weeks. If plants are grown in a random positioning machine during flower formation, for a time frame of 2-4 weeks, the effect of zero net gravity on floral monosymmetry should be visually detectable in mature

flowers. Alternatively, a clinostat rotating plants horizontally at 1-3 rpm during flower formation should disrupt gene expression patterns (Silver 1976). Loss of monosymmetry would indicate positional monosymmetry, potentially corresponding to a change in gene expression pattern. The role of *TCP1* expression patterns associated with any changes in morphology can be assessed by embedding floral tissue in paraplast in preparation for *in situ* hybridization, or by extracting RNA from floral dissections of abaxial and adaxial petals stored at -80° C for later use in semi-quantitative RT-PCR and qPCR experiments.

Interaction between floral symmetry, insect pollinators, and plant fitness.

TCP1-like genes underlie a unique floral presentation in angiosperms that both increases attractiveness of flowers to animal pollinators (Moller 1995; Rodriguez et al. 2004) and increases efficiency of pollen transfer (Endress 1999). This direct effect of floral morphology on plant fitness is implicated in speciation and diversification of angiosperm lineages with monosymmetric flowers (Sargent 2004), including radiations of monosymmetric taxa from polysymmetric ancestors (Reeves and Omstead 1998; Ree and Donoghue 1999). The short branches length on the backbone of the Cleomaceae phylogeny is consistent with a rapid speciation event that parallels diversification of floral monosymmetry in the family. Considering the difference between floral symmetry types between Cleomaceae and Brassicaceae, there is considerable potential to investigate a role of floral monosymmetry in the rapid diversification of basal lineages of Cleomaceae. However, the Brassicaceae has also undergone an independent rapid speciation event (Beilstein et al. 2010).

Understanding interactions between flowers and pollinators is necessary to assess effects of floral morphology on plant fitness. The most monosymmetric flowers of *Erysimum mediohispanicum*, a species of Brassicaceae, exhibit natural variation in floral morphology. Some individuals produce flowers with characteristics associated with monosymmetry in a typical cleomoid flower, including spatial orientation and differentiation between size of petal pairs (Gomez et al. 2006), while others produce flowers with cruciform flowers typical

of Brassicaceae (Gomez et al. 2006). The more monosymmetric flowers attract more pollinators, and produce a larger number of viable seeds (Gomez et al. 2006). It is possible to change the degree of floral monosymmetry of *C. violacea* by altering expression of a single gene, *ClevioTCP1.1* (Mankowski unpublished). Applying a pollinator study to assess differences between visitation frequency between monosymmetric and polysymmetric *C. violacea* flower shapes would link molecular, morphological, and ecological levels of evolution. Furthermore, pollinators in wild populations in Spain and Middle East are unknown. Simple pollinator surveys to identify wild pollinators will identify interactions that influence pollen transfer, and subsequently assumptions regarding the role of floral monosymmetry in rapid radiation of taxa within Cleomaceae.

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